Sam68 from an immortalised B-cell line associates with a subset of SH3 domains

Peter M. Finan**, Alison Hall, Stuart Kellie*

Yamanouchi Research Institute, Littlemore Hospital, Oxford OX4 4XS, UK

Received 15 February 1996; revised version received 1 May 1996

Abstract The binding of proteins from an immortalised B-cell line to a panel of SH3 domains was investigated in vitro. One of the most prominent SH3 domain binding proteins was a 68 kD polypeptide which strongly associated with the SH3 domains of c-src, p85α and p47^{phox} and weakly with the SH3 domain of PLCγ and n-src with undetectable binding to the other SH3 domains tested. Immunoblotting identified this protein as human Sam68. The ability of proline-rich peptides homologous to the Sam68 primary sequence to inhibit the binding of Sam68 to SH3 domains was investigated. Only one peptide inhibited binding of Sam68 to the p85α SH3 domain, whereas several peptides inhibited binding of Sam68 to c-src SH3 domain, suggesting that Sam68 uses different proline-rich motifs to bind to different SH3 domains. A peptide derived from residues 32–44 of Sam68 which fits the class II SH3 domain binding consensus sequence inhibited binding of Sam68 to both p85α SH3 domain and c-src SH3 domain, but with differential potency, suggesting a differential affinity of these SH3 domains for this proline-rich motif.

Key words: SH3 domain; Sam68; B-cells

1. Introduction

It has become clear that intracellular associations are necessary for signal transduction within cells, and in many cases this is mediated by the binding of specific SH2 domains to defined phosphotyrosine-containing peptide sequences [1,2]. The interaction of distinct SH2 domains with phosphotyrosyl peptides is determined by the residues immediately carboxy terminus to the phosphotyrosine residue, and the binding specificities of over 20 SH2 domains have now been characterised [3–5]. Many signalling molecules also contain SH3 domains; however, less is known about the biological functions and associations of SH3 domains. The first report of an SH3-binding motif described a proline-rich sequence [6], and a number of SH3 ligands have now been identified. All SH3 domain ligands to date have a PXXP motif, and these fall into two general classes: class I ligands contain an Arg residue N-terminal to the PxxP motif, whereas the Arg residue lies C-terminal to the core motif in Class II ligands. Interestingly, PPII helices are pseudo-symmetrical and Class I and Class II ligands have been predicted to fit into the SH3 domain binding site in two orientations [7–9]. SH3 domains are not restricted to signalling molecules, since cytoskeletal proteins such as myosin I and α-spectrin also contain these homologies. In addition the proteins p40^{phox}, p67^{phox} and p47^{phox}, which are cytoplasmic components of the phagocyte NADPH oxidase, contain SH3 domains, the latter two components each having two SH3 domains and one SH3-binding motif, and the cytoplasmic tail of the membrane component of the NADPH oxidase, p22^{phox}, also has an SH3-binding motif [10,11]. These regions are responsible for specific intermolecular interactions which regulate the assembly and activation of this enzyme, leading to the generation of superoxide. Since these components become detergent insoluble after activation, and are regulated by rac, it is possible that they might fit the criteria for cytoskeletal SH3 domain-containing proteins. EBV-transformed B-cells also have an NADPH oxidase identical to that found in phagocytes, but also have a number of signalling molecules and cytoskeletal proteins common to many cells. In an attempt to further investigate the role of SH3 domain-mediated interactions in B-cells we have investigated the ability of proteins from an EBV-transformed B-cell line to bind to distinct SH3 domains and report here the specific interaction of Sam68 with the SH3 domains derived from several proteins including c-src, p85α, PLCγ and p47^{phox}. We have also identified sequences on Sam68 responsible for these interactions.

2. Materials and methods

2.1. Reagents

The bacterial expression vector pGEX-4T-1 was purchased from Pharmacia. Glutathione-agarose beads were obtained from Sigma. Synthetic peptides were synthesised by Zinsser Analytic, Maidenhead, UK and Alta Bioscience, Birmingham, UK. Rabbit polyclonal antisera raised against a carboxy terminal epitope of Sam68 (residues 424–443) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

2.2. Cell culture

HL60 and Namalwa cells were cultured in RPMI 1640 and 10% heat-inactivated fetal calf serum containing 2 mM L-glutamine at 37°C in 5% CO_2. HL60 cells were differentiated into neutrophilic cells by the addition of DMSO to a final concentration of 1.25% for 5 days.

2.3. Glutathione S-transferase fusion proteins

DNA sequences encoding the N-terminal SH3 domains of p47^{phox} (aa 157–215) and p67^{phox} (aa 237–398) were amplified by PCR from a human neutrophil phage library (Clontech) and cloned into the pGEX-4T-1 expression vector. PCR primers corresponding to each end of the SH3 domains contained *Eco*RI and *Sal*I sites (p47N-SH3) or *Eco*RI and *Xho*I sites (p67N-SH3) to facilitate cloning. The pGEX constructs were sequenced using the Sequenase system (US Biochemicals). The remaining pGEX-SH3 constructs have been described previously [11,22]. Constructs were transformed into *Escherichia coli* XL1-Blue and expression of GST fusion proteins performed as described previously [23].

*Corresponding author. Fax: (44) (1865) 748974.
E-mail: skellie@yam-res.co.uk

**Present address: Protein Phosphorylation Laboratory, Imperial Cancer Research Fund Laboratories, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK.

0014-5793/96/$12.00 © 1996 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(96)00552-2

2.4. GST-SH3 binding assays

Affinity matrices were prepared by immobilising 25 μg of fusion protein on 50 μl of glutathione-agarose beads (Sigma). Differentiated HL60 cells and Namalwa cells were pelleted and washed twice in phosphate buffered saline. Cell pellets were solubilised in lysis buffer (50 mM Tris pH 7.5, 5 mM EGTA, 2% (v/v) Triton X-100, 75 mM NaCl, 0.5 mM PMSF) and clarified by centrifugation at $14000 \times g$ for 15 min at 4°C. Cell lysates from the equivalent of 5×10^6 cells for silver staining or 10^6 cells for Western blotting were mixed with the GST-SH3 affinity matrices for 3 h at 4°C. The beads were then washed extensively in wash buffer (50 mM Tris pH 7.5, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol). Binding proteins were eluted by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE and visualised by immunoblotting with an antibody to Sam68 or by silver staining. In addition, binding assays were performed in the presence of proline-rich synthetic peptides at concentrations indicated in the relevant figure legends.

3. Results

Initial experiments were performed to compare the SH3 domain-binding protein profiles of two haemopoietic cell lines: neutrophil-differentiated HL60 cells and Namalwa B-cells. Triton X-100 lysates were prepared from these cells and the proteins allowed to bind to immobilised SH3 domains. Fig. 1A shows the SH3 domain-binding protein profiles visualised by silver staining. Whilst there were several differences between the binding of HL60 proteins and Namalwa proteins, the most prominent was a 68 kD polypeptide from Namalwa cells which strongly associated with the SH3 domains of p85α and $p47^{phox}$ and weakly with the SH3 domain of PLCγ. This was characterised using a battery of antibodies against 68 kD proteins, and p68 was found to cross-react with an antibody against Sam68 [18,19]. The identity of p68 as Sam68 was confirmed by Western blotting of the SH3-binding proteins, showing the SH3-binding profile of Sam68 was identical to that of p68 visualised by silver staining (Fig. 1B). The specificity of Sam68 binding was further investigated by studying its association to a larger panel of SH3 domains. Western blotting of bound proteins revealed that there was intense staining of Sam68 bound to the SH3 domains from c-src, p85α, c-fgr and the N-terminal SH3 domain of $p47^{phox}$. There was less intense staining to the SH3 domains of n-src and PLCγ, and binding was undetectable to the other SH3 domains tested (Fig. 2), indicating that Sam68 from Namalwa cells bound to a restricted set of SH3 domains.

The absence of Sam68 binding from HL60 lysates was investigated. Both Namalwa cells and HL60 cells contained comparable amounts of Sam68 when total cell lysates were blotted and in fact contained higher levels than human fibroblasts (Fig. 3). However, only the Triton X-100 lysate from Namalwa cells contained Sam68 (Fig. 1B, lanes marked LYS), therefore the apparent absence of Sam68 binding in HL60 lysates was due to the lack of solubility of Sam68 from HL60 cells into Triton-containing buffer. The reason for this differential extraction is unknown but it is possible that there are functional differences between Sam68 from these two cell

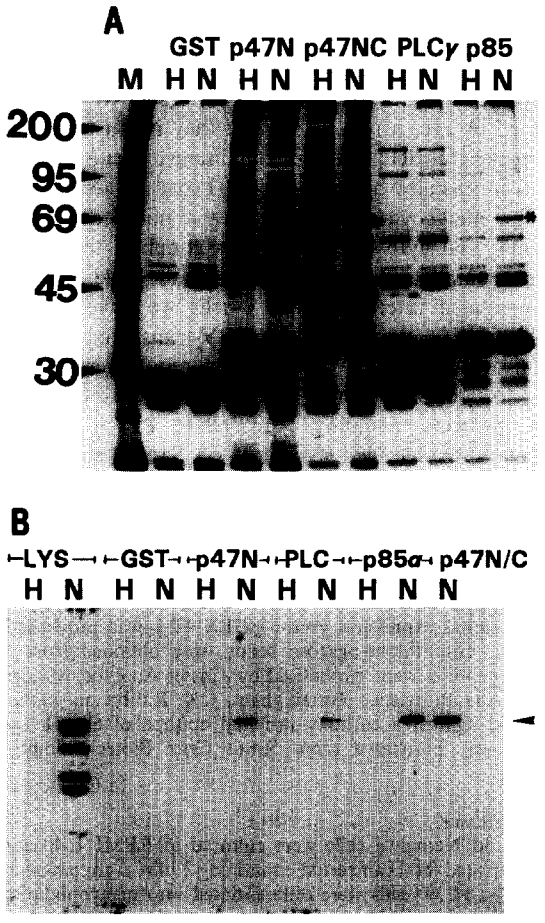

Fig. 1. (A) In vitro binding of detergent-soluble proteins from Namalwa cells (N) or differentiated HL60 cells (H) to immobilised GST-SH3 fusion proteins. Lysates were incubated with glutathione-agarose beads bound with GST or GST fusion proteins containing the N-terminal SH3 domain of $p47^{phox}$ (p47N), the tandem SH3 domains of $p47^{phox}$ (p47NC), the phospholipase Cγ SH3 domain (PLCγ) or the SH3 domain of the p85α subunit of phosphatidylinositol 3-kinase (p85). Bound proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE and visualised by silver staining. (B) Identification of p68 as Sam68 by Western blotting. SH3 domain-binding proteins were purified as described for (A) Following SDS-PAGE, proteins were transferred to nitrocellulose and probed with an antibody to Sam68 as described in Section 2. The arrowhead shows the position of Sam68.

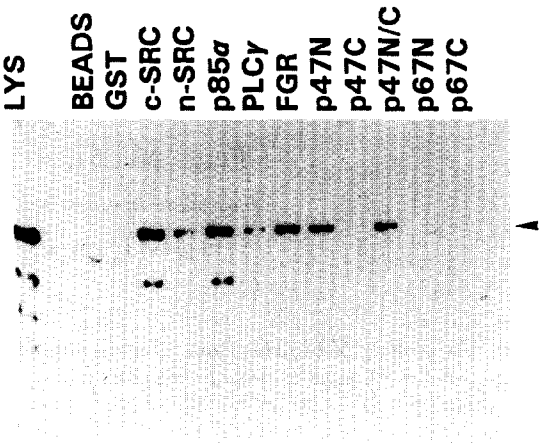

Fig. 2. In vitro binding of Sam68 to a panel of immobilised SH3 domains. Triton X-100 lysates of Namalwa cells were bound to GST-SH3 domains and Western blotting with anti-Sam68 performed as described for Fig. 1. Additional tracks are: LYS, total Triton X-100 cell lysate before binding; BEADS, glutathione beads with no immobilised fusion GST fusion protein; GST, immobilised GST; c-SRC, the SH3 domain of c-src; n-SRC, the SH3 domain of neuronal (n)-src; FGR, the SH3 domain of c-fgr; p67N, the N-terminal SH3 domain of $p67^{phox}$; p67C, the carboxy-terminal domain of $p67^{phox}$. The arrowhead shows the position of Sam68.

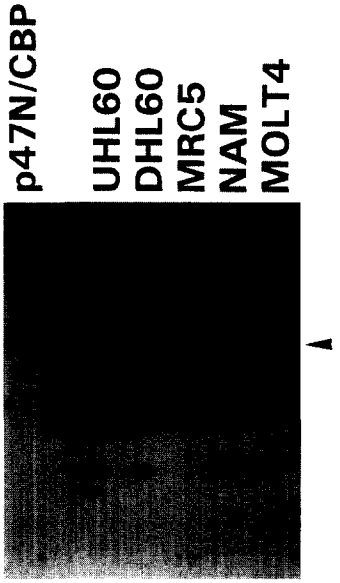

Fig. 3. Detection of Sam68 in haemopoietic cells. Cell pellets were solubilised into SDS-PAGE sample buffer and the extracted proteins subjected to Western blotting with anti-Sam68. p47/CBP, tandem $p47^{phox}$ SH3 domain binding protein; UHL60, undifferentiated HL60 cells; DHL60, differentiated HL60 cells; MRC5, MRC5 human fibroblasts; NAM, Namalwa cells; MOLT4, human T-cells. The arrowhead shows the position of Sam68.

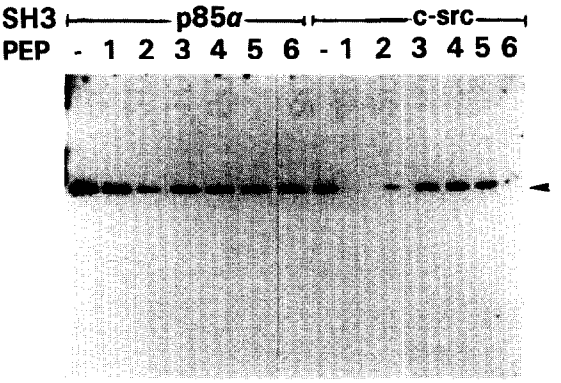

Fig. 4. Inhibition of Sam68 binding to p85α or c-src SH3 domains by proline-rich peptides derived from Sam68 sequence. Namalwa cell lysates were incubated with p85α or c-src SH3 domains in the presence of 500 μM Sam68-derived peptides as described in Table 1 and Sam68 binding detected by Western blotting. The arrowhead shows the position of Sam68.

types, related to cell proliferation or other aspects of activation such as superoxide production.

Several proline-rich putative SH3 binding domains were identified in Sam68. Six peptides corresponding to these regions were synthesised (see Table 1) and their ability to block the binding of Sam68 to immobilised SH3 domains was examined. At 500 μM only peptide P2 significantly inhibited the binding of Sam68 to p85α, whereas peptides P1, P2 and P6 inhibited the binding of Sam68 to c-src SH3 domain (Fig. 4). Peptide P2 appeared to inhibit the binding of Sam68 to c-src greater than to p85α SH3 domain, and this was confirmed by dose-response experiments. Whilst the IC_{50} of peptide P2 for Sam68 binding to c-src SH3 was < 50 μM, the IC_{50} of peptide P2 for Sam68 binding to p85α SH3 was > 400 μM (Fig. 5), which suggests that c-src SH3 domain binds to this proline-rich motif with greater affinity than p85α SH3 domain.

4. Discussion

We have identified a 68 kD SH3 domain-binding protein from Namalwa cells as Sam68 [18,19]. The cDNA for Sam68 was originally thought to encode a 62 kD RasGAP-associated protein (p62); however, recent experiments have confirmed that this cDNA does indeed encode the 68 kD Sam68 protein and not p62. The cDNA for p62 has yet to be cloned and is unlikely to be related to Sam68 [18]. Sam68 was originally identified as a c-src-binding protein in fibroblasts whose association was upregulated during mitosis [13,14] or oncogenic transformation [15]. This association was shown to be dependent on tyrosine phosphorylation of the carboxy-terminal domain which then directs binding of Sam68 to specific SH2 domains of several proteins including src, fyn, lyn, RasGAP, p85α, and grb2 [13–17]. In contrast to SH2 domain binding, Sam68 from either asynchronous or mitotic fibroblasts binds constitutively to the SH3 domain of c-src [13,14], thus the SH3 domain association of Sam68 may be functionally distinct from its SH2 domain binding. Whilst Sam68 has homology to heteroribonucleoprotein K (hnRNPK), and can bind to RNA in vitro, its function within cells at present is unknown [12,13,18,19]. Despite earlier reports it does not associate with RasGAP [20], and it has now been shown that p62 and Sam68 are distinct unrelated proteins [14,18]. The results presented here demonstrating the binding of Sam68 from asynchronous transformed B-cells to distinct SH3 domains are consistent with previous findings in fibroblasts, and extend previous studies to show constitutive binding to a number of SH3 domains. Furthermore, Sam68 from growing B-cells had different extraction properties from nonproliferating differentiated HL60 cells. Haemopoietic cells appeared to express more Sam68 than fibroblasts and the reason for this is unknown. Namalwa Sam68 bound with apparently high efficiency to the SH3 domains of c-src, p85α and fgr, and bound with lower apparent efficiency to the SH3 domain of PLCγ. This is in broad agreement with a previous study [17], with the exception that no binding of Sam68 to the SH3 domain of fgr

Table 1
Summary of the inhibition of binding of Sam68 to SH3 domains by proline-rich peptides derived from human Sam68 sequence

Peptide	Sequence (Res)	Inhibition of: p85SH3/Sam68	c-srcSH3/Sam68
P1	ASPATQPPPLLPPSA (57–71)	–	+++
P2	QTPSRQPPLPHRS (32–44)	+	++
P3	GGPAPTPLLPPSA (79–91)	–	–
P4	RIPLPPPPAPET (354–365)	–	–
P5	AAPPPPPVPRG (293–303)	–	–
P6	TRGVPPPPTVRGAPA (330–344)	–	+

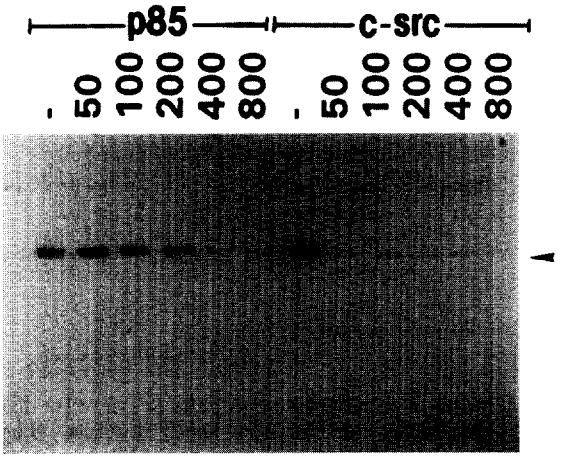

Fig. 5. Dose response of inhibition of Sam68 binding to p85α or c-src SH3 domains in the presence of increasing concentrations of peptide P2. See Fig. 4 for details of methodology.

was found. Others have also reported binding of a p62-related protein to PLCγ [16,21]. In addition we have shown that Sam68 associates with the N-terminal SH3 domain of $p47^{phox}$. The significance of this is unknown; however, $p47^{phox}$ is a component of the phagocyte NADPH oxidase complex, which is also functional in Namalwa cells (P. Bennett and S. Kellie, unpublished).

The use of proline-rich peptides based on the Sam68 sequence has allowed partial elucidation of the SH3 domain binding sites. Namalwa Sam68 binding to p85α SH3 domain could be inhibited only by peptide P2, whereas binding of Sam68 to c-src SH3 domain was inhibited by peptides P1, P2 and P6, suggesting that Sam68 uses only residues 32–44 to bind to p85α SH3 but uses this motif plus at least two others for binding to c-src SH3. In agreement with our results that peptide P6 encompassing residues 330–344 inhibits Sam68 binding to c-src SH3 is the report that Sam68 deleted in this region is partially defective in c-src SH3 binding [16]. Peptide P2, which inhibited binding to both c-src and p85α SH3 domains contains the sequence ..QPPLPHR.., which fits exactly the XPXL/VPXR sequence of class II SH3-binding motifs, suggesting that at least one of the Sam68 motifs might bind both SH3 domains in reverse orientation [7–9]. A recent report has described a peptide homologous to our P4 which partially inhibits Sam68 binding to c-src SH3 domain and a longer peptide with homology to P5 which blocked binding of Sam68 to c-src SH3 [17]; however, these differences may be due to different experimental techniques since these workers used in vitro translated Sam68 for binding studies whereas we have used Sam68 from cell lysates, which might be post-translationally modified or differently folded to confer different binding characteristics, for example by masking specific proline-rich motifs. However, we unequivocally demonstrate in this report that a peptide comprising residues 32–44 strongly inhibits the association of Sam68 with c-src SH3 domain. Whilst a similar peptide was reported to be ineffective [17], in that report the peptide tested was truncated before the arginine residue, so destroying the class II binding motif. This same sequence is mutated to ..PSPLPHR.. in murine Sam68, again destroying the class II binding motif, and this might explain the failure to detect this as a functional binding motif in murine Sam68 [16]. In summary, this paper describes an interaction between Sam68 from B-cells and specific SH3 domains, including a component of the NADPH oxidase, and further defines a putative novel binding site for the association of Sam68 with the SH3 domains of c-src and p85α.

Acknowledgements: We are grateful to I. Gout and M.D. Waterfield for PGEX vectors containing c-src, n-src, p85α, PLCγ and fgr, and to L. Wilson and J. Lackie for helpful discussions.

References

[1] Cohen, G.B., Ren, R. and Baltimore, D. (1995) Cell 80, 237–248.
[2] Pawson, T. and Schlessinger, J. (1993) Curr. Biol. 3, 343–442.
[3] Panoyotou, G., Gish, G., End, P., Truong, O., Gout, I., Dhand, R., Fry, M.J., Hiles, I., Pawson, T. and Waterfield, M.D. (1993) Mol. Cell. Biol. 13, 3567–3576.
[4] Songyang, Z., Shoelson, S.E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X.R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., et al. (1994) Mol. Cell. Biol. 14, 2777–2785.
[5] Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser W.G. et al. (1993) Cell 72, 767–778.
[6] Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D. (1993) Science 259, 1157–1161.
[7] Lim, W.A., Richards, F.M. and Fox, R.O. (1994) Nature 372, 375–379.
[8] Feng, S., Chen, J.K., Yu, H., Simon, J.A. and Schreiber, S.L. (1994) Science 266, 1241–1247.
[9] Yu, H., Chen, J.K., Feng, S., Brauer, A.W. and Schreiber, S.L. (1994) Cell 76, 933–945.
[10] McPhail, L.C. (1994) J. Exp.Med. 180, 2011–2015.
[11] Finan, P.M., 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.
[12] Wong, G., Muller, O., Clark, R., Conroy, L., Mora, N.F., Polakis, P. and McCormick, F. (1992) Cell 69, 551–558.
[13] Taylor, S.J. and Shalloway, D. (1994) Nature 368, 867–871.
[14] Fumigalli, S., Totty, N.F., Hsuan, J.J. and Courtneidge, S.A. (1994) Nature 368, 871–874.
[15] Weng, Z., Thomas, S.M., Rickles, R.J., Taylor, J.A., Brauer, A.W., et al. (1994) Mol. Cell. Biol. 14, 4509–4521.
[16] Richard, S., Yu, D., Blumer, K.J., Hausladen, D., Olsowsky, M.W., Connelly, P.A. and Shaw, S.S. (1995) Mol. Cell. Biol. 15, 186–197.
[17] Tayler, S.J., Anafi, M., Pawson, T. and Shalloway, D. (1995) J. Biol. Chem. 270, 10120–10124.
[18] Lock, P., Fumigalli, S., Polakis, P., McCormick, F. and Courtneidge, S.A. (1996) Cell 84, 23–24.
[19] Courtneidge, S.A. and Fumigalli, S. (1995) Trends Cell Biol. 4, 345–347.
[20] Ellis, C., Moran, M., McCormick, F. and Pawson, T. (1990) Nature 343, 377–381.
[21] Maa, M.C., Leu, T.H., Trandel, B.J., Chang, J.H. and Parsons, S.J. (1994) Mol. Cell. Biol. 14, 5466–5473.
[22] Gout, I., Dhand, R., Hiles, I.D., Fry, M.J., Panayatou, G., Das, P., Truong, O., Totty, N.F., Hsuan, J., Booker, G.W., Campbell, I.D. and Waterfield, M.D. (1993) Cell 75, 25–36.
[23] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.