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Structure of 85 kDa Subunit of Human Phosphatidylinositol 3-Kinase Analyzed by Using Monoclonal Antibodies

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An 85 kDa subunit (p85α) of phosphatidylinositol 3-kinase (PI-3K) has one SH3 and two SH2 regions [SH2(N) and SH2(C)], which direct protein-protein interaction. We have established eighteen hybridomas producing monoclonal antibodies against p85α to study the structure-function relationship of this protein. Epitope mapping using a series of deletion mutants expressed in *E. coli* showed that the monoclonal antibodies bound to at least 5 distinct epitope regions, which were well dispersed on p85α except for its carboxyl-terminus. Monoclonal antibodies against amino-terminal regions and polyclonal antibodies against carboxyl-terminal regions immunoprecipitated p85α expressed in human cells and in *E. coli*. On the other hand, monoclonal antibodies against the central part of p85α failed to immunoprecipitate p85α efficiently; however, they could immunoprecipitate p85α mutants with deletion of either the amino- or the carboxyl-terminal region. Similar results were obtained by immunocytochemistry using confocal microscopy. These results suggested that steric hindrance prevents binding of monoclonal antibodies to the central part of p85α where SH2(N) is located. The SH2(N) may have a distinct function from SH2(C), which is located at the carboxyl-terminal region and has been shown to mediate the binding of PI-3K to activated growth factor receptors.

Key words: Phosphatidylinositol 3-kinase — Signal transduction — Monoclonal antibody

Phosphatidylinositol-3 kinase (PI-3K[6]) was first reported as a phosphatidylinositol kinase activity associated with polyoma middle-T antigen/pp60$^{c\text{-}src}$ complex.[1] Unlike classical phosphatidylinositol kinase which phosphorylates the D-4 position of the inositol ring, PI-3K phosphorylates the D-3 position of PI, PI-4P, and PI-3,4P_2 to yield PI-3P, PI-3,4P_2, and PI-3,4,5P_3, respectively,[2,3] the biological roles of which are still unknown. The products of PI-3K are not hydrolyzed by known phosphoinositide-specific phospholipase C's (PLC),[4] suggesting that PI-3K regulates a signaling pathway distinct from the PLC-mediated protein kinase C/calcium ion system in the intact cells.

There is substantial evidence that PI-3K is involved in signal transduction via tyrosine kinases. PI-3K phosphorylated on tyrosines by receptor-type tyrosin kinases such as platelet-derived growth factor (PDGF) receptor, insulin receptor, colony stimulating factor-1 receptor, epidermal growth factor receptor and hepatocyte growth factor receptor, and appears to be activated upon ligand stimulation.[5–13] PI-3K is also tyrosine-phosphorylated by non-receptor type tyrosine kinases such as v-Src, v-Abl, v-Fms, v-Ros, v-Yes, and v-Fps.[14–19] We and others have reported that PI-3K is involved in signaling mediated by membrane-bound immunoglobulins or by IL-2 receptors in lymphocytes and also that c-Lyn or c-Fyn are associated with PI-3K activities.[20–24]

Purification of PI-3K from various sources[25–27] has demonstrated that this enzyme consists of two subunits, 110 kDa and 85 kDa proteins (p110 and p85), which are the catalytic and regulatory subunits, respectively. cDNAs of the 85 kDa protein have been isolated from bovine, murine, and human cells, and indicate that two forms of p85, α-type (p85α) and β-type (p87β), are present in mammalian cells.[28–30] Amino acid sequence analysis showed that both p85α and p85β contain SH2/SH3 regions which are regions conserved among proteins involved in signal transduction, such as phospholipase C-γ (PLC-γ), *ras* GTPase activating protein (GAP), non-receptor type tyrosine kinases, protein tyrosine phosphatase 1C, *akt* serine-threonine kinase, and the *crk* oncogene product.[31–39] The role of the SH2 sequence has been studied extensively. We found that SH2 sequences bind to a variety of phosphotyrosine-containing peptides.[40] This finding suggests that the signaling molecules

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[6] Abbreviations: PI-3K, phosphatidylinositol 3-kinase; p85α, 85 kDa α-type subunit; p87β, 87 kDa β-type subunit; PLC, phospholipase C; GAP, *ras* GTPase activating protein; GST, glutathione *S*-transferase; MAb, monoclonal antibody.

with SH2 domains may bind to autophosphorylated growth factor receptors upon ligand stimulation. The receptor may then phosphorylate the signaling molecules to activate them.

The mode of association of PI-3K with PDGF receptor has been studied extensively. PI-3K binds to the PDGF receptor at a peptide sequence that includes a phosphorylated tyrosine residue, 751, which lies within the kinase insert region of the protein-tyrosine kinase domain.[41] The carboxyl-terminal SH2 region [SH2(C)] of p85α has higher affinity for the receptor than the amino-terminal SH2 region [SH2(N)].[42-45]

Here we report the establishment of hybridomas producing monoclonal antibodies (MAbs) against p85α. These MAbs have been characterized and found to be versatile for studies of the tissue distribution and function of PI-3K.

MATERIALS AND METHODS

Cloning and expression of the cDNA for human PI-3K p85α The 5′ and 3′ halves of the cDNA coding for PI-3 kinase were amplified from total RNA of HeLa cells by the reverse transcriptase-mediated polymerase chain reaction (PCR) technique with synthetic oligomers, CAGATTTGCAAACATGAGTGCTGAG, CAGAGAAGCCATATTTCCCATCTCG, ACTAAAATGCATGGTGATTATACTC, and AGTAAGCGCTTCATCGCCTCTGCTGTGC, used as primers. The PCR products were cloned into the *Hinc*II site of the pUC19 vector. The sequence of the inserts was identical to that reported previously,[30] except that A at the nucleotide position 1031 in their numbering system was substituted by G, and A at the nucleotide position 1599 by G. The former resulted in a change in amino acid from Asn to Asp. Because this substitution was found in all of the clones isolated independently, we concluded that this was not an artifact produced by the PCR technique, but that the gene of HeLa cells has this substitution. The two halves of the p85α cDNA were combined at the *Vsp*I site found at the center of the coding sequence.

The p85α cDNA was transferred into the expression vector pGEX2T (Pharmacia) between the *Sma*I and *Eco*RI sites to form pG85α. *E. coli* JM109 was used for gene expression. A fusion protein containing glutathione *S*-transferase (GST) and p85α was produced from pG85α, purified over glutathione-Sepharose beads, and cleaved at the fused site with thrombin as described elsewhere.[46]

p85α mutants for epitope mapping We prepared a series of deletion mutants of p85α as fusion proteins with GST to determine the epitope regions of MAbs. Plasmids for the expression of these mutants were constructed by a combination of restriction enzyme cleavage and ligation. The amino acid sequences present in mutants are as follows: ΔSna, 81–724; ScaL, 264–724; ΔRV, 341–724; PvuS, 573–724; ScaPvu, 264–562; ΔXhoSau, 1–338 and 434–724; ΔDraPvu, 1–512 and 573–724; PvuL, 1–562.

Preparation of monoclonal antibodies Hybridomas were prepared according to standard protocols.[47,48] Female BALB/c mice were immunized with the recombinant human p85α expressed in *E coli*. Spleen cells of immunized mice and SP2/O myeloma cells were fused with polyethyleneglycol. Hybridomas were selected with HAT medium as described previously.[49] Tissue culture supernatants of the hybridomas were examined for reactivity with the recombinant p85α and GST by enzyme-linked immunosorbent assay (ELISA). Hybridomas positive for p85α and negative for GST were further cloned by the limiting dilution method to homogeneity. Isotype of specific antibodies secreted from the cloned hybridomas was determined by using a mouse monoclonal antibody isotyping kit (Amersham Corp.).

Immunoblotting 143B human osteosarcoma cells (ATCC CRL 8803) and COS7 monkey kidney cells (ATCC CRL 1651) were washed with Dulbecco's phosphate-buffered saline (PBS) and lysed immediately with Laemmli's buffer.[50] *E. coli* expressing p85α was lysed in RIPA buffer (10 m*M* Tris hydrochloride, pH 7.5, 5 m*M* EDTA, 150 m*M* NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 50 μM Na_3VO_4, 5 μM Na_2MoO_4, 100 m*M* NaF, 1 m*M* PMSF, and 10 μg/ml aprotinin). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDE membranes (Millipore Corp.). The membranes were pre-incubated with PBS containing 2% skim milk and 0.05% Tween 20 for 1 h, and then with the culture supernatants of the hybridomas for 2 h. The membranes were subsequently rinsed in PBS containing 0.05% Tween 20, incubated with anti-mouse antibody conjugated with alkaline phosphatase (TAGO Inc.) for 1 h and washed in PBS. The antibodies bound to the membrane were visualized with the chromogenic substrate, bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT).[48] In some experiments, peroxidase-conjugated anti-mouse antibody (TAGO Inc.) and the ECL chemiluminescence system (Amersham Corp.) were used for detection of mouse antibodies.

Transient expression of p85α and p87β in COS7 cells COS7 cells were transfected with the expression vectors for p85α, p87β (kindly provided by Dr. M. Waterfield) and p85α mutants, which were derived from a pcDL-SRα296(*Bgl*II) replication-competent expression vector[51] by the DEAE-dextran method.[52] Cells were analyzed by immunoblotting 48 h after transfection.

Immunoprecipitation 143B cells were lysed RIPA buffer. Cell lysates containing 50 μg of protein were incubated with 50 μl of culture supernatants of hybridomas for 2 h. Proteins bound to antibodies were precipitated with

Protein G Sepharose 4B (Pharmacia), separated by 8% SDS-PAGE, transferred to PVDF membrane, and probed with anti-p85α rabbit antibody raised against the recombinant p85α expressed in *E. coli*. Antibodies bound to the membrane were visualized as described above.

In some experiments, 143B cells grown on 6 cm dishes were labeled with 200 μCi of [^{35}S]methionine in 2 ml of methionine-free MEM and immunoprecipitated. Proteins were dissociated from Protein G-Sepharose in 30 μl of 8 *M* urea, diluted in RIPA buffer and immunoprecipitated again with the addition of antibodies and Protein G-Sepharose, separated on 7% SDS-PAGE, and detected by fluorography.

PI-3 kinase assay PI-3 kinase assay was carried out as described previously[17] except for washing of immunoprecipitants with PBS.

Immunocytochemistry COS7 cells were transfected with the p85α expression vector. Twenty-four hours later, cells were replated on glass slides coated with fibronectin. Forty-eight hours after transfection, cells were fixed either in PBS containing 4% formalin for 5 min at 25°C, in 95% methanol-5% acetone for 5 min at 4°C, in 80% methanol for 30 min at 4°C, or in acetone for 5 min at 4°C. When cells were fixed in 4% formalin, they were permeabilized for 5 min either in PBS containing 0.2% Triton X-100 or methanol. Cells were incubated with hybridoma supernatants for 2 h at 25°C and washed in PBS, followed by incubation with FITC-conjugated anti-mouse immunoglobulin antibody (TAGO). We used confocal microscopy (BioRad) to observe the cells.

RESULTS

Generation and screening of anti-p85α MAbs Human p85α was expressed in *E. coli* and purified as described in "Materials and Methods." We used this recombinant p85α to immunize BALB/c mice and to screen hybridomas by ELISA.

Tissue culture supernatants of eighteen hybridomas which were positive for the recombinant p85α by ELISA were further examined for specificity by immunoblotting using a total cell lysate of the 143B human osteosarcoma cell line. An 85 kDa band was detected with all the culture supernatants positive on ELISA (Fig. 1). Ten of the hybridomas also detected another protein which ran slightly more slowly than p85 in SDS-PAGE; this protein appeared to be p87β, as will be described later.

Cross-reactivities of anti-p85α MAbs with p87β In an attempt to examine whether the 87 kDa band detected by immunoblotting might be p87β, cDNAs coding for p85α and p87β were expressed in COS7 cells (Fig. 2). MAb AB6, which detected a single band at 85 kDa, reacted only with p85α expressed in COS7 cells (lanes 1 to 3). In contrast, MAb CB2, which detected two bands at 85 and

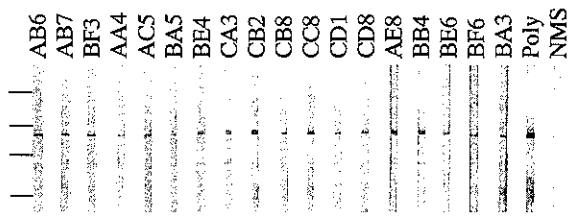

Fig. 1. Specific recognition of p85α by anti-p85α MAbs. 143B human osteosarcoma cells were directly lysed with Laemmli's buffer, separated by 8% SDS-PAGE, transferred to PVDF membrane, and probed with the culture supernatants of hybridomas. Bound antibodies were detected with the anti-mouse goat antibody conjugated with alkaline phosphatase and BCIP/NBT as substrates. The names of hybridomas are indicated at the top. Poly: polyclonal mouse antibody against p85α ($\times$1,000 dilution). NMS: normal mouse serum ($\times$1,000). Bars indicate the positions of prestained molecular weight markers 0f 200, 97, 68, and 46 kDa.

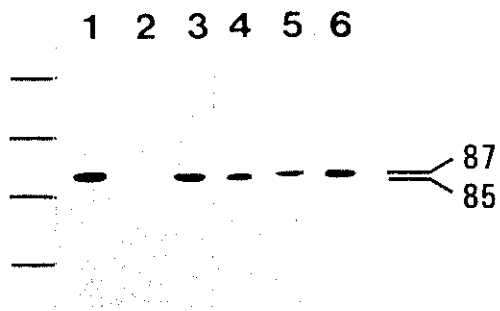

Fig. 2. Cross-reactivity of anti-p85α MAbs with p87β. p85α and p87β expressed in COS7 cells were analyzed by immunoblotting as described in the legend to Fig. 1, except that they were visualized with an ECL chemiluminescence system. MAbs AB6 (lanes 1, 2, 3) and CB2 (lanes 4, 5, 6) were used as probes. Lanes 1 and 4, p85α; lanes 2 and 5, p87β; lanes 3 and 6, mixture of p85α and p87β. Bars indicate the positions of prestained molecular weight markers of 200, 116, 80, and 50 kDa.

87 kDa, reacted with both p85α and p87β expressed in COS7 cells (lanes 4 and 5). We could not distinguish two bands corresponding to p85α and p87β in lane 6, probably due to abundunt expression of these proteins in COS7 cells. The p85α and p87β expressed in COS7 cells co-migrated with the 85 and 87 kDa proteins of 143B cells (data not shown), strongly suggesting that these are identical proteins.

Epitope mapping of anti-p85α MAbs The reactivity of the anti-p85α MAbs in immunoblotting experiments suggests that these MAbs recognize the primary amino acid

sequences. This enabled us to determine the epitope regions for the MAbs on seven mutant p85α proteins expressed in *E. coli* (Fig. 3a). According to the structures of these mutant proteins, the epitope regions were arbitrarily divided into seven regions, designated as regions A to G. Their amino acid positions were as follows: A, 1–79; B, 81–262; C, 264–338; D, 339–429; E, 431–512; F, 513–562; G, 564–724. Epitopes for the MAbs were mapped in five regions (A, B, C, D, and F) according to the patterns of immunoblotting (Fig. 3b), however, it is possible that some of these MAbs recognize boundaries of these epitope regions.

Immunoprecipitation of native p85α with the anti-p85α MAbs We examined the reactivity of the anti-p85α MAbs to the native p85α in eukaryocytes using immunoprecipitation (Fig. 4a, Table I). Saturating amounts of MAbs were incubated with 50 μg of 143B cell lysate. All MAbs against epitope regions A and B immunoprecipitated p85α, although the efficiency was slightly different with each MAb. The MAbs against epitope regions C, D, and F did not immunoprecipitate p85α. To examine whether the antibodies against the carboxyl-terminus of p85α (epitope G) could immunoprecipitate p85α, we prepared polyclonal mouse antibody by immunization of PvuS protein expressed in *E. coli* (epitope G, Fig. 3a). This antibody precipitated p85α from COS7 cells expressing human p85α cDNA (Fig. 4b, lane 5) and from 143B cells (data not shown) as did MAbs against epitope regions A and B (Fig. 4b, lanes 1 and 2). Neither mouse polyclonal antibody against GST (lane 6) nor MAbs against regions C and D (lanes 3 and 4) immunoprecipitated p85α. This result suggests that the central region of p85α is not exposed in the native conformation, possibly owing to the steric hindrance of p110 or of the other region of p85α itself.

Immunoprecipitation of p85α expressed in *E. coli* with the anti-p85α MAbs We utilized mutant p85α proteins expressed in *E. coli* (Fig. 3a) to examine the possibility that the central region of p85α was masked by other domains of p85α itself. MAbs against regions A and B and GST immunoprecipitated the authentic fusion protein which consisted of p85α and GST (Fig. 5, lanes 1, 2 and 5). MAbs against regions C and D (lanes 3 and 4) did not immunoprecipitate the p85α. Deletion of the A

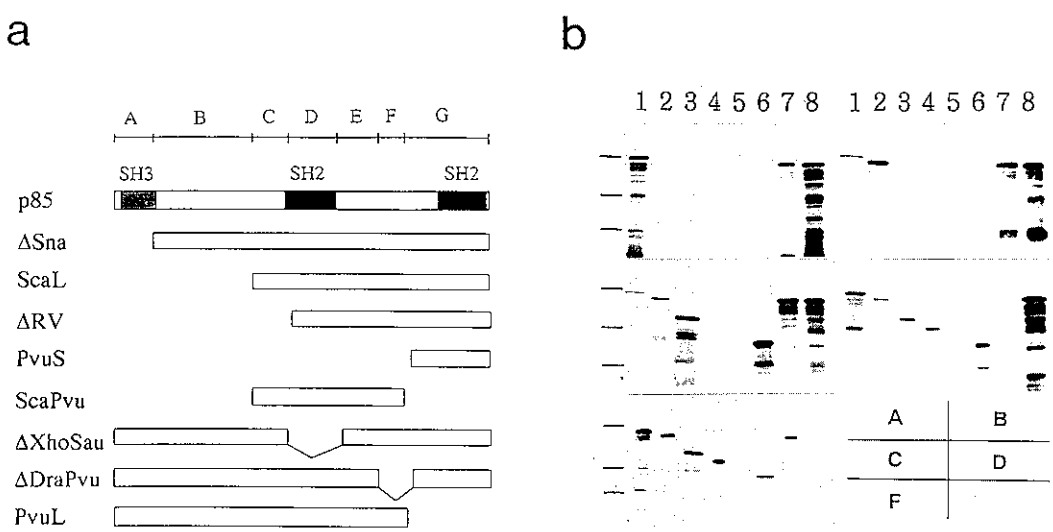

Fig. 3. Epitope mapping of anti-p85α MAbs. a, p85α was arbitrarily divided into seven epitope regions based on the restriction sites, A to G, as indicated at the top. One SH3 and two SH2 regions are shown as a hatched box and closed boxes, respectively. Seven deletion mutants were used for epitope mapping (ΔSna, ScaL, ΔRV, PvuS, ScaPvu, ΔXhoSau, and ΔDraPvu). PvuL was used in the experiments described in the legend to Fig. 5. The precise amino acid positions of mutant p85α are described in "Materials and Methods." b. p85α and the mutants were expressed in *E. coli* as fusion proteins with GST. Cells were lysed with Laemmli's buffer, separated by 8% SDS-PAGE, transferred to PVDF membrane, and probed with MAbs. An ECL chemiluminescence system was used for detection of bound antibodies. MAbs were divided into five groups based on their reactivity to the mutant p85α's. Typical patterns of immunoblotting for each epitope region are shown here. MAbs AB6 (panel A), CC8 (panel B), BB4 (panel C), BE6 (panel D) and BA3 (panel F) were used as the representatives of epitope regions A, B, C, D and F, respectively. The position of the panels are shown in the right side of the lower part. Lane 1, p85α; lane 2, ΔSna; lane 3, ScaL; lane 4, ΔRV; lane 5, PvuS; lane 6, ScaPvu; lane 7, ΔXhoSau; lane 8, ΔDraPvu. Bars indicate the positions of prestained molecular weight markers of 116, 80, and 50 kDa.

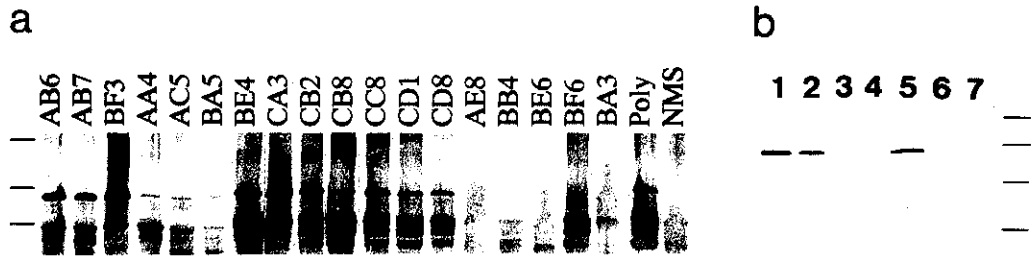

Fig. 4. Immunoprecipitation of human p85α by anti-p85α MAbs. a, Lysates of 143B human osteosarcoma cells were immunoprecipitated with the MAbs and polyclonal mouse sera shown at the top. Poly: mouse polyclonal antibody against p85α. NMS: normal mouse serum. The bound proteins were separated by 8% SDS-PAGE and probed with a rabbit anti-p85α antibody, followed by anti-rabbit goat antibody conjugated with alkaline phosphatase and BCIP/NBT. Bars indicate the positions of prestained molecular weight markers of 200, 97, and 68 kDa, b, COS7 cells expressing p85α were labeled with [^{35}S]methionine, lysed with RIPA buffer, and precipitated with anti-p85α antibodies: AB6 (epitope A, lane 1), CC8 (epitope B, lane 2), BB4 (epitope C, lane 3), BE6 (epitope D, lane 4), anti-C-terminus polyclonal antibody (epitope G, lane 5), anti-GST antibody (lane 6), and anti-feline immunodeficiency virus MAb (lane 7). Bars indicate the positions of molecular weight markers at 116, 93, 68, and 46 kDa.

Table I. Characterization of Monoclonal Antibodies against p85α

Clone	Epitope region[a)]	Immuno-precipitation[b)]	Binding to p110	Cross-reactivity with β	Isotype
AB6	A	++	+	−	IgG1, κ
AB7	A	++	+	−	IgG1, κ
BF3	A	++	+	−	IgG1, κ
AA4	B	+	ND[c)]	+	IgG1, κ
AC5	B	+	ND	−	IgG1, κ
BA5	B	+	ND	−	IgG1, κ
BE4	B	++	+	+	IgG1, κ
CA3	B	++	+	+	IgG1, κ
CB2	B	++	+	+	IgG2b, κ
CB8	B	++	+	−	IgG1, κ
CC8	B	++	+	−	IgG1, κ
CD1	B	++	+	+	IgG1, κ
CD8	B	++	+	+	IgG1, κ
AE8	C	−	ND	+	IgG1, κ
BB4	C	−	ND	+	IgG1, κ
BE6	D	−	ND	−	IgG1, κ
BF6	D	−	ND	−	IgG1, κ
BA3	F	−	ND	+	IgM, κ

a) p85α was divided into seven epitope, regions, labeled A to G (Fig. 3a).
b) The relative amounts of p85α which was immunoprecipitated by anti-p85α MAbs are indicated by ++ and + (Fig. 4a).
c) ND, not determined.

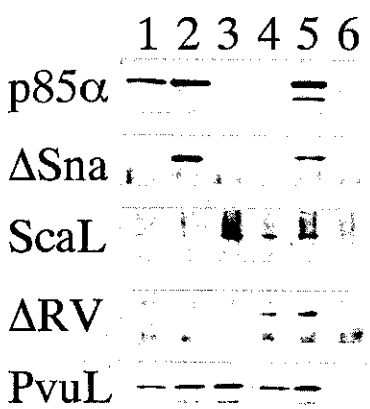

Fig. 5. Immunoprecipitation of p85α and its mutants with anti-p85α MAbs. p85α, N-terminus deletion mutants (ΔSna, ScaL, ΔRV), and a C-terminus deletion mutant (PvuL) were expressed in *E. coli*. Cells were lysed in RIPA buffer and immunoprecipitated with the anti-p85α MAbs AB6 (epitope A, lane 1), CC8 (epitope 8, lane 2), BB4 (epitope C, lane 3), and BE6 (epitope D, lane 4), or by anti-GST (lane 5), or by anti-feline immunodeficiency virus MAb. Immunoprecipitates were analyzed by immunoblotting with anti-p85α rabbit serum used as described in Fig. 3.

region (ΔSna) gave similar results to the authentic p85α, except that no signal was detected with the anti-A region MAb. However, further deletion of the B and C regions (ScaL and ΔRV, respectively) made the mutants reactive with the MAbs against regions C and D. A mutant which lacked the carboxyl-terminal G region (PvuL) also exhibited reactivity with MAbs against regions C and D. These results indicate that MAbs against regions C and D can immunoprecipitate the p85α if the epitopes are exposed and that both amino- and carboxyl-termini block the binding of MAbs to the central part of p85α.

Immunocytochemical analysis of p85α mutants expressed in eucaryotic cells To confirm our result that SH2(N) of p85α is masked in the authentic p85α protein, we examined the reactivity of anti-p85α MAbs to p85α mutants expressed in COS cells by immunocytochemistry (Fig. 6). Use of confocal microscopy enabled us to analyze the reactivity quantitatively. MAbs to the amino-terminal regions (epitopes A and B) reacted with the authentic p85α (Fig. 6, panels a) and carboxyl-terminal mutants (c and d), but not to the amino-terminal mutant (b). Anti-epitope C MAb showed only weak reactivity, demonstrated as yellow to blue color, with the authentic and amino-terminal-mutant p85α's (Fig. 6, a and b). In contrast, the deletion of carboxyl-terminus of p85α significantly increased the reactivity, demonstrated as red color, with the anti-epitope C MAb (c and d). An MAb against the central part of p85α (epitope D) reacted only with mutant p85α's lacking either the amino or carboxyl terminus (b to d), but not with the authentic p85α (a). These results support the idea that the central part of p85α is not exposed in the native conformation.

We detected p85α in the cytoplasm, predominantly around the nuclei in COS7 cells that expressed p85α cDNA. The nuclei and cytoplasmic membranes were not stained. Polyclonal rebbit and mouse antibodies against p85α yielded similar results (data not shown). Alternative fixation with reagents such as methanol or acetone did not change the pattern of staining (data not shown).

Co-precipitation of p110 and PI-3K activity with p85α The association between p85α and p110 was examined

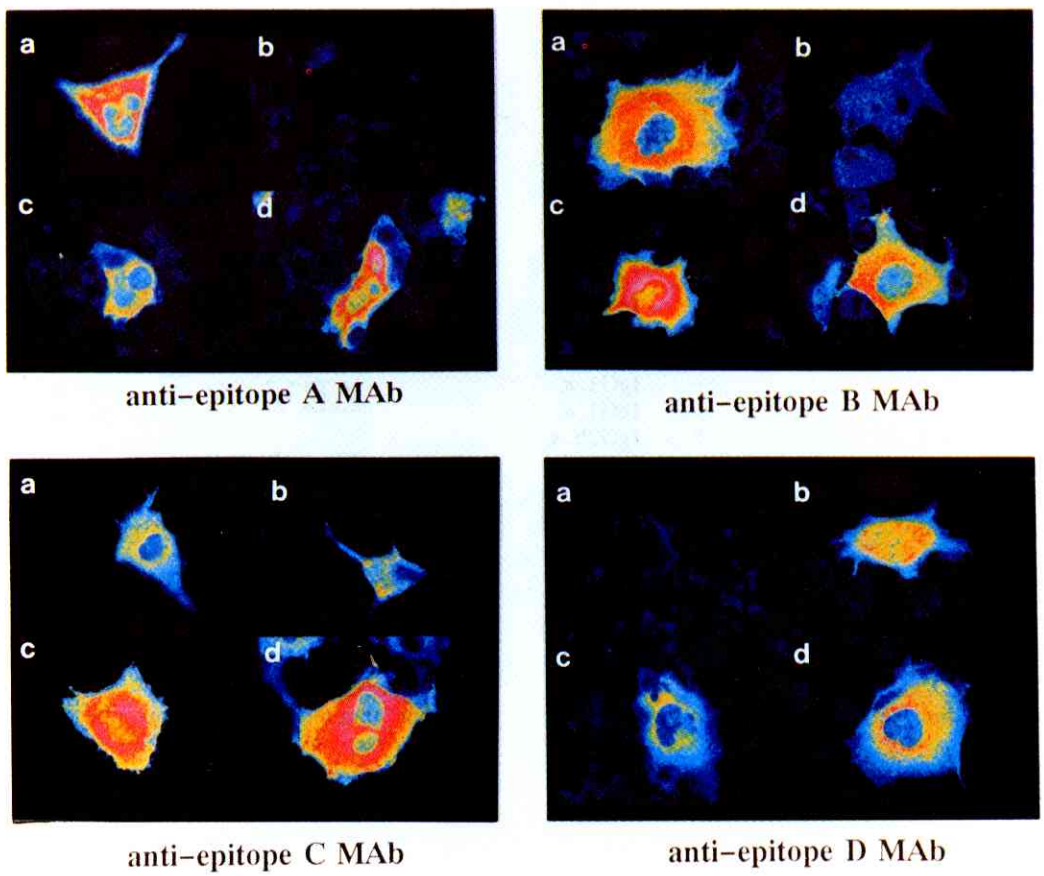

Fig. 6. Immunocytochemical analysis of p85α and its mutants expressed in COS7 cells. COS7 cells transfected with expression vectors for p85α and its mutants were fixed with 80% methanol for 15 min at 4°C and incubated with anti-p85α MAbs (anti-epitope A, AB6; anti-epitope B, CC8; anti-epitope C, AE8; anti-epitope D, BE6) followed by FITC-conjugated anti-mouse immunoglobulin antibody. Cells were observed by confocal microscopy (magnification, ×800). The intensity of the signals is indicated by the use of false colors: red, high; yellow, intermediate; blue, low. Mutants of each panel are as follows: a, authentic p85α (amino acid position 1–724); b, ScaL (111–724); c, ΔC5 (1–656); d, ΔC9 (1–579).

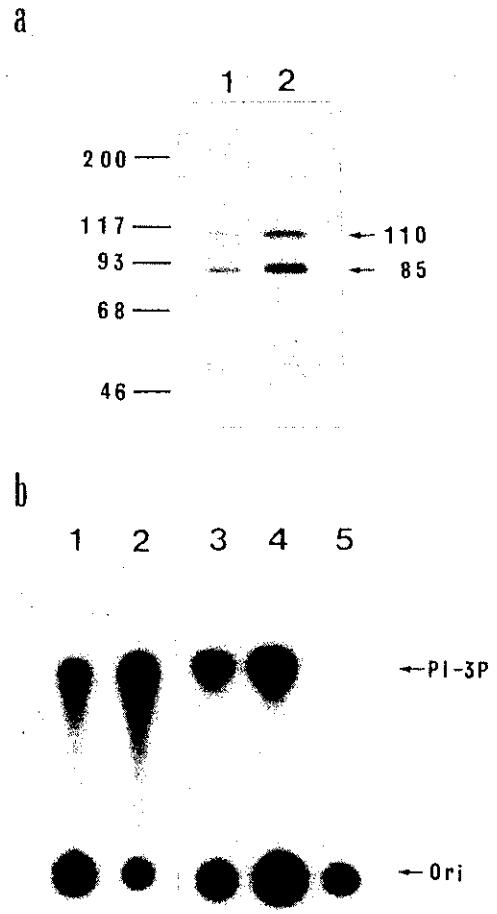

Fig. 7. Association of p85α with p110 subunit and PI-3K activity. a, 143B cells were labeled with [^{35}S]methionine, lysed with RIPA buffer, and immunoprecipitated with anti-p85α MAbs AB6 (lane 1) and CB2 (lane 2) as described in "Materials and Methods." Proteins were detected by fluorography. b, 143B cells were lysed with NP-40 buffer and immunoprecipitated with anti-p85α MAbs AB6 (lane 1) and AB7 (lane 2); anti-p85α mouse serum (lane 3); anti-p85α rabbit serum (lane 4); anti-feline immunodeficiency virus MAb (lane 5). Immunoprecipitates were applied to the PI-3K assay as described in "Materials and Methods."

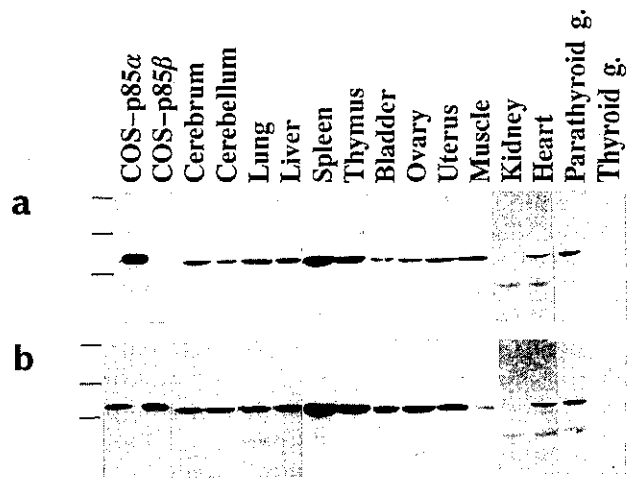

Fig. 8. Immunoblot analysis of p85α and p87β in various tissues of *Mastomys coucha*. Twenty micrograms of protein extracted from tissues was separated by 8% SDS-PAGE, transferred to PVDF membrane and probed with either AB6 (a), which reacts only with p85α or CB2 (b), which reacts with both p85α and p87β. p85α and p87β expressed in COS cells were used as positive controls. Bars indicate the positions of prestained molecular weight markers of 200, 116, and 80 kDa.

by immunoprecipitation of p85α from [^{35}S]methionine-labeled cell lysates (Fig. 7a, Table I). A 100 kDa protein was the only protein co-precipitated with p85α, supporting previous finding that PI-3K consists of two subunits of p85 and p110. All antibodies which efficiently reacted with p85α co-precipitated p110 (Table I). We confirmed that these antibodies co-precipitated PI-3K activity (Fig. 7b).

Tissue distribution of p85α by immunoblotting We examined the tissue distribution of p85α protein by immunoblotting with anti-p85α MAbs reactive only with p85α or with both p85α and p87β (Fig. 8 a and b). Twelve out of fourteen organs of multimammate mouse, *Mastomys coucha*, contained p85α at a detectable level. Among them, higher expression of p85α was detected in the spleen and thymus. We could not detect either p85α or p87β in the kidney and thyroid gland. Because we could not discriminate p87β from p85α of *Mastomys coucha* by SDS-PAGE, the distribution of p87β could not be examined.

DISCUSSION

We have established a series of hybridomas producing MAbs against the p85α subunit of human PI-3K and determined their epitope regions. These MAbs covered at least 5 distinct epitopes which were dispersed on the amino-terminal two-thirds of p85α. Although the whole p85α protein was used for immunization, no hybridoma producing antibodies specific for the carboxyl-terminus was obtained. Polyclonal antibodies obtained from the mice used to produce the hybridomas did not detect the carboxyl-terminal region of p85α expressed in *E. coli* by immunoblotting (data not shown). We immunized mice with the PvuS mutant, which consists of the carboxyl-terminal region of p85α. However, the titer of antisera to detect p85α was much lower than that obtained immuni-

zation of whole p85α. These results appear to reflect weak antigenicity of the carboxyl-terminus of p85α.

The variety of MAbs enabled us to study the structure of p85α. MAbs against the amino-terminal (epitopes A and B) and a polyclonal antibody against the carboxyl-terminal regions immunoprecipitated p85α from 143B cell lysates, whereas MAbs against the central region (epitope C, D, and F) failed to do so. This was also the case when we used p85α overexpressed in *E. coli* or in COS7 cells. In 143B cells, a substantial part of p85α was bound to p110 (S. Tanaka and M. Matsuda, unpublished observation). Therefore, the observation would primarily reflect the p85α bound to p110. Since overexpression of p85α results in large pools of the free form of p85α, these results indicate that not only p85α bound to p110, but also the free form of p85α cannot be immunoprecipitated with MAbs against the central region of p85α. These results indicate that the central region of p85α (epitope regions C, D, and F) is masked in both forms of p85α, bound to and not bound to p110. Furthermore, we confirmed this by examining the reactivity of MAbs with p85α and its deletion mutants expressed in COS7 monkey cells by using confocal microscopy. Again, the reactivity of MAbs against epitopes C and D significantly increased upon deletion of either the amino or the carboxyl terminus of p85α.

An explanation as to why MAbs that did not immunoprecipitate p85α showed positive signals in the ELISA system awaits further experiments. Binding of p85α to ELISA plates, which is thought to be mediated by hydrophobic interaction, might have changed the conformation of p85α, exposing the central region.

Our preliminary results suggest that the central part of p85α is required for binding to p110 and that the presence of both amino- and carboxyl-termini of p85α blocks the binding (F. Shibasaki in preparation). This finding supports the idea that the p110 prevents immunoprecipitation with MAbs against the central part of p85α.

Recently, Williams and his colleagues showed that SH2(C) has stronger affinity for the autophosphorylated site of PDGF receptor than SH2(N).[42] This can be understood from our results suggesting that SH2(N) might be masked by p110 or the amino and carboxyl termini of p85α itself, and that SH2(C) was exposed in the native p85α.

PI-3K has two SH2 regions, as do GAP and PLC-γ. Because each SH2 region binds to a distinct set of phosphotyrosine-containing proteins,[25, 53–55] the two SH2 regions of p85α might allow binding to a broader range of target proteins. In the case of PLC-γ, the SH2(N) reinforces the binding mediated by SH2(C). Although the SH2(C) of PLC-γ has only a weak capacity to bind phosphotyrosine-containing proteins, it enhances the binding of phosphotyrosine-containing proteins by SH2-

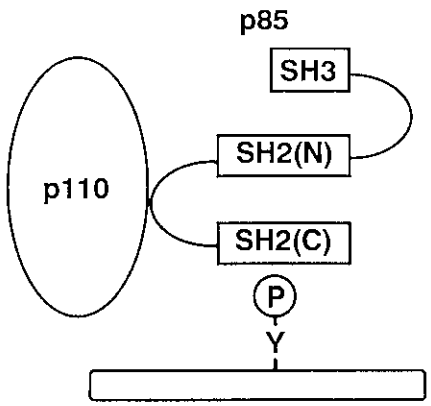

Fig. 9. Structure of PI-3K. The SH2(N) is masked by SH3, SH2(C), and p110 subunit of PI-3K. Only SH2(C) mediates the binding to the phosphotyrosine-containing proteins.

(N).[53, 55] However, our results here indicate that the functions of the two SH2 regions may be distinct in PI-3K because SH2(N) is not exposed in the native conformation (Fig. 9). The SH2(C) might be involved in binding to the growth factor receptors and SH2(N) possibly in binding to p110 or in maintenance of conformation of p85 and p110. Another possibility is that the SH2(N) might bind to a phosphotyrosine- or phosphothreonine-containing peptide sequence intramolecularly. Similar models have been proposed for p60$^{c\text{-}src}$ [56] and the BCR protein.[57] We are currently investigating whether or not some modification, such as dephosphorylation, might be necessary for exposure of SH2(N).

The amino acid sequences of p85α and p87β are highly homologous throughout the coding sequence. This apparently results in the production of anti-p85α MAbs that are cross-reactive with p87β. All MAbs examined thus far cross-reacted to p85α from rat and simian cell lines (data not shown), indicating high homology among p85α's of mammalian species.

Immunocytochemistry with confocal microscopy demonstrated p85α was localized predominantly in the cytoplasm, particularly around the nucleus. This finding is consistent with previous biochemical analyses in which most of the PI-3K activity was recovered in the cytosolic fraction.[26, 27, 58] Although we observed patchy enrichment of p85α in the cytoplasm, the details of this finding await further examination. By using this system, we are currently studying the subcellular localization of p85 before and after the activation of tyrosine kinases.

We found that spleen and thymus contained higher amount of p85α protein than the other tissues. This supports recent findings suggesting a significant role of PI-3K in the signal transduction of lymphocytes.[20–24]

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REFERENCES

1) Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L. and Roberts, T. M. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. *Nature,* **315,** 239–244 (1985).
2) Whitman, M., Downes, C. P., Keeler, T. and Cantley, L. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature,* **332,** 644–646 (1988).
3) Auger, K. R., Carpenter, C. L., Cantley, L. C. and Varticovski, L. Phosphatidylinositol 3-kinase and its novel product, phosphatidylinositol 3-phosphate, are present in *Saccharomyces cerevisiae. J. Biol. Chem.,* **264,** 20181–20184 (1989).
4) Lips, D. L., Majerus, P. W., Gorga, F. R., Young, A. T. and Benjamin, T. L. Phosphatidylinositol 3-phosphate is present in normal and transformed fibroblasts and is resistant to hydrolysis by bovine brain phospholipase C II. *J. Biol. Chem.,* **264,** 8759–8763 (1989).
5) Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. and Roberts, T. M. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. *Cell,* **50,** 1021–1029 (1987).
6) Coughlin, S. R., Escobedo, J. A. and Williams, L. T. Role of phosphatidylinositol kinase in PDGF receptor signal transduction. *Science,* **243,** 1191–1194 (1989).
7) Endemann, G., Yonezawa, K. and Roth, R. A. Phosphatidylinositol kinase or an associated protein is a substrate for the insulin receptor tyrosine kinase. *J. Biol. Chem.,* **265,** 396–400 (1990).
8) Ruderman, N. B., Kapeller, R., White, M. F. and Cantley, L. C. Activation of phosphatidylinositol 3-kinase by insulin. *Proc. Natl. Acad. Sci. USA,* **87,** 1411–1415 (1990).
9) Varticovski, L., Druker, B., Morrison, D., Cantley, L. C. and Roberts, T. M. The colony stimulating factor-1 receptor associates with and activates phosphatidylinositol-3 kinase. *Nature,* **342,** 699–702 (1989).
10) Reedijk, M., Xingquan, L. and Pawson, T. Interactions of phosphatidylinositol kinase, GTPase-activating protein (GAP), and GAP-associated proteins with the colony-stimulating factor 1 receptor. *Mol. Cell. Biol.,* **10,** 5601–5608 (1990).
11) Shurtleff, S. A., Downing, J. R., Rock, C. O., Hawkins, S. A., Roussel, M. F. and Sherr, C. J. Structural features of the colony stimulating factor 1 receptor that affect its association with phosphatidylinositol 3-kinase. *EMBO J.,* **9,** 2415–2421 (1990).
12) Bjorge, J. D. Activated type I phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation. *Proc. Natl. Acad. Sci. USA,* **87,** 3816–3820 (1990).
13) Graziani, A., Gramaglia, D., Cantley, L. C. and Comoglio, P. M. The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. *J. Biol. Chem.,* **266,** 22087–22090 (1991).
14) Fry, M. J., Gebhardt, A., Parker, P. J. and Foulkes, J. G. Phosphatidylinositol turnover and transformation of cells by Abelson murine leukemia virus. *EMBO J.,* **4,** 3173–3178 (1985).
15) Jackowski, S., Rettenmier, C. W., Sherr, C. J. and Rock, C. O. A guanine Nucleotide-dependent phosphatidylinositol 4,5-diphosphate phospholipase C in cells transformed by the v-fms and v-fes oncogenes. *J. Biol. Chem.,* **261,** 4978–4985 (1986).
16) Fukui, Y., Kornbluth, S., Jong, S.-J., Wang, L.-H. and Hanafusa, H. Phosphatidylinositol kinase type I activity associates with various oncogene phoducts. *Oncogene Res.,* **4,** 283–292 (1989).
17) Fukui, Y., Saltiel, A. R. and Hanafusa, H. Phosphatidylinositol-3 kinase is activated in v-src, v-yes, and f-fps transformed chicken embryo fibroblasts. *Oncogene,* **6,** 407–411 (1991).
18) Gutkind, J. S., Lacal, P. M. and Robbins, K. C. Thrombin-dependent association of phosphatidylinositol-3 kinase with p60c-src and p59fyn in human platelets. *Mol. Cell. Biol.,* **10,** 3806–3809 (1990).
19) Fukui, Y. and Hanafusa, H. Phosphatidylinositol kinase activity associates with viral p60*src* protein. *Mol. Cell. Biol.,* **9,** 1651–1658 (1989).
20) Yamanashi, Y., Fukui, Y., Wongsasant, B., Kinoshita, Y., Ichimori, Y., Toyoshima, K. and Yamamoto, T. Activation of Src-like protein-tyrosine kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA,* **89,** 1118–1122 (1992).
21) Gold, M. R., Chan, V. W. F., Turck, C. W. and Defranco, A. L. Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in lymphocytes-B. *J. Immunol.,* **148,** 2012–2022 (1992).
22) Merida, I., Diez, E. and Gaulton, G. N. IL-2 Binding activates a tyrosine-phosphorylated phosphatidylinositol-

3-kinase. *J. Immunol.*, **147**, 2202–2207 (1991).
23) Remillard, B., Petrillo, R., Maslinski, W., Tsudo, M., Strom, T. B., Cantley, L. and Varticovski, L. Interleukin-2 receptor regulates activation of phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **266**, 14167–14170 (1991).
24) Augustine, J. A., Sutor, S. H. and Abraham, R. T. Interleukin 2- and polyomavirus middle T antigen-induced modification of phosphatidylinositol 3-kinase activity in activated T lymphocytes. *Mol. Cell. Biol.*, **11**, 4431–4440 (1991).
25) Carter, R. H., Tuveson, D. A., Park, D. J., Rhee, S. G. and Fearon, D. T. The CD19 complex of lymphocytes-B – activation of phospholipase-C by a protein tyrosine kinase-dependent pathway that can be enhanced by the membrane IgM complex. *J. Immunol.*, **147**, 3663–3671 (1991).
26) Howell, B. W. STY, a tyrosine-phosphorylating enzyme with sequence homology to serine/threonine kinases. *Mol. Cell. Biol.*, **11**, 568–572 (1991).
27) Shibasaki, F., Homma, Y. and Takenawa, T. Two types of phosphatidylinositol 3-kinase from bovine thymus – monomer and heterodimer form. *J. Biol. Chem.*, **266**, 8108–8114 (1991).
28) Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J. and Waterfield, M. D. Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src compexes, and PI3-kinase. *Cell*, **65**, 91–104 (1991).
29) Escobedo, J. A., Navankasattusa, S., Kavanaugh, W. M., Milfay, D., Fried, V. A. and Williams, L. T. cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF b-receptor. *Cell*, **65**, 75–82 (1991).
30) Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A. and Schlessinger, J. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell*, **65**, 83–90 (1991).
31) Stahl, M. L., Ferenz, C. R., Kelleher, K. L., Kriz, R. W. and Knopf, J. L. Sequence similarity of phospholipase C with the non-catalytic region of src. *Nature*, **332**, 269–272 (1988).
32) Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. and Rhee, S. G. Inositol phospholipid-specific phospholipase C: complete cDNA and protein sequences and sequence homology to tyrosine kinase-related oncogene products. *Proc. Natl. Acad. Sci. USA*, **85**, 5419–5423 (1988).
33) Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nakanishi, O., Suzuki, K. and Takenawa, T. A second type of rat phosphoinositide-specific phospholipase C containing a src-related sequence not essential for phosphoinositide-hydrolyzing activity. *J. Biol. Chem.*, **264**, 21885–21890 (1989).
34) Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S. and Gibbs, J. B. Cloning of bovine GAP and its interaction with oncogenic ras p21. *Nature*, **335**, 90–93 (1988).
35) Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G. A., Landner, M., Long, C. M., Crosier, W. J., Watt, K., Koths, K. and McCormick, F. Molecular cloning of two types of GAP complementary DNA from human placenta. *Science*, **242**, 1697–1700 (1988).
36) Sadowski, I., Stone, J. C. and Pawson, T. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus $P130^{gag\text{-}fps}$. *Mol. Cell. Biol.*, **6**, 4396–4408 (1986).
37) Fischer, E. H., Charbonneau, H. and Tonks, N. K. Protein tyrosine phosphatases – a diverse family of intracellular and transmembrane enzymes. *Science*, **253**, 401–406 (1991).
38) Bellacosa, A., Testa, J. R., Staal, S. P. and Tsichlis, P. N. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*, **254**, 274–277 (1991).
39) Mayer, B. J., Hamaguchi, M. and Hanafusa, H. A novel viral oncogene with structural similarity to phospholipase C. *Nature*, **332**, 272–275 (1988).
40) Matsuda, M., Mayer, B. J., Fukui, Y. and Hanafusa, H. Binding of oncoprotein, $P47^{gag\text{-}crk}$, to a broad range of phosphotyrosine-containing proteins. *Science*, **248**, 1537–1539 (1990).
41) Kazlauskas, A. and Cooper, J. A. Phosphorylation of the PDGF receptor b subunit creates a tight binding site for phosphatidylinositol 3 kinase. *EMBO J.*, **9**, 3279–3286 (1990).
42) Klippel, A., Escobedo, J. A., Fantl, W. J. and Williams, L. T. The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor-beta receptor. *Mol. Cell. Biol.*, **12**, 1451–1459 (1992).
43) Fantl, W. J., Escobedo, J. A. and Williams, L. T. Mutations of the platelet-derived growth factor receptor that cause a loss of ligand-induced conformational change, subtle changes in kinase activity, and impaired ability to stimulate DNA synthesis. *Mol. Cell. Biol.*, **9**, 4473–4478 (1989).
44) Pagano, M., Draetta, G. and Jansendurr, P. Association of cdk2 kinase with the transcription factor-E2F during S-phase. *Science*, **255**, 1144–1147 (1992).
45) Mcglade, C. J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A. D., Panayotou, G., End, P., Bernstein, A., Kazlauskas, A., Waterfield, M. D. and Pawson, T. SH2 domains of the p85alpha subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. *Mol. Cell. Biol.*, **12**, 991–997 (1992).
46) Smith, D. B. and Johnson, K. S. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, **67**, 31–40 (1988).
47) Kohler, G. and Milstein, C. Continuous cultures of fused

cells secreting antibody of predefined specificity. *Nature,* **256,** 495–497 (1975).
48) Harlow, E. and Lane, E. "Antibodies. A Laboratory Manual" (1988). Cold Spring Harbor Laboratory Press, New York.
49) Nagata, S., Yamamoto, K., Ueno, Y., Kurata, T. and Chiba, J. Preferential generation of monoclonal IgG-producing hybridomas by use of vesicular stomatitis virus-mediated cell fusion. *Hybridoma,* **10,** 369–378 (1991).
50) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680–685 (1970).
51) Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. and Arai, N. SRa promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.,* **8,** 466–472 (1988).
52) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning: A Laboratory Manual" (1989). Cold Spring Harbor Laboratory Press, New York.
53) Anderson, D., Koch, C. A., Grey, L., Ellis, C., Moran, M. F. and Pawson, T. Binding of SH2 domains of phospholipase Cr1, GAP, and src to activated growth factor receptors. *Science,* **250,** 979–982 (1990).
54) Matsuda, M., Mayer, B. J. and Hanafusa, H. Identification of domains of the v-crk oncogene product sufficient for association with phosphotyrosine-containing proteins. *Mol. Cell. Biol.,* **11,** 1607–1613 (1991).
55) Matsuda, M., Reichman, C. T. and Hanafusa, H. Biological and biochemical activity of v-CrK chimeras containing the SH2/SH3 regions of phosphatidylinositol-specific phospholipase C-gamma and Src. *J. Virol.,* **66,** 115–121 (1992).
56) Roussel, R. R., Brodeur, S. R., Shalloway, D. and Laudano, A. P. Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. *Proc. Natl. Acad. Sci. USA,* **88,** 10696–10700 (1991).
57) Pendergast, A. M., Clark, R., Kawasaki, E. S., McCormick, F. P. and Witte, O. N. Baculovirus expression of functional p210 BCR-ABL oncogene product. *Oncogene,* **4,** 759–766 (1989).
58) Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S. and Pawson, T. Src homology region 2 domains direct protein-protein interactions in signal transduction. *Proc. Natl. Acad. Sci. USA,* **87,** 8622–8626 (1990).