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Human platelets contain p110δ phosphoinositide 3-kinase☆

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Abstract

The phosphoinositide 3-kinase (PI3K) catalytic subunit p110δ, the most recently discovered member of the heterodimeric Class IA PI3K family, has been detected uniquely in leukocytes, but not in one member of the leukocyte family: platelets. We have examined freshly prepared isolates of human platelets for the presence of this enzyme, realizing that p110δ is highly susceptible to proteolytic degradation. We have utilized p110δ-directed Western blotting, RT-PCR, PI3K activity assays, and immunoprecipitations of PI3K Class IA subunits p85α, p85β, and p110δ from lysed human platelets, as well as Triton X-100-insoluble cytoskeletal preparations from resting and thrombin receptor-activated platelets. We report that p110δ is present in association with p85α and p85β in platelets, both in cytosolic and cytoskeletal fractions. The latter finding is consistent with the proposed role of p110δ in cytoskeletal function.

Keywords: PI3K; Platelet; Cytoskeleton; Leukocyte; p110; p85

The phosphoinositides PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are important second messengers in most eukaryotes, serving as tethering targets and regulators of a variety of signal transduction proteins (reviews [1–4]). Their synthesis is largely under the control of Class I PI3Ks, which phosphorylate the 3-OH position of the myoinositol ring of PI lipids. Class IA PI3Ks are heterodimeric proteins, consisting of a 110 kDa catalytic and a regulatory subunit, usually of 85 kDa. Three types of Class IA p110 subunit, α, β, and δ, have been described to date. Whereas p110α and β are broadly distributed, the p110δ subunit, associated with p85α and β adaptors, has been reported to be restricted to leukocytes [5]. The same report detected no p110δ in platelets, which are white cells involved in hemostasis, but also noted that p110δ was very susceptible to degradation. To add to our knowledge of which PI3Ks are available to play a role in platelets [6–9], we decided to re-examine these cells for the presence of p110δ.

Materials and methods

Anti-p110δ (N-terminally directed, aa 73–90 Mab, and C-terminally directed, aa 1030–1044 polyAb) and anti-p110α (N-terminally directed MAb) were purchased from BD BioSciences or generated in the Vanhaesebroeck lab [5]. Anti-p85α (U13) and p85β (T4) MAbs were gifts from Dr. I. Gout (Ludwig Institute for Cancer Research, UK). Anti-mouse IgG-HRP, anti-rabbit IgG-HRP, and ECL reagents were purchased from Amersham. Anti-mouse IgG-agarose, anti-rabbit IgG-agarose, and Histopaque1077 were from Sigma. Standard proteins for p110α, β, and δ were expressed as described [10]. ACK lysis buffer was from Biosource. Triton X-100 and BCA protein assay reagents were from Pierce. Prestained protein markers and TRIzol Reagent were from Gibco-BRL. AMV reverse transcriptase kit and RNasin were from Promega. PCR-DNA polymerase kit was from Eppendorf. SFLLRN and Oligo(dT) 18-mers/PCR 20-mer primers were synthesized by the Kimmel Cancer Center's Peptide and Nucleic Acid Facilities, respectively. [γ-^{32}P]ATP and ATP were purchased from NEN and Boehringer, respectively. PtdIns(4,5)P_2 and PtdSer were from Sigma. Other reagents were from Fisher and Bio-Rad.

☆ *Abbreviations:* PCR, polymerase chain reaction; PI, phosphoinositide; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PI3K, phosphoinositide 3-kinase; bZIP, basic region leucine zipper; CSK, cytoskeleton; SDS–PAGE, SDS-polyacrylamide gel electrophoresis; dNTP, deoxyribonucleoside trisphosphate; MAb, monoclonal antibody; polyAb, polyclonal antibody.

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Preparations of platelets and leukocytes from fresh human blood. Human platelets were prepared freshly as described [6–8], and suspended in GFB pH 7.3 (132 mM NaCl, 12 mM sodium citrate, 10 mM dextrose, 20 mM HEPES, 2.8 mM KCl, 8.9 mM $NaHCO_3$, and 0.86 mM $MgCl_2$). The platelet preparation was checked by microscopy (non-platelets were <0.1%). Human leukocytes were prepared from the buffy coat of the same fresh human blood, and were purified with Histopaque 1077 to make a non-platelet leukocyte (a mix of myeloid and lymphocytic cells) layer, and with ACK lysis buffer to remove red cell contamination [11,12]. The purified leukocytes were suspended in GFB as described above for platelet preparations.

Lysis of platelets and other leukocytes. Preparations of purified platelets and non-platelet leukocytes were each lysed on ice for 5 min with an equal volume of ice-cold pH 7.4, 2× lysis buffer, containing 2% Triton X-100, 50 mM Tris–HCl, 10% glycerol, 200 mM NaCl, 24 mM EGTA, 10 mM $MgCl_2$, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 4 mM Na_3VO_4. Some platelet lysates were stored (2–3 days) at −70 °C. Protein contents were assayed, and equal amounts of lysate protein were then mixed with SDS–PAGE sample buffer [6–8] and applied to 7.5% SDS–PAGE gels for subsequent Western blotting as described below.

Preparation of platelet 1%Triton X-100-insoluble (CSK) and soluble (SOL) fractions. Platelets (4×10^9/ml) +/− SFLLRN (50 μM)/fibrinogen (400 μg/ml)/Ca^{2+} (6.4 mM), or +/− thrombin (2 U/ml) were incubated up to120 s at 37 °C. After lysis of incubated platelet suspensions, CSK and SOL were separated by centrifugation (15,000*g*, 5 min, at 4 °C). The pellets (CSK) were washed three times with GFB, suspended in GFB 7.3 containing the inhibitors described above, and sonicated briefly on ice. The protein contents were determined [6–8].

Western blots. In some studies, MAbs (25 μl, p85α or p85β) or p110δ polyAb (2 μl) and platelet SOLs (100 μl), for immunoprecipitations, were incubated at 4 °C overnight with gentle shaking. Anti-mouse IgG-agarose (25/100 μl initial SOL, for MAbs) or anti-rabbit IgG-agarose (50/100 μl) was added, and mixtures incubated at 4 °C for 2 h and centrifuged at 2000 rpm 4 °C for 1 min. The sediments were washed several times with phosphate-buffered saline in 0.1% Triton X-100 containing protease inhibitors, and were digested with SDS–PAGE sample buffer for 5 min at 100 °C prior to electrophoresis and Western blotting for p110δ, p110α, p85α, or p85β. In other studies, after electrophoresis of platelet or non-platelet leukocyte lysates, platelet CSK/SOL fractions, or expressed p110α, β, or δ on SDS–PAGE gels, resolved proteins were transferred onto nitrocellulose membranes under the same conditions and blocked [8], and nitrocellulose lanes were cut for blotting with the MAbs p110δ (N-terminally directed) or p110α (0.5 μg/ml Tris buffered saline Tween with azide), or the polyAb p110δ (C-terminally directed; 1 μg/ml TBST with azide) in comparison with high MW standards. Membranes and antibodies were incubated at 4 °C overnight with shaking. The blots were washed several times, and anti-mouse IgG-linked HRP (diluted 1:10,000) for MAbs, or anti-rabbit IgG-HRP (diluted 1:5000) for the polyAb, were added, and the mixtures were incubated for 1–2 h at room temperature with shaking. The blots were washed and developed with ECL [8] for up to 2 min at room temperature.

PI3K activity assays. Platelet SOL was used for immunoprecipitations with C-terminally directed polyAb to p110δ. This antibody neutralizes the activity of recombinant p110δ by about 98–99%, but sensitive radioactive assays make it possible to detect this residual p110δ activity in immunoprecipitates. The N-terminally directed MAb was ineffective for immunoprecipitations. Controls, which were "mock" immunoprecipitations, omitted the primary antibody. Precipitates were washed as above, and finally, 3× with 2× PI3K buffer (40 mM Tris–HCl, 200 mM NaCl, 2 mM EGTA, pH 7.4). Washed beads were resuspended in 40 μl 2× PI3K buffer for assay. Assays, and subsequent resolution and quantitation of products by HPLC, were conducted as described previously [8,13]. Assays contained 40 μl pellet suspensions, 10 μl lipid substrate PtdIns(4,5)P_2/PtdSer, and 50 μl ATP/buffer. Final concentrations were: 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM lipid, 225 μM ATP, 10 μC_i [γ-^{32}P]ATP.

Isolation and purification of platelet RNA. Fresh purified human platelets were frozen in liquid N_2 and ground with a pestle in a liquid N_2-filled mortar. The total RNA was isolated from such frozen platelet powders with TRIzol Reagent and centrifuged at 4 °C 12,000*g* for 10 min. Chloroform (0.2 ml/ml of TRIzol used initially) was added to the 12,000*g*-supernatant, incubated at 15–30 °C for 2–3 min, and centrifuged again. The RNA in the aqueous phase was precipitated with isopropanol (0.5 ml/ml TRIzol used initially), washed with 75% ethanol (1 ml/ml TRIzol used initially), and traces of ethanol were removed by airflow. The purified RNA was re-suspended in DEPC-treated water and the RNA concentration was determined prior to RT-PCR.

RT-PCR of platelet RNA. For reverse transcription, 3 μg total platelet RNA + 2 μl oligo(dT) 18-mers (10.7 pmol/μl) + nuclease-free water to 10 μl was heated for 5 min at 70 °C, after which AMV reverse transcriptase mix (5 μl of 10× AMV buffer + 2.5 μl dNTP mix [stock with 2.5 mM each dNTP] + 1 μl [40 U] RNAse inhibitor + 2.5 μl sodium pyrophosphate [stock of 40 mM pre-warmed to 42 °C] + 3 μl [30 U] AMV reverse transcriptase) was added. The mixture was chilled on ice for 5 min, incubated at 42 °C for 60 min, 92 °C for 10 min, and cooled to 4 °C. For PCR, the cDNA template was mixed with the following reagents: 4 μl$MgCl_2$ (25 mM stock), 5 μl 10× buffer, 2.5 μl dNTP mix (stock with 2.5 mM each dNTP), appropriate amount of primers (10 pmol each), 1 U Taq DNA polymerase, and nuclease-free water to 50 μl. Primers were based on the proline-rich region and bZIP-like domain in human p110δ, not present in PI3K p110α and β [5]. PCR primers: forward, 5′-CCTGCCCCCCAGGTCCAGAA-3′ (20 bp); reverse, 5′-GAGGCAGCGTTCCCCGGTCT-3′ (20 bp). PCR cycles were: 1× 94 °C 30 s, 40× (94 °C 15 s, 59 °C 45 s, 72 °C 1 min), 1×72 °C 2 min, then 4 °C. The size of the resulting cDNA was checked by electrophoresis on 1.2% agarose gels and its sequence was analyzed by BigDye terminator reaction chemistry on the ABI Prism 3700 DNA analyzer at the nucleic acid facility of KCC.

Results and discussion

Using p110δ-specific antibodies (Fig. 1), we have observed that p110δ is indeed present in platelets at a level comparable, on a protein basis, to that in myeloid/lymphocytic leukocytes (Fig. 2). Storage of the frozen lysate, however, leads to a substantial loss of this protein (not shown). It seems likely that deterioration of p110δ in stored platelet samples accounts for the previous failure to detect this enzyme in platelets [5]. Storage is also known to lead to decreased platelet responsiveness to a variety of platelet agonists. When we examined the distribution of p110δ between platelet CSK and SOL fractions, we found CSK to be somewhat enriched in p110δ, both in resting or activated platelets (Fig. 3). CSK-associated p110δ was detected when Western blots were probed with either N-terminally directed MAb or C-terminally directed polyAb (Fig. 4). Although each antibody detected some bands of differing patterns, in addition to p110, the C-terminally directed polyAb conspicuously detected additional bands of lower (approximately 10–20 kDa) molecular weight than p110. These will be the subject of further investigation. The presence of p110δ was most pronounced, i.e., without the slightly lower molecular weight species, when detected with N-terminally directed (aa 73–90) MAb, raising the possibility that there could be some

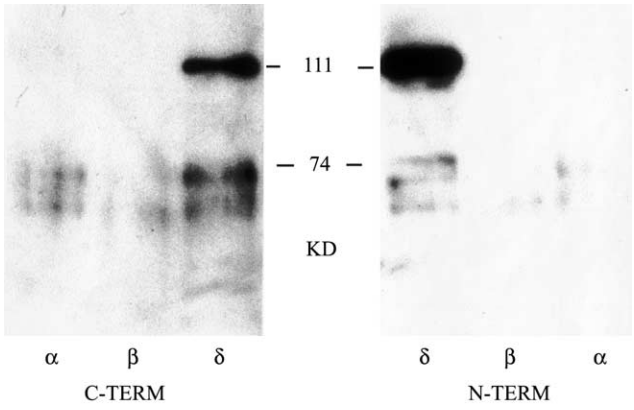

Fig. 1. Western blots of p110 standards by p110δ-directed antibodies. Recombinant p110α, β, and δ proteins, expressed in Sf9 insect cells, were run on SDS–PAGE gels and Western blotted with polyAb (C-TERM) or MAb (N-TERM) antibodies to p110δ. Duplicate gels were run and blotted with MAb to p110α, which detected only recombinant p110α at p110 (not shown).

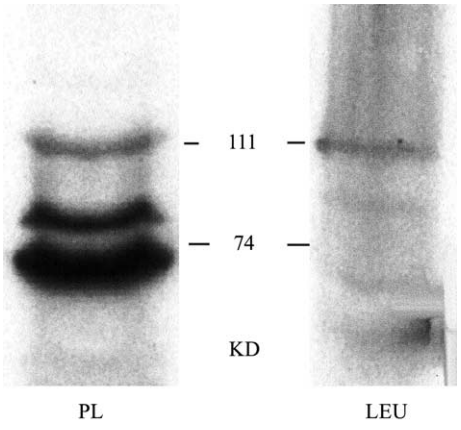

Fig. 2. Western blot of p110δ in human platelet and non-platelet leukocyte lysates. Lysates were prepared, resolved on SDS–PAGE gels, and Western blotted with MAb to p110δ as described. Fresh platelet (PL) and non-platelet (myeloid + lymphocytic cell) leukocyte (LEU) lysate = 217 μg protein/lane. Results are typical of three experiments.

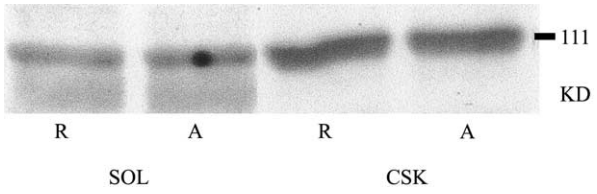

Fig. 3. Western blot of p110δ in platelet fractions. Triton-insoluble (CSK; 40 μg protein/lane) and soluble (SOL; 80 μg protein/lane) fractions from resting (R) or activated (2 min SFLLRN; A) platelets were resolved by SDS–PAGE and Western blotted with MAb to p110δ. Results are representative of four experiments.

alteration, possibly by proteolytic calpain, of some of p110δ at the N-terminus in platelet CSK. This would allow selective detection of lower molecular weight species by the C-terminally directed (aa 1030–1044) polyAb. Since the amount of CSK protein increases in

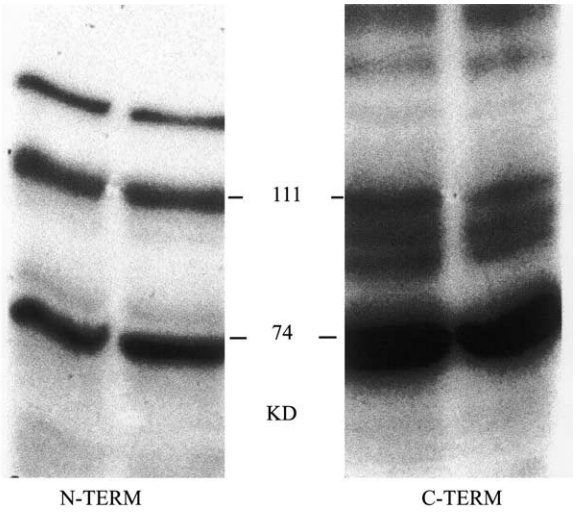

Fig. 4. Western blot of p110δ, detected in CSK by N-terminally directed MAb or C-terminally directed polyAb. Resolved proteins of platelet CSK (resting) were probed with MAb (N-TERM) or polyAb (C-TERM) antibodies to p110δ. Results are representative of two experiments.

activated platelets [7], CSK-associated p110δ could account for a substantial portion of that activity in platelets after stimulation of the thrombin receptor, as long as any deletion at the N-terminus of p110δ covered less than the N-terminal p85 binding site, to maintain catalytic activity (Vanhaesebroeck, unpublished results). In confirmation of p110δ's status as a member of the Class IA PI3K family, we observed that p110δ was associated with immunoprecipitated p85α and p85β adaptor subunits (Fig. 5). The p110δ immunoprecipitated from SOL also displayed p85α and p85β when blotted for these subunits (not shown). The amounts of p110δ were similar in p85α and p85β immunoprecipitates, which is interesting because the ratio of p85α/p85β in platelets is approximately 6:1 [7]. CSK also contained p85α and p85β, but more p85α and p85β were found in SOL, when resting platelet fractions were examined (not shown). We have reported [7] that activation of platelets leads to increased p85α and p85β in CSK. Slightly more p110α, however, was found associated with p85α than with p85β in immunoprecipitates from SOL (Fig. 5). It thus appears likely that p85β-containing PI3K heterodimers are relatively (with respect to p85α) enriched in

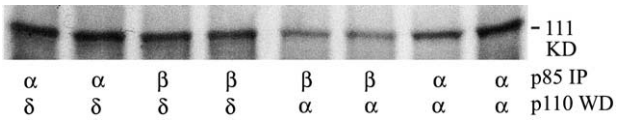

Fig. 5. Association of p110δ or p110α with p85α or p85β in human platelets. Platelet SOL was used for immunoprecipitations (IPs) of p85α or p85β. IPs were solubilized and resolved by SDS–PAGE, and then Western blotted (WB) with MAbs to p110δ or p110α. Results are representative of three experiments.

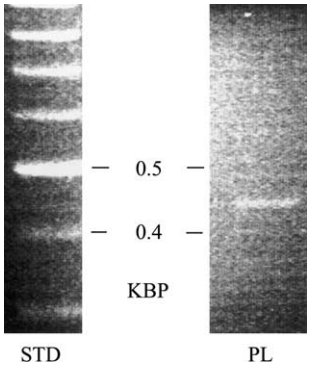

Fig. 6. Resolution of cDNA on agarose gels. RT-PCR was performed on platelet RNA, using primers to the region of p110δ encompassing the unique Pro-rich and bZIP regions, and the resulting cDNA was resolved in comparison with base pair ladder standards. The cDNA migrated at the position expected for 444 bp.

p110δ. We verified the immunological evidence for the presence of p110δ in human platelets at the level of mRNA, as well as by assays of enzymatic activity. After RT-PCR, a 444 bp cDNA (Fig. 6), having a sequence in agreement with that encompassing the unique Pro-rich (874–933) and bZIP (1198–1317) motifs of p110δ (5; GenBank Y10055), was observed. Further, although enzymatically impaired by the presence of the immunoprecipitating antibody, immunoprecipitated p110δ exhibited, in duplicate assays, 1.6× more activity in converting PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 than did mock (primary antibody-free) precipitates containing traces of PI3K adsorbed to the agarose pellets (control $= 1778 \pm 154$ dpm vs. p110δ IP $= 2803 \pm 30$ dpm).

Given the original failure to detect p110δ in platelets and the restriction of p110δ to leukocytes, Vanhaesebroeck et al. [5] speculated that p110δ might contribute uniquely to transendothelial migration of lymphoid and myeloid cells. Further, it was proposed that p110δ might participate in cytoskeletal rearrangements [5]. We would suggest that cytoskeletal function in activated platelets [14] and platelet spawning from megakaryocytes might also be influenced by p110δ, variants on the functions proposed earlier [5]. Any such role would need to be verified by knock-out studies.

The identification of p110δ as another member of the Class I PI3K family that is present in human platelets contributes to our knowledge of the entities potentially responsible for the production of the important second messengers PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, which have been shown to accumulate in stimulated platelets [15]. These studies should lead to additional strategies for dissecting which PI3K species act, and at what locations, when platelets are exposed to different receptor-directed agonists.

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References

[1] M. Wymann, L. Pirola, Structure and function of phosphoinositide 3-kinases, Biochim. Biophys. Acta 1436 (1998) 127–150.
[2] D.A. Fruman, R.E. Meyers, L.C. Cantley, Phosphoinositide kinases, Annu. Rev. Biochem. 67 (1998) 481–507.
[3] T.O. Chan, S.E. Rittenhouse, P.N. Tsichlis, AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation, Annu. Rev. Biochem. 68 (1999) 965–1014.
[4] B. Vanhaesebroeck, S.J. Leevers, K. Ahmadi, J. Timms, R. Katso, P.C. Driscoll, R. Woscholski, P.J. Parker, M.D. Waterfield, Synthesis and function of 3-phosphorylated inositol lipids, Annu. Rev. Biochem. 70 (2001) 535–602.
[5] B. Vanhaesebroeck, M.J. Welham, K. Kotani, R. Stein, P.H. Warne, M.J. Zvelebil, K. Higashi, S. Volinia, J. Downward, M.D. Waterfield, p110δ, a novel phosphoinositide 3-kinase in leukocytes, PNAS USA 94 (1997) 4330–4335.
[6] J. Zhang, M.J. Fry, M.D. Waterfield, S. Jaken, L. Liao, J.E. Fox, S.E. Rittenhouse, Activated phosphoinositide 3-kinase associates with membrane skeleton in thrombin-exposed platelets, J. Biol. Chem. 267 (1992) 4686–4692.
[7] J. Zhang, J. Zhang, S.J. Shattil, M.C. Cunningham, S.E. Rittenhouse, Phosphoinositide 3-kinase γ and p85/phosphoinositide 3-kinase in platelets, J. Biol. Chem. 271 (1996) 6265–6272.
[8] J. Zhang, H. Banfic, F. Straforini, L. Tosi, Volinia, S.E. Rittenhouse, A type II phosphoinositide 3-kinase is stimulated via activated integrin in platelets. A source of phosphatidylinositol 3-phosphate, J. Biol. Chem. 273 (1998) 14081–14084.
[9] T.O. Chan, U. Rodeck, A.M. Chan, A.C. Kimmelman, S.E. Rittenhouse, G. Panayotou, P.N. Tsichlis, Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit, Cancer Cell 1 (2002) 181–191.
[10] B. Vanhaesebroeck, G.E. Jones, W.E. Allen, D. Zicha, R. Hooshmand-Rad, C. Sawyer, C. Wells, M.D. Waterfield, A.J. Ridley, Distinct PI(3)Ks mediate mitogenic signaling and cell migration in macrophages, Nat. Cell Biol. 1 (1999) 69–71.
[11] A. Boyum, Separation of leukocytes from blood and bone marrow, Scand. J. Clin. Lab. Invest. 21 (Suppl. 97) (1968) 77.
[12] X. Bossuyt, G.E. Marti, T.A. Fleischer, Comparative analysis of whole blood lysis methods for flow cytometry, Cytometry 30 (1997) 124–133.
[13] A.N. Carter, R.-S. Huang, A. Sorisky, C.P. Downes, S.E. Rittenhouse, Phosphatidylinositol (3,4,5)-trisphosphate is formed from phosphatidylinositol (4,5)-bisphosphate in thrombin-stimulated platelets, Biochem. J. 301 (1994) 415–420.
[14] A. Rotman, Actin transformations, in: H. Holmsen (Ed.), Platelet Responses and Metabolism, vol. III, CRC Press, Boca Raton, FL, 1987, pp. 245–255.
[15] H. Banfic, C.P. Downes, S.E. Rittenhouse, Biphasic activation of PKBα/Akt in platelets: evidence for stimulation by both PtdIns(3,4)P_2, produced via a novel pathway, and by PtdIns(3,4,5)P_3, J. Biol. Chem. 273 (1998) 11630–11637.