PKB/Akt interacts with inosine-5′ monophosphate dehydrogenase through its pleckstrin homology domain

Evan Ingley[a,b], Brian A. Hemmings[a,*]

[a] *Friedrich Miescher-Institut, Postfach 2543, Basel CH-4002, Switzerland*
[b] *Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Level 6, MRF Building, Rear 50 Murray St., Perth, W.A. 6000, Australia*

Received 6 June 2000; revised 10 June 2000; accepted 11 June 2000

Edited by Matti Saraste

Abstract The pleckstrin homology (PH) domain of the proto-oncogenic serine/threonine protein kinase PKB/Akt can bind phosphoinositides. A yeast-based two-hybrid system was employed which identified inosine-5′ monophosphate dehydrogenase (IMPDH) type II as specifically interacting with PKB/Akts PH domain. IMPDH catalyzes the rate-limiting step of de novo guanosine-triphosphate (GTP) biosynthesis. Using purified fusion proteins, PKB/Akts PH domain and IMPDH associated in vitro and this association moderately activated IMPDH. Purified PKB/Akt also associated with IMPDH in vitro. We could specifically pull-down PKB/Akt or IMPDH from mammalian cell lysates using glutathione-*S*-transferase (GST)–IMPDH or GST–PH domain fusion proteins, respectively. Additionally, PKB/Akt and IMPDH could be co-immunoprecipitated from COS cell lysates and active PKB/Akt could phosphorylate IMPDH in vitro. These results implicate PKB/Akt in the regulation of GTP biosynthesis through its interaction with IMPDH, which is involved in providing the GTP pool used by signal transducing G-proteins.

Key words: Inosine-5′ monophosphate dehydrogenase; Pleckstrin homology domain; Protein kinase B; Akt; Guanosine-triphosphate

1. Introduction

The PKB/Akt subfamily of serine/threonine protein kinases, consisting of α, β and γ isoforms [1–3], is closely related to the cAMP-dependent protein kinase (PKA) and Ca^{2+}/phospholipid-dependent protein kinase (PKC) families in its kinase domain and contains an amino-terminal pleckstrin homology (PH) domain [4]. The human PKB/Aktα isoform is the protooncogenic form of *v-akt*, which encodes a complete PKB/Akt with the addition of a truncated *gag* sequence at the amino-terminus providing a myristoylation sequence [5].

Originally, the PH domain was identified as an internal repeat, present at the amino- and carboxyl-termini of pleckstrin, a 47 kDa protein which is the major PKC substrate in activated platelets [6]. The superfamily of PH domain-containing molecules consists of over 90 members, including serine/threonine protein kinases (PKB/Akt, Nrk, βARK, PKCμ), tyrosine protein kinases (Btk, Tec, Itk), GTPase regulators (ras-GAP, ras-GRF, Vav, SOS, BCR), all known mammalian phospholipase Cs, cytoskeletal proteins (β-spectrin, AFAP-110, syntrophin), 'adapter' proteins (GRB-7, 3BP2) and kinase substrates (pleckstrin, IRS-1) [4,7–12].

Structures have been solved for Bruton's tyrosine kinase (Btk), β-spectrin, pleckstrin, dynamin and phospholipase Cδ-1 PH domains [13–17] and all are similarly folded. The presence of PH domains in many signaling and cytoskeletal proteins implicates it in the mediation of signaling processes. Indeed, the PH domain of the β-adrenergic receptor kinase (βARK) appears partly responsible for its binding to the βγ-subunits of the heterotrimeric G-proteins associated with the β-adrenergic receptor [18,19]. While the PH domain of Btk appears to mediate an interaction with PKC [20], more recently, several PH domains have been shown to bind phosphoinositides (PIs) [21–25] and signaling pathways that are initiated by PI 3-kinase appear to activate PKB/Akt. Previously, we have shown that the PH domain of PKB/Akt seems to be pivotal in localizing it to the plasma membrane after growth factor (insulin or IGF-1) stimulation [26,27], by virtue of its ability to bind the PI 3-kinase product PtdIns(3,4,5,)P_3 [25], where upon it becomes activated by at least one protein kinase (USK/PKBK) [28,29]. Fully activated PKB/Akt is phosphorylated on Thr308 and Ser473 [26,27] and is known to phosphorylate and inactivate components of the apoptotic machinery, BAD [30,31], Caspase9 [32] and FKHRL1 [33]. PKB/Akt then translocates to the nucleus [26,27] presumably to phosphorylate other specific protein targets.

Considering the importance of ascertaining the function of PH domains and noting that we have previously shown PKB/Akts PH domain specifically binds PIs [25] while some other PH domains appear to mediate protein–protein interactions [18–20], we used the yeast two-hybrid system [34,35] to determine if PKB/Akts PH domain could function by forming specific associations with other proteins. In the present study we have identified PKB/Akts PH domain as interacting with inosine-5′ monophosphate dehydrogenase (IMPDH) type II in in vitro and in vivo binding assays. PKB/Akts PH domain also appears to moderately stimulate IMPDH activity and constitutively active PKB/Akt can phosphorylate IMPDH in vitro. In conjunction with the known role of IMPDH in guanosine-triphosphate (GTP) biosynthesis our findings suggest a role for PKB/Akt in regulating the GTP pool used by signal transducing G-proteins.

*Corresponding author. Fax: (41)-61-697 3976.
E-mail: hemmings@fmi.ch

PII: S0014-5793(00)01866-4

2. Materials and methods

2.1. Bacterial and yeast strains

All yeast strains and plasmids for yeast two-hybrid experiments were obtained from Clontech (Palo Alto, CA, USA) as components of the Matchmaker Two-Hybrid System or from Dr. D. Nathans (Howard Hughs Medical Institute, Baltimore, ML, USA). Yeast strains SFY526 (*MATa*, *ura3–52*, *his3–200*, *ade2–101*, *lys2–801*, *trp1–901*, *leu2–3*, *112*, *can*^{r}, *gal4–542*, *gal80–538*, *URA3::GAL1-lacZ*), HF7c (*MATa*, *ura3–52*, *his3–200*, *lys2–801*, *ade2–101*, *trp1–901*, *leu2–3*, *112*, *gal4–542*, *gal80–538*, *LYS2::GAL1-HIS3*, *URA3::*(*GAL4 17-mer*)$_3$-CYC1-lacZ) and PCY2 [36] (*MAT*α, *his3–200*, *ade2–101*, *lys2–801*, *trp1–63*, *leu2–3*, *gal4–542*, *gal80–538*, *URA3::GAL1-lacZ*) were used to assay for protein–protein interactions. The yeast strain HF7c was used for library screening. Yeast techniques including transformation were performed according to the instructions in the Matchmaker Two-Hybrid System manual and as previously described [37]. The bacterial strains XL1-blue and JM109 (Stratagene) were employed in the cloning of plasmids and the production of glutathione-*S*-transferase (GST) fusion proteins. The bacterial strains JM109(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE (Invitrogen) were used for the production of $(His)_6$-tagged proteins. General molecular biological techniques were performed as previously described [38,39].

2.2. Plasmid construction

Yeast vector plasmids containing the GAL4 DNA binding domain (amino acids 1–147, pGBT9) and the GAL4 activation domain (amino acids 768–881, pGAD424) as well as the control plasmids pCL1 (full-length GAL4 gene), pVA3 (p53 gene), pTD1 (SV40 large T antigen), and pLAM5′ (human lamin C gene) were from Clontech. The yeast vector pPC62, containing the GAL4 DNA binding domain, was a gift from Dr. D. Nathans. The GST fusion vector pGEX-2T was a gift from Dr. P. Matias (FMI). The baculovirus transfer vector (pVL1392) and the $(His)_6$-tag vector (pRSET-A) were from Invitrogen. pGBT-PH(1–127), pGBT-PH(1–150), pGBT-PH(1–47) and pGBT-PH(47–127) contained in-frame fusions of amino acids 1–127, 1–150, 1–47, 47–127 of the human PKB/Aktα PH domain, respectively, with the GAL4 DNA binding domain. They were constructed by subcloning polymerase chain reaction (PCR) fragments generated with specific oligonucleotides into the *Eco*RI–*Bam*HI sites of pGBT9.

pGEX-PH131 contained an in-frame fusion of amino acids 1–131 of human PKB/Aktα with GST. It was constructed by subcloning a PCR fragment generated with specific oligonucleotides into the *Bam*HI–*Eco*RI sites of pGEX-2T. pGBT-PH-Kin(1–411), pGBT-PH-Kin-COOH(1–480), pGBT-Kin-COOH(147–480), pGBT-Kin(147–411) and pGBT-COOH(411–480) contained in-frame fusions of amino acids 1–411, 1–480, 147–480, 147–411 and 411–480 of human PKB/Aktα, respectively, with the GAL4 DNA binding domain. They were constructed by subcloning the appropriate *Bam*HI–*Eco*RI fragments from corresponding pGEX constructs into the *Pst*I–*Xba*I sites of pPC62 using *Pst*I–*Bam*HI and *Eco*RI–*Xba*I adapters. The *Xho*I–*Xba*I fragments from the resultant pPC62 plasmids were then isolated and subcloned into the *Xho*I–*Eco*RI sites of pGBT9 using an *Xba*I–*Eco*RI adapter. The plasmids pGAD-IMPDH(1–481), pGAD-IMPDH(1–427), pGAD-IMPDH(1–325), pGAD-IMPDH(28–514), pGAD-IMPDH(70–514), pGAD-IMPDH(1–40) and pGAD-IMPDH(428–514) contained in-frame fusions of amino acids 1–481, 1–427, 1–325, 28–514, 70–514, 1–40 and 428–514 of human IMPDH type II, respectively, with the GAL4 activation domain. They were constructed by subcloning PCR fragments generated with specific oligonucleotides into the *Bam*HI–*Sal*I sites of pGAD424.

pGEX-IMPDH contained an in-frame fusion of the complete human IMPDH type II cDNA with GST. It was constructed by subcloning the *Sma*I–*Xho*I fragment of IMPDH from pGADGH-IMPDH into the *Sma*I site of pGEX-2T using a *Xho*I–*Sma*I adapter. pVL1392-hPKB/Aktα contained the *Eco*RI fragment from WI38x-Akt71 [1] encompassing the full coding region of human PKB/Aktα. pRSET-PHQKKK [40] contains an in-frame fusion of amino acids 1–116 of human PKB/Aktα with an amino-terminal $(His)_6$-tag, and the addition of three lysines at the carboxyl terminus. It was constructed by subcloning an *Nde*I–*Pfl*MI fragment from pRK-RAC [1] into the *Bam*HI–*Eco*RI sites of pRSET-A using *Bam*HI–*Nde*I and *Pfl*MI–*Eco*RI adapters. The eukaryotic expression constructs of hemagglutinin (HA)-tagged PKB/Aktα, wild-type (pCMV5-HA-PKB/Akt), kinase inactive (pCMV5-HA-PKB/Akt-K197A) and constitutively active (pCMV5-HA-PKB/Akt-T308D/S473D) forms have been described previously [26]. All plasmid constructions were confirmed by restriction fragment analysis and sequencing. Further details of plasmid constructs are available upon request.

2.3. Library screening

The human HeLa S3 Matchmaker cDNA library was purchased from Clontech. pGBT-PH127 was transformed into HF7c with and without the control plasmids (pGAD424, pCL1 and pTD1). Colonies from this transformation were tested for HIS3 and LacZ expression to confirm that the PH domain alone does not activate transcription. The HF7c yeast containing just pGBT-PH127 was then transformed with enough of the HeLa S3 cDNA library inserted into the two-hybrid activation vector pGADGH to produce 1.0×10^6 yeast Leu^+/Trp^+ transformants. Doubly transformed cells were plated onto Leu^-/Trp^-/His^- plates and incubated at 30°C for 8 days. Positive colonies were picked, restreaked onto Leu^-/Trp^-/His^- plates and assayed for LacZ activity by the filter assay. Library clones that were His^+ and $LacZ^+$ were then cured of the pGBT-PH127 plasmid and tested again for His auxotrophy and LacZ activity. Cured clones that were negative in both assays were then mated to PCY2 yeast containing either pGBT9, pGBT-PH127, pGBT-KIN-CT, pLAM5′ or pTD1. Clones corresponding to the diploids which had became positive for both His auxotrophy and LacZ activity only in the presence of pGBT-PH127 were designated as true positives and then sequenced.

2.4. In vitro binding studies

GST fusions produced from the plasmids pGEX-2T, pGEX-PH131 and pGEX-IMPDH were expressed in *Escherichia coli* XL-1 blue cells by induction with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) for 2 h at 24°C. The fusion proteins were purified as previously described [41] except that the cells were lysed in a French Press. Full-length human PKB/Aktα was expressed and purified from the baculovirus system. Briefly, a baculovirus was constructed by co-transfection of Sf9 cells with pVL1392-RACα and wild-type baculovirus AcMNPV DNA. The resultant recombinant virus was then purified by limiting dilution and detected by dot-blot hybridization. The purified virus was used to produce human PKB/Aktα in Sf9 cells. The human PKB/Aktα was purified by sequential anion exchange, phenyl-Sepharose and gel filtration chromatography. The human PKB/Aktα PH domain $(His)_6$-tagged fusion produced by Bl21(DE3)pLysS cells transformed with pRSET-PHQKKK was expressed and purified as described [40]. Briefly, cells were induced with 0.2 mM IPTG for 2 h at 24°C before harvesting. Cell pellets were lysed in a French Press and the soluble PH domain was purified sequentially on Ni(II) affinity, cation exchange and gel filtration columns. Binding studies were performed using GST fusions (2.5 μg) coupled to glutathione-agarose beads in binding buffer (20 mM phosphate buffer pH 7.2, 150 mM NaCl, 1% Triton X-100, 5 mM DTT) containing 2.5 μg of $(His)_6$-tagged PH domain or baculovirus produced human PKB/Aktα in a total volume of 100 μl. The samples were incubated at 4°C for 1–2 h with agitation every 5 min. The beads were then washed three times with buffer (20 mM phosphate buffer pH 7.2, 150 mM NaCl) before being analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue R-250 or followed by Western blot analysis using a human PKB/Aktα-specific antiserum [1]. The secondary antibody was a horse radish peroxidase coupled anti-rabbit antibody (Amersham) which was detected using the enhanced chemiluminescence (ECL) method (Amersham) by autoradiography.

2.5. In situ pull-down assay

MCF-7 cells were lysed in buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 30 mM pNPP, 25 mM β-glycerol phosphate, 15 mM PPi, 25 mM NaF, 1 mM vanadate, 20 μM PAO, 1 mM benzamidine, 0.1 mM PMSF) using 12 strokes of a Dounce homogenizer. Soluble protein from the supernatant of lysates centrifuged at $14\,000\times g$ for 15 min at 4°C was added to GST, GST–PH or GST–IMPDH protein (5 μg) attached to glutathione-agarose beads and incubated at 4°C for 2 h with continuous agitation. The beads were then washed four times with lysis buffer before being analyzed by Western blotting as described above with the human PKB/Aktα-specific antiserum or an IMPDH-specific antiserum [42].

2.6. In vivo binding assay

Monkey COS cells were transfected with the plasmids pCMV5-HA-PKB/Akt (wild-type HA-tagged PKB/Akt), pCMV5-HA-PKB/Akt-K197A (kinase inactive HA-tagged PKB/Akt) or pCMV5-HA-PKB/Akt-T308D/S473D (constitutively active HA-tagged PKB/Akt) using Lipofectamine 2000 (Gibco BRL) according to the manufacturers instructions. After 48 h the cells were harvested, lysed in buffer (see Section 2.5) and immunoprecipitated with HA (BAbCo, Berkeley, CA, USA) or IMPDH-specific antibodies. The IMPDH-specific antibody was generated against the peptide YSGELKFEKRTSSAQ-VEGGVHSLHSYEKRLF. Immunoprecipitates were collected with protein-G beads (Sigma) and washed three times with lysis buffer. Immunoprecipitates were then analyzed by SDS–PAGE followed by Western blotting using HA, IMPDH or a human PKB/Aktα-specific antiserum [1]. The secondary antibody was a horse radish peroxidase coupled anti-rabbit antibody (Amersham) which was detected using the ECLplus method (Amersham) by autoradiography.

2.7. In vitro kinase assay

COS cells transfected with pCMV5-HA-PKB/Akt-K197A or pCMV5-HA-PKB/Akt-T308D/S473D were lysed and immunoprecipitated with HA-specific antibodies (BAbCo) as described in Section 2.6. The immunoprecipitated HA-PKB/Akt-K197A or HA-PKB/Akt-T308D/S473D protein was then used in kinase assays with purified GST or GST–IMPDH as substrate. Purified GST fusions were prepared as described in Section 2.4. Kinase reaction conditions were similar to those previously described [1] and were carried out in buffer (50 mM Tris–HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 1 μM PKI, 3.3 μM [γ-^{32}P]ATP) with 5 μg of GST fusion and 20 μl of immunoprecipitated PKB/Akt protein. Reactions were carried out at 30°C for 30 min before analysis by SDS–PAGE, transfer to PVDF membrane (Amersham) and quantitated on a phosphoimager 445-SI (Molecular dynamics, Sunnyvale, CA, USA).

2.8. IMPDH enzyme assay

Assays for IMPDH activity were performed essentially as described [43] monitoring the production of XMP by absorbance at 286 nm. The IMPDH was produced as a GST fusion and purified on glutathione-agarose beads and eluted as soluble protein with reduced glutathione. IMPDH activity was tested in the presence of either soluble GST (produced from pGEX-2T) or PH domain (produced from pGEX-PH131) at a molar ratio of IMPDH to GST/PH domain of 1:5.

3. Results

To determine if PKB/Akts PH domain could interact with other proteins we fused it to the GAL4 DNA binding domain and screened a HeLa cDNA library fused to the GAL4 transcriptional activation domain in the yeast reporter strain HF7c. In our screen of 1.0×10^6 primary transformants we

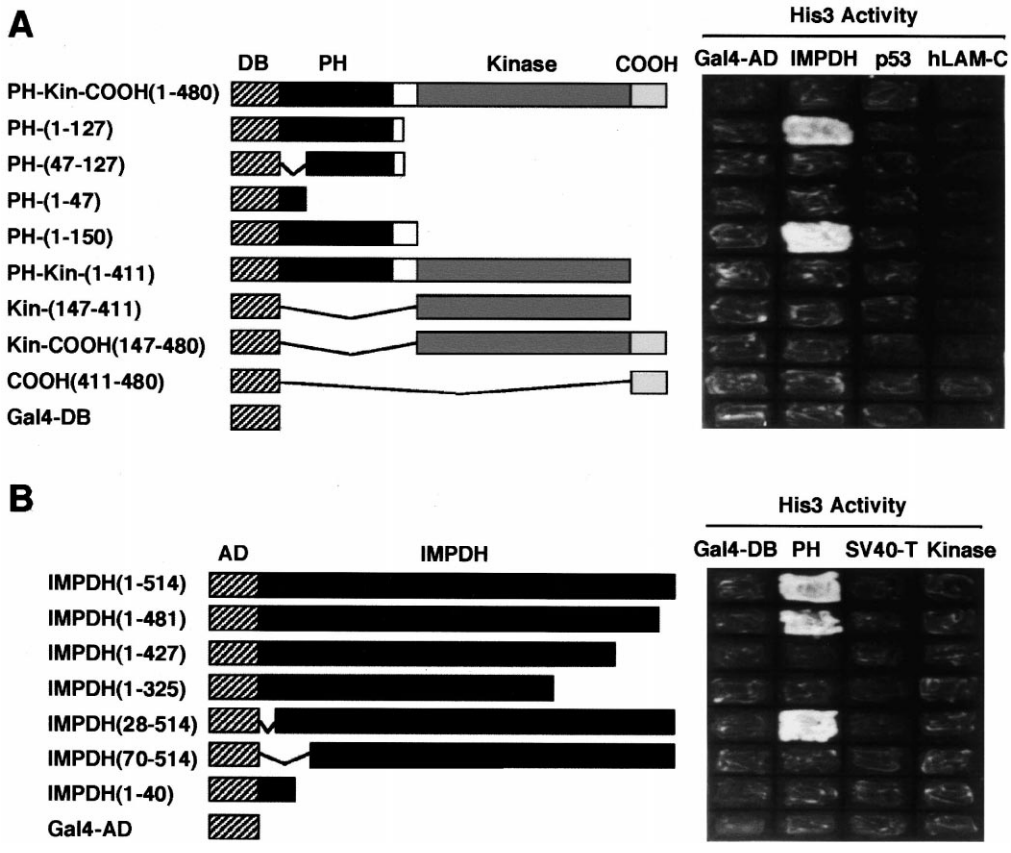

Fig. 1. Interaction of the PKB/Aktα with IMPDH type II in the yeast two-hybrid system. A: Co-transformants of Gal4 DNA binding constructs pGBT9 (GAL4-DB), pGBT-PH127 (PH(1–127)) pGBT-PHIV-VI (PH(47–127)), pGBT-PHI-III (PH(1–47)), pGBT-PH150 (PH(1–150)), pGBT-PH-KIN (PH-Kin(1–411)), pGBT-PH-KIN-CT (PH-Kin-COOH(1–480)), pGBT-KIN (Kin(147–411)), pGBT-KIN-CT (Kin-COOH(147–480)) and pGBT-CT (COOH(411–480)) with either pGAD424 (Gal4 AD), pGADGH-IMPDH (IMPDH), pVA3 (p53) or pLAM5′ (hLAM-C) in HF7c cells were selected on Leu^-/Trp^- plates and then replated onto $Leu^-/Trp^-/His^-$ plates to detect activation of the HIS3 reporter. Liquid β-gal assays of the above co-transformants were also performed in SFY526 cells. Assays were performed in triplicate. B: Delineation of the PKB/Aktα PH domain binding region of IMPDH type II using the yeast two-hybrid system. Co-transformants of the Gal4 activation domain constructs pGAD424 (GAL4-AD), pGADGH-IMPDH (IMPDH(1–514)), pGAD-IMPDH1–481 (IMPDH(1–481)), pGAD-IMPDH1–427 (IMPDH(1–427)), pGAD-IMPDH1–325 (IMPDH(1–325)), pGAD-IMPDH28–514 (IMPDH(28–514)), pGAD-IMPDH70–514 (IMPDH(70–514)), pGAD-IMPDH1–40 (IMPDH(1–40)) and pGAD-IMPDH428–514 (IMPDH(428–514)) were selected and assayed as described in (A). See Section 2 for plasmid construction details.

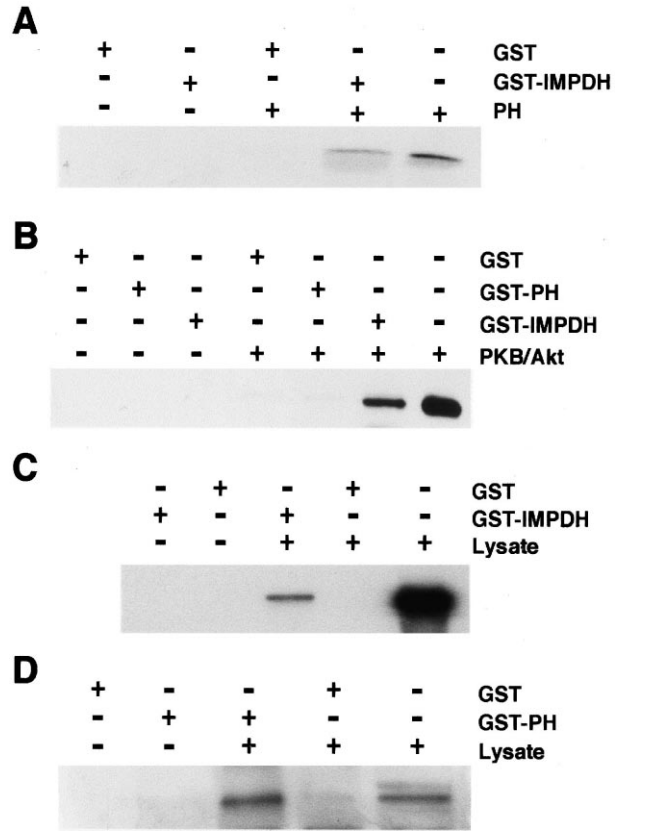

Fig. 2. Detection of IMPDH-PKB/Akt association in in vitro binding assays and in in situ pull-down assays from MCF-7 cell lysates. A: Binding of a $(His)_6$-tagged human PKB/Aktα PH domain to GST–IMPDH coated beads. GST or GST–IMPDH beads were incubated with or without the $(His)_6$-tagged PH domain as indicated. After washing, the protein attached to the beads was analyzed by Coomassie staining of an SDS–PAGE gel. B: Binding of baculovirus produced human PKB/Aktα to GST–IMPDH coated beads. GST, GST–PH or GST–IMPDH beads were incubated with or without the baculovirus produced human PKB/Aktα as indicated. After washing, the protein attached to the beads was analyzed by SDS–PAGE followed by Western blotting using a human PKB/Aktα-specific antiserum as described in Section 2. C: Association of human PKB/Aktα from MCF-7 cell extracts with GST–IMPDH and not GST. GST or GST–IMPDH beads were incubated with or without MCF-7 cell extracts as indicated. After washing, the protein attached to the beads was analyzed by SDS–PAGE followed by Western blotting using a human PKB/Aktα-specific antiserum as described in Section 2. D: Association of human IMPDH from MCF-7 cell extracts with GST–PH and not GST. GST or GST–PH beads were incubated with or without MCF-7 cell extracts as indicated. After washing, the protein attached to the beads was analyzed by SDS–PAGE followed by Western blotting using an IMPDH-specific antiserum as described in Section 2.

identified 37 clones which showed specific interaction with PKB/Akts PH domain, by activation of the reporters for His auxotrophy and LacZ activity (Fig. 1A). These clones could be subdivided into six different cDNA classes, based on the size of the cDNA insert. Upon sequencing all clones where found to encode human IMPDH type II inclusive of the initiator methionine through to the termination codon of the previously cloned cDNA [42]. IMPDH catalyzes the rate-limiting step of de novo GTP biosynthesis in the NAD-dependent conversion of IMP to XMP [42,43].

The interaction we have identified requires a complete PH domain as constructs containing either subdomains I–III (amino acids 1–47) or subdomains IV–VI (amino acids 47–127) alone did not show any interaction with IMPDH. The lack of interaction with subdomains IV–VI is significant as this region has previously been shown to interact weakly with the $\beta\gamma$-subunits of heterotrimeric G-proteins [19]. This interaction of IMPDH and PKB/Akts PH domain is, however, inhibited in the two-hybrid system with constructs containing PKB/Akts kinase domain juxtaposed to PKB/Akts PH domain as occurs in its natural context. These constructs were expressed correctly in the yeast as they interacted with a control protein known to interact with the PKB/Akt carboxyl-terminal region (results not presented). This inhibition of interaction could be due to an intramolecular interaction of PKB/Akts PH domain with itself or another region of PKB/Akt. Inclusion of amino acids between the PH and kinase domains (including the first four amino acids of the kinase domain) didn't inhibit the interaction. We also fused PKB/Akts PH domain to the GAL4 activation domain to test if it could interact with any of the human PKB/Aktα GAL4 DNA binding constructs. We could detect no such interaction, indicating that the PH domain doesn't self associate or form a complex with other regions of the PKB/Akt molecule in this system (Fig. 1A). The inhibition of interaction, ob-

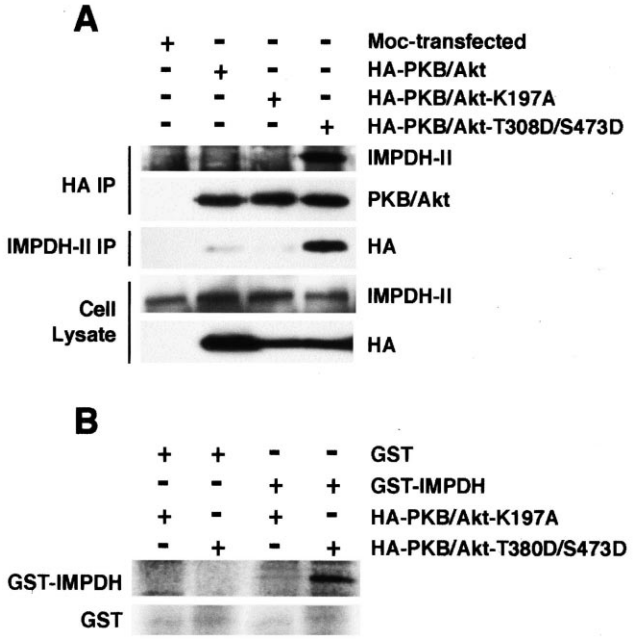

Fig. 3. Detection of IMPDH-PKB/Akt association in vivo and phosphorylation of IMPDH by PKB/Akt in vitro. A: Co-immunoprecipitation of IMPDH and HA-tagged PKB/Aktα, wild-type (HA-PKB/Akt), kinase inactive (HA-PKB/Akt-K197A) and constitutively active (HA-PKB/Akt-T308D/S473D) forms expressed in COS cells. Cell lysates from cells expressing the various constructs indicated, or moc-transfected, were immunoprecipitated (IP) with HA-specific or IMPDH-specific antibodies. The immunoprecipitates were then analyzed by SDS–PAGE and Western blotting using HA-, IMPDH-, or PKB/Akt-specific antibodies as described in Section 2. B: Phosphorylation of GST–IMPDH by constitutively active PKB/Aktα and not kinase inactive PKB/Aktα. Kinase inactive (K197A) and constitutively active (T380D/S473D) forms of HA-tagged PKB/Akt were immunoprecipitated from COS cell lysates and then added to purified GST or GST–IMPDH with [γ-^{32}P]ATP in a kinase buffer as described in Section 2. Kinase reactions were then analyzed by SDS–PAGE followed by transfer to PVDF membrane and the radioactivity was quantitated on a phosphoimager 445-SI (Molecular dynamics, Sunnyvale, CA, USA).

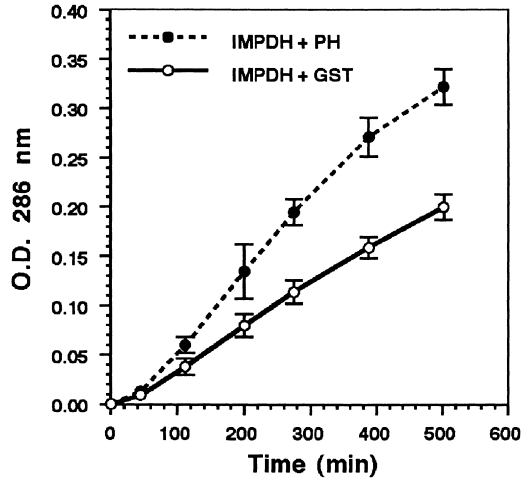

Fig. 4. Effect of the PKB/Akt PH domain on IMPDH activity in vitro. IMPDH expressed and purified as a GST fusion was incubated with a five-fold molar excess of either GST–PH domain fusion protein or GST alone under IMPDH assay conditions as described in Section 2. The production of XMP was monitored by measuring the change in optical density at 286 nm. Each time point was assayed in triplicate and error bars represent a 99% confidence interval.

served above, in the yeast two-hybrid system would thus appear to be due to steric hindrance.

We constructed nested amino- and carboxyl-terminal deletions of IMPDH to determine the region of the molecule responsible for interaction with PKB/Akts PH domain (Fig. 1B). This indicated that an almost intact IMPDH molecule was required for the interaction. The amino-terminal boundary of the PH interaction domain was found to lie between amino acids 28 and 70, while the carboxyl-terminal boundary lies between amino acids 427 and 481.

To test if IMPDH could interact directly with PKB/Akts PH domain we employed an in vitro binding assay system using GST fusions (Fig. 2A). In this assay we see that the $(His)_6$-tagged PH domain can bind to the GST–IMPDH fusion but not to GST alone. We also employed this assay system to test if full-length baculovirus purified human PKB/Aktα could directly interact with IMPDH (Fig. 2B). Here we see a specific interaction of the full-length PKB/Akt molecule with GST–IMPDH and not GST alone or the GST–PH fusion. Also, when we used the GST–IMPDH in a pull-down assay with MCF-7 cell extracts we see a specific association of human PKB/Aktα with the GST–IMPDH and not with GST (Fig. 2C). We could also perform the converse experiment, pulling down IMPDH from cell lysates using the GST–PH domain fusion protein (Fig. 2D). Thus, we have shown the existence of an association between human PKB/Aktα and human IMPDH type II in three heterologous systems, yeast two-hybrid, in vitro purified protein interactions and in situ pull-downs.

To test if PKB/Akt and IMPDH could interact in vivo we expressed HA-tagged wild-type, kinase inactive (K197A) and constitutively active (T308D/S743D) forms of PKB/Akt in COS cells and then immunoprecipitated PKB/Akt with HA-specific antibodies or IMPDH with a specific antiserum. The results (Fig. 3A) show that the constitutively active form of PKB/Akt (T308D/S743D) co-immunoprecipitated with IMPDH. This occurred when we immunoprecipitated PKB/Akt and probed for IMPDH and when we immunoprecipitated IMPDH and probed for PKB/Akt. We next examined if the constitutively active PKB/Akt immunoprecipitated from COS cells could phosphorylate IMPDH (Fig. 3B). These results show that indeed the constitutively active PKB/Akt is able to phosphorylate a GST fusion of IMPDH and not GST alone.

We then assayed the effect of this interaction on the activity of IMPDH. The addition of soluble PH domain as a GST fusion to IMPDH produced a moderate activation of the enzyme compared to the addition of GST alone (Fig. 4).

4. Discussion

Recently we have learned much about the involvement of PKB/Akt in PI3 kinase signaling pathways where PIs alter the subcellular localization of PKB/Akt [25,26] and other kinases (USK/PKBK) provide activating phosphorylations [28,29,44] for PKB/Akt. Considering the importance of ascertaining the full spectrum of molecules involved in the regulation of PKB/Akt we used the yeast two-hybrid system [34,35] to determine if PKB/Akts PH domain could function by forming specific interactions with other proteins. This method has found wide application in demonstrating interactions between Bcl-2 and R-Ras p23 [45], SOS1 and GRB2 [46], Ras and Raf [47,48], and Raf and 14-3-3 [49]. In the present study the two-hybrid system was able to demonstrate PKB/Akts PH domain as interacting specifically with an enzyme involved in the rate-limiting step of de novo guanine biosynthesis, IMPDH [42]. It also required an intact PH domain, and was not conferred by the region (subdomains IV–VI plus a carboxyl-terminal extension) which has previously been shown to bind weakly to the $\beta\gamma$-subunits of heterotrimeric G-proteins [19].

Interestingly, in the two-hybrid system we couldn't detect this interaction when full-length PKB/Akt was used instead of just its PH domain. This could suggest that the PH domain may be interacting with part of the PKB/Akt molecule itself and thus masking its interaction with IMPDH. However, when we tested the ability of PKB/Akts PH domain (amino acids 1–127) to interact with the various PKB/Akt domain constructs in the two-hybrid system we could find no support for such a hypothesis. Recent evidence, however, suggests that there are intramolecular interactions within PKB/Akt [50,51], demonstrated with a PKB/Akt PH domain-containing construct (amino acids 1–147). However, Datta et al. [51] demonstrate a lack of interaction between PKB/Akt and just the PH domain (amino acids 1–109), suggesting the $\sim$40 amino acid carboxyl terminal extension was in part responsible for the binding. Our PH domain constructs contained only half of this extended region, which could explain our inability to find any intramolecular interaction. Alternatively, the inability to observe an interaction of IMPDH type II with full-length PKB/Akt in the yeast two-hybrid system could be due to steric hindrance. The constructs with a domain flanking both ends of the PH domain failed to interact with IMPDH, while those flanked at only one end did. Another possible explanation would be that there is a mode of regulation of the interaction, by an auxiliary molecule or post-translational modification, which is in the non-permissive state in the two-hybrid system when the full-length PKB/Akt molecule is used.

We were able to reproduce this interaction in an in vitro binding system using a GST fusion of IMPDH and a $(His)_6$-

tagged PH domain. This corroboration in a heterologous system lends weight in support of the interaction being biologically significant. This in vitro interaction also appeared to produce a moderate activation of IMPDH. Due to the lack of detectable interaction in the two-hybrid system between full-length PKB/Akt and IMPDH we checked to see if we could detect any such interaction in our in vitro binding system. Indeed, we could show that a baculovirus produced human PKB/Aktα could interact with IMPDH, again supporting a significance of the observed interaction. We could also show that this interaction occurs using an in situ pull-down assay with GST fusions and cell lysates from MCF-7 cells. Further, we have demonstrated an in vivo association of IMPDH and PKB/Akt by co-immunoprecipitation from COS cells expressing a constitutively active form of PKB/Akt. Finally, we were able to show that this constitutively active PKB/Akt could phosphorylate IMPDH in vitro. The above results thus support the view that this novel interaction between human PKB/Aktα and human IMPDH type II is of significance and is promoted by PKB/Akts PH domain.

In our delineation of the region of IMPDH responsible for binding to PKB/Akts PH domain we found we could only delete small regions of the amino- and carboxyl-termini while retaining interaction; producing a core protein of some 453 amino acids being required for the binding. This suggests that the PH domain binding component of IMPDH is composed of disperse elements distributed widely along its primary sequence. These elements may, however, be clustered into a spatially small region in the three-dimensional structure of IMPDH. This contrasts with the binding elements of the SH2 [52] and SH3 [53] domains, which are composed of short peptides of 10–20 amino acids, tyrosine phosphorylated in the case of SH2 and proline rich in the case of SH3. The crystal structure of human IMPDH type II has recently been solved [54] and shows the presence of a catalytic domain (amino acids 1–113 and 232–514) with an internal flanking domain (amino acids 113–232). It is conceivable that the flanking domain could be the region responsible for binding the PKB/Akt PH domain. Small truncations at either end of the IMPDH molecule would affect the catalytic core and may prevent the correct folding of the rest of the molecule including the internal flanking domain. This scenario would be consistent with our observations.

IMPDH is a highly conserved enzyme (41% amino acid identity between bacterial and mammalian sequences) involved in the rate-limiting step of guanine biosynthesis. In mammals there are two isoforms (type I and type II) 84% identical, which are differentially expressed [55]. Type I is constitutively expressed at low levels while the type II protein is up-regulated during cellular proliferation [56]. IMPDH activity levels are also elevated during rapid proliferation in many cells [57].

By measuring the metabolic fluxes, the proliferative index of intact cancer cells has been shown to be linked with the preferential channeling of IMP into guanylate biosynthesis [55]. Inhibition of cellular IMPDH activity results in an abrupt cessation of DNA synthesis and a cell-cycle block at the G_1–S interface leading to apoptosis [58]. The specific inhibition of IMPDH by tiazofurin and the subsequent decline in the GTP pool, results in the down-regulation of the G-protein ras [59], which is involved in many signal transduction pathways leading to cellular proliferation [60]. These results implicate IMPDH as a central player in regulating normal cellular proliferation and the neoplastic state.

Interestingly, p53 has been implicated in regulating IMPDH activity levels [61]. Here, a moderate over-expression of p53 (3–6-fold) induces a profound growth arrest which is rescued by purine nucleotide precursors. Indeed, the p53 over-expression induces a specific block in IMP to XMP conversion, and a diminished activity level of IMPDH. The p53 block doesn't affect the rate of RNA synthesis, nor is the phenotype rescued by deoxynucleotides indicating that a lack of precursors for DNA synthesis is also not the cause of the block. It would seem most likely that this effect is mediated through a down-regulation of the GTP pool required by G-proteins such as ras.

The above observations suggest that IMPDH type II is primarily involved in producing XMP which is then channeled into the GTP pool and is crucial for the regulation of G-proteins involved in signal transduction such as ras. It may be that the type I enzyme provides a basal level of XMP that is channeled into the GTP/dGTP pools required for RNA and DNA synthesis. Changes in IMPDH type II activity would alter the GTP/GDP ratio by specifically altering the GTP component which could greatly affect ras signaling pathways as ras is sensitive to small changes in the GTP/GDP ratio [62]. Thus, the findings presented here, of an interaction between IMPDH type II and the protooncogenic serine/threonine protein kinase PKB/Akt, could have significance in the regulation of ras and other G-protein linked signal transduction pathways. This interaction could also be important for regulation of the apoptotic machinery as both molecules appear to be involved with this mechanism of cell death.

With our finding that a constitutively active form of PKB/Akt could phosphorylate IMPDH in vitro, the potential for PKB/Akt to regulate IMPDH in vivo through phosphorylation must be considered. Interestingly, it has recently been shown [63] that in the pyrimidine biosynthesis pathway of mammalian cells there is a cell-cycle regulation of thymidine kinase by phosphorylation as well as by transcription and translation [64]. Our findings are provocative of suggesting a role of phosphorylation in the regulation of the purine biosynthetic pathway. In conjunction with the known role of IMPDH in GTP biosynthesis our findings suggest a role for PKB/Akt in regulating the GTP pool which is required by signal transducing G-proteins. It is provocative that activation of the PKB/Akt pathway promotes cyclin D1 translation allowing G_1–S phase progression [65], and that IMPDH type II activity is required at the G_1–S interface. Thus, the interplay between PKB/Akt and IMPDH reported here could suggest that PKB/Akt activation leads to IMPDH type II activation which in turn prepares the cell for entry into S phase.

Acknowledgements: The authors wish to thank Dr. F.R. Collart (Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, IL, USA) for the generous gift of the IMPDH rabbit antiserum.

References

[1] Jones, P.F., Jakubowicz, T., Pitossi, F.J., Maurer, F. and Hemmings, B.A. (1991) Proc. Natl. Acad. Sci. USA 88, 4171–4175.
[2] Jones, P.F., Jakubowicz, T. and Hemmings, B.A. (1991) Cell Regul. 2, 1001–1009.
[3] Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y.,

Kameyama, K., Haga, T. and Kikkawa, U. (1995) Biochem. Biophys. Res. Commun. 216, 526–534.
[4] Haslam, R.J., Koide, H.B. and Hemmings, B.A. (1993) Nature 363, 309–310.
[5] Bellacosa, A., Testa, J.R., Staal, S.P. and Tsichlis, P.N. (1991) Science 254, 274–277.
[6] Tyers, M., Rachubinski, R.A., Stewart, M.I., Varrichio, A.M., Shorr, R.G., Haslam, R.J. and Harley, C.B. (1988) Nature 333, 470–473.
[7] Mayer, B.J., Ren, R., Clark, K.L. and Baltimore, D. (1993) Cell 73, 629–630.
[8] Musacchio, A., Gibson, T., Rice, P., Thompson, J. and Saraste, M. (1993) Trends Biochem. 18, 343–348.
[9] Parker, P.J., Hemmings, B.A. and Gierschik, P. (1994) Trends Biochem. 19, 54–55.
[10] Ingley, E. and Hemmings, B.A. (1994) J. Cell. Biochem. 56, 436–443.
[11] Gibson, T.J., Hyvonen, M., Musacchio, A., Saraste, M. and Birney, E. (1994) Trends Biochem. Sci. 19, 349–353.
[12] Frech, M., Ingley, E., Andjelkovic, M. and Hemmings, B.A. (1995) Biochem. Soc. Trans. 23, 616–618.
[13] Baraldi, E., Carugo, K.D., Hyvonen, M., Surdo, P.L., Riley, A.M., Potter, B.V., O'Brien, R., Ladbury, J.E. and Saraste, M. (1999) Structure 7, 449–460.
[14] Yoon, H.S., Hajduk, P.J., Petros, A.M., Olejniczak, E.T., Meadows, R.P. and Fesik, S.W. (1994) Nature 369, 672–675.
[15] Ferguson, K.M., Lemmon, M.A., Schlessinger, J. and Sigler, P.B. (1994) Cell 79, 199–209.
[16] Macias, M.J., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M. and Oschkinat, H. (1994) Nature 369, 675–677.
[17] Ferguson, K.M., Lemmon, M.A., Schlessinger, J. and Sigler, P.B. (1995) Cell 83, 1037–1046.
[18] Koch, W.J., Inglese, J., Stone, W.C. and Lefkowitz, R.J. (1993) J. Biol. Chem. 268, 8256–8260.
[19] Touhara, K., Inglese, J., Pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994) J. Biol. Chem. 269, 10217–10220.
[20] Yao, L., Kawakami, Y. and Kawakami, T. (1994) Proc. Natl. Acad. Sci. USA 91, 9175–9179.
[21] Harlan, J.E., Hajduk, P.J., Yoon, H.S. and Fesik, S.W. (1994) Nature 371, 168–170.
[22] Hyvonen, M., Macias, M.J., Nilges, M., Oschkinat, H., Saraste, M. and Wilmanns, M. (1995) EMBO J. 14, 4676–4685.
[23] Lemmon, M.A., Ferguson, K.M., O'Brien, R., Sigler, P.B. and Schlessinger, J. (1995) Proc. Natl. Acad. Sci. USA 92, 10472–10476.
[24] Garcia, P., Gupta, R., Shah, S., Morris, A.J., Rudge, S.A., Scarlata, S., Petrova, V., McLaughlin, S. and Rebecchi, M.J. (1995) Biochemistry 34, 16228–16238.
[25] Frech, M., Andjelkovic, M., Ingley, E., Reddy, K.K., Falck, J.R. and Hemmings, B.A. (1997) J. Biol. Chem. 272, 8474–8481.
[26] Andjelkovic, M., Alessi, D.R., Meier, R., Fernandez, A., Lamb, N.J., Frech, M., Cron, P., Cohen, P., Lucocq, J.M. and Hemmings, B.A. (1997) J. Biol. Chem. 272, 31515–31524.
[27] Meier, R., Alessi, D.R., Cron, P., Andjelkovic, M. and Hemmings, B.A. (1997) J. Biol. Chem. 272, 30491–30497.
[28] Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B. and Cohen, P. (1997) Curr. Biol. 7, 261–269.
[29] Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F. and Hawkins, P.T. (1997) Science 277, 565–570.
[30] Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) Cell 91, 231–241.
[31] del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997) Science 278, 687–689.
[32] Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C. (1998) Science 282, 1318–1321.
[33] Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E. (1999) Cell 96, 857–868.
[34] Fields, S. and Song, O. (1989) Nature 340, 245–247.
[35] Chien, C.T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) Proc. Natl. Acad. Sci. USA 88, 9578–9582.
[36] Chevray, P.M. and Nathans, D. (1992) Proc. Natl. Acad. Sci. USA 89, 5789–5793.
[37] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) Current Protocols in Molecular Biology, Wiley, New York.
[38] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[39] Davis, L.G., Dibner, M.D. and Batty, J.F. (1986), Basic Methods in Molecular Biology, Elsevier, New York.
[40] Ingley, E. and Hemmings, B.A. (1999) Protein Exp. Purif. 17, in press.
[41] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[42] Collart, F.R. and Huberman, E. (1988) J. Biol. Chem. 263, 15769–15772.
[43] Antonino, L.C. and Wu, J.C. (1994) Biochemistry 33, 1753–1759.
[44] Alessi, D.R. and Cohen, P. (1998) Curr. Opin. Genet. Dev. 8, 55–62.
[45] Fernandez-Sarabia, M.J. and Bischoff, J.R. (1993) Nature 366, 274–275.
[46] Chardin, P., Camonis, J.H., Gale, N.W., van Aelst, L., Schlessinger, J., Wigler, M.H. and Bar-Sagi, D. (1993) Science 260, 1338–1343.
[47] Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Cell 74, 205–214.
[48] Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993) Proc. Natl. Acad. Sci. USA 90, 6213–6217.
[49] Freed, E., Symons, M., Macdonald, S.G., McCormick, F. and Ruggieri, R. (1994) Science 265, 1713–1716.
[50] Hemmings, B.A. (1997) Science 275, 628–630.
[51] 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.
[52] Marengere, L.E., Songyang, Z., Gish, G.D., Schaller, M.D., Parsons, J., Stern, M.J., Cantley, L.C. and Pawson, T. (1994) Nature 369, 502–505.
[53] Finan, P., Shimizu, Y., Gout, I., Hsuan, J., Truong, O., Butcher, C., Bennett, P., Waterfield, M.D. and Kellie, S. (1994) J. Biol. Chem. 269, 13752–13755.
[54] Colby, T.D., Vanderveen, K., Strickler, M.D., Markham, G.D. and Goldstein, B.M. (1999) Proc. Natl. Acad. Sci. USA 96, 3531–3536.
[55] Natsumeda, Y., Ohno, S., Kawasaki, H., Konno, Y., Weber, G. and Suzuki, K. (1990) J. Biol. Chem. 265, 5292–5295.
[56] Konno, Y., Natsumeda, Y., Nagai, M., Yamaji, Y., Ohno, S., Suzuki, K. and Weber, G. (1991) J. Biol. Chem. 266, 506–509.
[57] Collart, F.R., Chubb, C.B., Mirkin, B.L. and Huberman, E. (1992) Cancer Res. 52, 5826–5828.
[58] Cohen, M.B. and Sadee, W. (1983) Cancer Res. 43, 1587–1591.
[59] Olah, E., Natsumeda, Y., Ikegami, T., Kote, Z., Horanyi, M., Szelenyi, J., Paulik, E., Kremmer, T., Hollan, S.R., Sugar, J. and Weber, G. (1988) Proc. Natl. Acad. Sci. USA 85, 6533–6537.
[60] Avruch, J., Zhang, X.F. and Kyriakis, J.M. (1994) Trends Biochem. Sci. 19, 279–283.
[61] Sherley, J.L. (1991) J. Biol. Chem. 266, 24815–24828.
[62] Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) Nature 349, 117–127.
[63] Chang, Z.F., Huang, D.Y. and Hsue, N.C. (1994) J. Biol. Chem. 269, 21249–21254.
[64] Sherley, J.L. and Kelly, T.J. (1988) J. Biol. Chem. 263, 375–382.
[65] Muise-Helmericks, R.C., Grimes, H.L., Bellacosa, A., Malstrom, S.E., Tsichlis, P.N. and Rosen, N. (1998) J. Biol. Chem. 273, 29864–29872.