A Type II Phosphoinositide 3-Kinase Is Stimulated via Activated Integrin in Platelets

A SOURCE OF PHOSPHATIDYLINOSITOL 3-PHOSPHATE*

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We have observed that aggregation of human platelets, caused by activation of integrin αIIbβ3 and its consequent binding of fibrinogen, stimulates a novel pathway for synthesis of phosphatidylinositol 3,4-bisphosphate, thereby activating protein kinase B/Akt. Such synthesis depends upon both the generation of phosphatidylinositol 3-phosphate (PtdIns3P), which is sensitive to wortmannin (IC50 7 nM) and calpain inhibitors, and the phosphorylation of PtdIns3P by PtdIns3P 4-kinase. We now report that a recently characterized C2 domain-containing phosphoinositide 3-kinase isoform (HsC2-P13K) is present in platelets and a leukemic cell line (CHRF-288) derived from megakaryoblasts, and is likely to be responsible for the stimulated synthesis of PtdIns3P observed in platelets. HsC2-P13K, identifiable by Western blotting and immunoprecipitable activity, is sensitive to wortmannin (IC50 6–10 nM), requires Mg2++, and shows strong preference for PtdIns over PtdIns4P or phosphatidylinositol 4,5-bisphosphate as substrate. HsC2-P13K is activated severalfold when platelets aggregate in an αIIbβ3-dependent manner or when platelet or CHRF-288 lysates are incubated with Ca2++. Activation is prevented by calpain inhibitors. CHRF-288, which cannot undergo activation of αIIbβ3 and thereby aggregate in response to platelet agonists, do not generate PtdIns3P or activate HsC2-P13K under conditions that stimulate other phosphoinositide 3-kinases. HsC2-P13K may thus be an important effector for integrin-dependant signaling.

Activation of phosphoinositide 3-kinase (PI3K)† is an important cell signaling event that has been linked causally to a variety of physiologic changes, including proliferative responses to growth factors (1), differentiation (2), anti-apoptosis (3), cytoskeletal rearrangements and integrin activation (4, 5), and integrin-mediated cell motility and carcinoma invasion (6, 7). The second messengers involved in these events are thought to be PtdInsP3 and/or PtdIns(3,4)P2, which are capable of stimulating the activity of protein kinases such as PKB/Akt (8), and some protein kinase C isoforms (9–11), but may act in additional ways. Most of the PI3Ks studied in these contexts are, or have been assumed to be, of the “Type I” class (see Ref. 12 for review), where classification is based upon structural homology, substrate specificity, and mode of regulation. The Type I PI3Ks are heterodimers, containing 110–130-kDa catalytic subunits associated with 50–85- or 101-kDa adaptor entities, small GTPases, or βγ subunits of heterotrimeric GTP-binding proteins (12). Type II PI3Ks are catalytic entities about twice the size of Type I catalytic subunits, and it is unknown whether they, like Type I and Type III, have adaptor proteins or whether their increased size provides a built-in adaptor. They contain a defining C2 domain at their C termini, as well as an N-terminal extension. Importantly, as well, they cannot utilize, or utilize poorly, PtdIns(4,5)P2, as a substrate, and their means of activation, or even whether they can be activated in a signal-transduction setting, has been unknown. The cloning and characterization of two new PI3Ks of the Type II class from human cells have been described recently (13, 14). These PI3Ks have been designated PI3KC2α (13) and HsC2-P13K (14). PI3KC2α (190 kDa), which has been expressed, is resistant to wortmannin (IC50 420 nM) and resembles (90% homology) mouse m-ckp (15) and p170 (16). It preferentially phosphorylates PtdIns and PtdIns4P, but can, albeit poorly, phosphorylate PtdIns(4,5)P2. HsC2-P13K is a closely related 185-kDa protein, except for its divergent 350 N-terminal residues, which encompass two proline-rich sequences appropriate for interaction with src-homology 3 domains (14). As described here, expressed HsC2-P13K phosphorylates PtdIns in strong preference to the other two known PI3K substrates, PtdIns4P and PtdIns(4,5)P2, and is very sensitive to wortmannin (IC50 10 nM), in contrast to PI3KC2α. Mammalian Type III PI3K (HsVPS34) contains a catalytic subunit similar in size to those of Type I and an associated protein of 150 kDa. HsVPS34 is inhibited by nanomolar wortmannin (IC50 6–10 nM) and calpain inhibitors, and have been found to be stimulated by numerous growth factors and heterotrimeric GTP-binding protein-coupled receptors. They can be activated, depending upon the subtype, cell, and receptor that has been stimulated, by tyrosine phosphorylation, association with tyrosine-phosphorylated or proline-rich domains, small GTPases, or βγ subunits of heterotrimeric GTP-binding proteins (12). Type II PI3Ks are catalytic entities about twice the size of Type I catalytic subunits, and it is unknown whether they, like Type I and Type III, have adaptor proteins or whether their increased size provides a built-in adaptor. They contain a defining C2 domain at their C termini, as well as an N-terminal extension. Importantly, as well, they cannot utilize, or utilize poorly, PtdIns(4,5)P2, as a substrate, and their means of activation, or even whether they can be activated in a signal-transduction setting, has been unknown. The cloning and characterization of two new PI3Ks of the Type II class from human cells have been described recently (13, 14). These PI3Ks have been designated PI3KC2α (13) and HsC2-P13K (14). PI3KC2α (190 kDa), which has been expressed, is resistant to wortmannin (IC50 420 nM) and resembles (90% homology) mouse m-ckp (15) and p170 (16). It preferentially phosphorylates PtdIns and PtdIns4P, but can, albeit poorly, phosphorylate PtdIns(4,5)P2. HsC2-P13K is a closely related 185-kDa protein, except for its divergent 350 N-terminal residues, which encompass two proline-rich sequences appropriate for interaction with src-homology 3 domains (14). As described here, expressed HsC2-P13K phosphorylates PtdIns in strong preference to the other two known PI3K substrates, PtdIns4P and PtdIns(4,5)P2, and is very sensitive to wortmannin (IC50 10 nM), in contrast to PI3KC2α. Mammalian Type III PI3K (HsVPS34) contains a catalytic subunit similar in size to those of Type I and an associated protein of 150 kDa. HsVPS34 is inhibited by nanomolar wortmannin, phosphorylates only PtdIns lipid substrate, and, in contrast to Type I and Type II kinases, is stimulated by Mn2++ versus Mg2++. Type III PI3Ks, based upon findings with the yeast homologue, VPSp, are thought to participate constitutively in secretory protein sorting to vacuoles, rather than...
being stimulated in response to cellular agonists.

We have observed (17, 18) that activation of platelet integrin αIIbβ3, via its consequent fibrinogen-dependent aggregation or clustering, stimulates a novel pathway for the generation of PtdIns(3,4)P2 in platelets that is a function of PtdIns3P production and phosphorylation of PtdIns3P by a 4-kinase. No PtdInsP2 is generated during this integrin-dependent event. In the present study, we have examined the nature of the PI3K involved in the integrin-activated pathway, which results in the activation of platelet PDKb/Akt (17, 18). Our data indicate that a PI3K of the Type II subclass, HsC2-PI3K, is most likely to be responsible for the generation of PtdIns3P that occurs.

**EXPERIMENTAL PROCEDURES**

Most reagents were obtained from sources described (17, 18). Calpain I and II inhibitors were purchased from Boehringer Mannheim. HsC2-PI3K cDNA was assembled using a combination of reverse transcription-polymerase chain reaction and standard cDNA screening, by using the sequence of HsC2-PI3K (14) cloned in the mammalian expression vector pBCKMV for transient expression in HEK 293 cells. Its activity after immunoprecipitation was assayed as described below. PI3K isoform-discriminant polyclonal antisera against the first 350 amino acid portion of HsC2-PI3K (14), expressed in *E. coli* as an N-terminal fused glutathione S-transferase protein, were raised in rabbits. These antisera were used for all immunoprecipitations and Western blots directed at HsC2-PI3K and do not detect or immunoprecipitate Type I PI3Ks, HsVPS34, or PI3KC2α. Antibodies to p85αβ subunits and PI3KC2α were the generous gifts of Drs. Ivan Gout and Jan Domini (Ludwig Institute, London). Antibody to HsVPS34 was prepared by Dr. Volinia (19).

**Activation of Platelets and CHRF-288 Labeled with [32P]Pi.** Washed human platelets were labeled to equilibrium with [32P]Pi, as described (17, 18). CRFF-288 cells were washed with DMEM, incubated overnight with 0.1% bovine serum albumin/DMEM in the absence of serum, and incubated in low P,-DMEM with [32P]Pi (1 mCi/ml), as reported (17). Lysates (platelet, CHRF-288, or HEK293) were spun at 90 min 4°C, HsC2-PI3K was immunoprecipitated from supernatants and Triton-soluble fractions and HsC2-PI3K immunoprecipitates were dissolved in SDS-reducing buffer and proteins resolved by one-dimensional SDS-polyacrylamide gel electrophoresis on 7.5% gels, prior to electroblotting to nitrocellulose for Western blotting and enhanced chemiluminescence detection, as described previously (5). CSK from Triton lysates before mixing with sample buffer, such that the same amount of CSK protein would be applied per lane. Labeled platelets were incubated as in (17, 18).

**Immunoprecipitations**—Nonlabeled platelets or CRFF-288 cells were incubated ± agonists/calcium inhibitors as above, and incubations were washed with ice-cold Triton lysis buffer containing 100 μg/ml calpeptin (17) or RIPA buffer, containing 50 μg/ml Tris, 150 μM NaCl, 1% Triton X-100, 0.5% Na+ deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and calpeptin, pH 7.6. After lysates (platelet, CRFF-288, or HEK293) were spun at 100,000 × g × 90 min 4°C, HsC2-PI3K was immunoprecipitated from 450 μl of supernatants with antibody and protein A/Sepharose. Immunoprecipitates were washed once with Triton lysis buffer, then three times with HEPES (5 mM)/EDTA (2 mM), pH 7.4, and re-suspended to 80 μl in HEPES/EDTA for PI3K activity assays (40 μl/100 μl assay) using PtdIns, PtdIns4P, or PtdIns4,5P2 (200 μM) substrates that had been suspended in HEPES/EDTA. In some cases, varied concentrations of wortmannin in MeSO/buffer vehicle were prepared freshly and added to washed immunoprecipitates for 5 min at 37°C prior to assay of activity, in comparison with the same amount of MeSO/buffer vehicle (17, 18). Assays contained [γ-32P]ATP (40 μM; 1 mCi/ml), MgCl2 (4 mM), or MnCl2 (4 mM), Tris (20 mM)/NaCl (100 mM)/EGTA (0.5 mM)/HEPES (3 mM)/EDTA (1.2 mM), pH 7.5, and were run at 30°C for 20 min. Activity with respect to PtdIns/Mg2+ was determined to be linear for 30 min. Lipids were extracted and resolved by HPLC as above. The PI3K, α5/PI3K, was also immunoprecipitated from Triton-soluble fractions using antibodies to the p85α/p50β subunits (5) for assay of kinase activity under the above conditions. In other studies, Triton lysates from unstimulated platelets or CRFF-288 cells, without calpeptin or EGTA, were incubated at room temperature for various periods up to 60 min ± calpeptin ± 2 mM Ca2+·EGTA. Incubations were terminated with 50 μg/ml calpeptin, 5 mM EGTA, pH 7.4, and samples chilled and centrifugated. HsC2-PI3K was immunoprecipitated from supernatants and PI3K activity assayed with PtdIns, as above.

**Western Blotting**—Platelet and CRFF-288 Triton-soluble and insoluble ("CSK") fractions and HsC2-PI3K immunoprecipitates were dissolved in SDS-reducing buffer and proteins resolved by one-dimensional SDS-polyacrylamide gel electrophoresis on 7.5% gels, prior to transfer to nitrocellulose for Western blotting and enhanced chemiluminescence detection, as described previously (5). CSK from Triton lysates were suspended in buffer at 4–11 times their concentrations in Triton lysates before mixing with sample buffer, such that the same amount of CSK protein would be applied per lane.

**RESULTS AND DISCUSSION**

Exposure of platelets to a variety of agonists, under conditions that promoted αIIbβ3 + FIB-dependent aggregation, led to

**Fig. 1. Effects of platelet exposure to activators of αIIbβ3 and inhibitors of calpain on accumulation of [32P]PtdIns3P and activation of HsC2-PI3K.** A. radiolabeled or nonlabeled platelets were incubated for various periods ± SFLLRN ± FIB and (at two time points) ± calpain inhibitors. Accumulation of [32P]PtdIns3P (squares, dotted line) and activation of HsC2-PI3K (circles, solid line) are presented as multiples of agonist-free controls, where the range of duplicates is contained within symbols. Effects of inhibitors are shown by isolated symbols. No activation was observed in the absence of FIB. B. platelets were incubated for 7 min ± LIBS, ± PMA, ± FIB, with (solid bars) or without (open bars) calpain inhibitors, and HsC2-PI3K activity was measured in immunoprecipitates from lysates using PtdIns substrate. C. radiolabeled platelets were incubated as in B and generation of [32P]PtdIns3P quantitated. Results for B and C are multiples of agonist-free controls, with the range of duplicates ± 0.06. Representative basal radioactivities were 6736 ± 102 dpm (platelet PtdIns3P) and 15,655 ± 749 dpm (immunoprecipitated HsC2-PI3K).
the activation of HsC2-PI3K and, in \textsuperscript{32}P-labeled platelets, transient accumulation of \textsuperscript{32}P\textunderscore PtdIns3P (Fig. 1). Both effects were inhibited by wortmannin (IC\textsubscript{50} 7 nM, for \textsuperscript{32}P\textunderscore PtdIns3P; IC\textsubscript{50} 6 nM for HsC2-PI3K), in keeping with the wortmannin sensitivity of expressed HsC2-PI3K (IC\textsubscript{50} 10 nM). As shown in Fig. 1A, the increased activity of HsC2-PI3K in immunoprecipitates from Triton-soluble fractions of platelets incubated with SFLLRN (directed to the thrombin receptor) was sustained for up to 14 min and slightly preceded the accumulation of [\textsuperscript{32}P]\textunderscore PtdIns3P in stimulated platelets. The transient accumulation of [\textsuperscript{32}P]\textunderscore PtdIns3P is probably attributable to the increased activity of PtdIns3P 4-kinase under these conditions (17, 18). In the presence of FIB (not shown), or in the presence of maximally effective concentrations of calpeptin (IC\textsubscript{50} 1 \mu M) or calpain I inhibitor (IC\textsubscript{50} 0.3 \mu M), the increase in PtdIns3P levels and activation of HsC2-PI3K were abolished. Calpain I inhibitor (90% inhibition at 1 \mu M) was more effective than calpain II inhibitor (18% inhibition at 1 \mu M). Similarly, increases in HsC2-PI3K activity (Fig. 1A) and [\textsuperscript{32}P]\textunderscore PtdIns3P (Fig. 1C) in response to PMA (activating protein kinase C) + FIB or LIBS (directly activating \(\alpha_{IIb}\beta_3\) + FIB) were blocked by calpain inhibition and did not occur in the absence of FIB. Both PMA and SFLLRN are known to activate p85/PI3K, whether or not FIB is present, and thereby contribute to “inside-out” signaling leading to the sustained activation of \(\alpha_{IIb}\beta_3\) (5). SFLLRN also activates another Type I enzyme, PI3K\textsubscript{\gamma}, which is dependent upon \(\beta y\) subunits of GTP-binding proteins, and apparently is not involved in signaling leading to \(\alpha_{IIb}\beta_3\) activation (5). LIBS by-passes such a pathway, promoting the FIB binding conformation of \(\alpha_{IIb}\beta_3\) by interaction with the \(\beta y\) subunit (21) in a wortmannin-insensitive manner, i.e. without the prerequisite of p85/PI3K activation or stimulated accumulation of 3-OH-phosphorylated phosphoinositides (5, 17). Once FIB binds to \(\alpha_{IIb}\beta_3\) and aggregation occurs, however, “outside-in” signaling that promotes calpain activation is triggered, leading to formation of PtdIns3P and PtdIns(3,4)P\textsubscript{2} (17, 18). Calpain, a Ca\textsuperscript{2+} - dependent thiol protease, is known to be activated intracellularly when FIB binds to \(\alpha_{IIb}\beta_3\) and aggregates platelets that have been stimulated in the presence of Ca\textsuperscript{2+} (22). To simulate these calpain-activating conditions, in the absence of integrin activation, unstimulated platelet lysates were incubated at room temperature in the presence of millimolar Ca\textsuperscript{2+}, and the activity of immunoprecipitated HsC2-PI3K was then assayed. It was found that activity rose transiently (2.49 ± 0.12-fold after 10 min, 4.08 ± 0.18-fold after 20 min, and 3.24 ± 0.17-fold after 60 min) and that the increase was blocked by omitting

**Fig. 2.** Effects of platelet stimulation on phosphoinositide 3-kinase activities in HsC2-PI3K immunoprecipitates. Immunoprecipitates of HsC2-PI3K from resting or activated platelets, as in Fig. 1, were assayed in the presence of PtdIns (open bars), PtdIns4P (gray bars), or PtdIns(4,5)P\textsubscript{2} (black bars). The ratio of 3-kinase activity (no other kinase activities were detectable) from unstimulated preparations were PtdIns: PtdIns4P = 490:1; PtdIns: PtdIns(4,5)P\textsubscript{2} = 270:1. The difference for PtdIns4P substrate versus PtdIns(4,5)P\textsubscript{2} was not statistically significant.

**Fig. 3.** Effects of stimulation of CHRF-288 cells on accumulation of 3-phosphorylated phosphoinositides and HsC2-PI3K activity. A, \textsuperscript{32}P-labeled CHRF-288 were stimulated for various periods with SFLLRN ± FIB, and labeled 3-phosphorylated phosphoinositides were quantitated. No differences were observed between SFLLRN alone and SFLLRN + FIB. Results are expressed as a multiple of agonist-free controls, where basal levels were 2111 dpm (PtdIns\textsubscript{3}), 2372 dpm (PtdIns(3,4)P\textsubscript{2}), and 11,128 dpm (PtdIns(3,4,5)P\textsubscript{3}). B, HsC2-PI3K was immunoprecipitated from CHRF-288 lysates, as in Fig. 1 and assayed with PtdIns substrate. Results are a multiple of control levels (range of duplicates was ±0.05) and did not vary with time of incubation with agonist.

**Fig. 4.** Western blots of HsC2-PI3K in platelet lysates. Samples from Triton-soluble (TS) fractions, Triton-soluble fractions after immunoprecipitation of HsC2-PI3K (PS), or immunoprecipitates (IP) were resolved by SDS-polyacrylamide gel electrophoresis and proteins transferred to nitrocellulose for immunodetection of HsC2-PI3K. Similarly, Triton-insoluble (CSK) fractions were examined. Variables included resting platelets (R), SFLLRN + FIB (S), or SFLLRN + FIB + calpain inhibitor (S + C)-treated platelets, and Triton lysates from resting platelets (R) that were then incubated with Ca\textsuperscript{2+} (+) or Ca\textsuperscript{2+} + calpain inhibitor (−), after which Triton-soluble and -insoluble CSK fractions were obtained and resolved on gels (the Triton-soluble fractions from incubations ± Ca\textsuperscript{2+} were more dilute than other Triton-soluble fractions). The position of HsC2-PI3K is indicated by an arrow.

Ca\textsuperscript{2+} or by including calpain inhibitors.

The increased 3-kinase activity in HsC2-PI3K immunoprecipitates from stimulated platelets was specific for PtdIns as a substrate (Fig. 2). As would be expected from the substrate specificity of 293 cell-expressed HsC2-PI3K, which exhibited a greater than 10 times preference for PtdIns (not shown), im-
munoprecipitated HsC2-PI3K from resting platelets strongly favored PtdIns(1′2′)P1dIns3P = 155,122 ± 6968 dpm) over PtdIns4P(1′2′)PtdIns3(4,3)P2 = 316 ± 55 dpm) or PtdIns(4,5)P2(1′2′)PtdIns3(4,3)P2 = 573 ± 41 dpm), and activity with respect to PtdIns was selectively increased by platelet stimulation. Thus, the increased PI3K activity seen with respect to PtdIns is not attributable to co-precipitating Type I kinase activity from activated platelets (5). Furthermore, the marked sensitivity of the activity to wortmannin and selectivity of HsC2-PI3K antibody against PI3KC2α (13) being co-precipitated, despite the fact that PI3KC2α is present in platelets and CHRF-288 cells.2 Finally, unlike the case for both expressed and native HsVPS34 (also present in platelets and CHRF-288 cells), which shows enhanced activity with Mn2+ versus Mg2+ as a co-factor (19), immunoprecipitated HsC2-PI3K was less than 10% as active when Mn2+ was substituted for Mg2+. Thus, the stimulated PI3K activity of HsC2-PI3K immunoprecipitates is not due to contaminating or co-precipitating Type III (HsVPS34) activity. Additionally, such other PI3Ks were not immunologically or enzymatically detectable in HsC2-PI3K immunoprecipitates (not shown).

As we have reported (20, 23), CHRF-288, a leukemic cell line derived from a platelet precursor cell, the megakaryoblast (24), can be stimulated by a variety of physiological agonists to accumulate PtdInsP3 and PtdIns(3,4)P2. These increases are sensitive to wortmannin, and CHRF-288 cells contain p85/P13K and PI3Kγ. Type I enzymes whose activities in lysates can be stimulated by guanosine 5′-O-(thiotriphosphate) and βγ subunits of GTP-binding proteins, respectively. Despite displaying apparently normal α1β1 subunits at their surface, however, CHRF-288 cells cannot undergo the activation of this integrin that leads to the binding of FIB (25) and aggregation. We found that CHRF-288 cells, after incubation with SFLLRN + FIB (or PMA + FIB, not shown) accumulated no [32P]PtdIns3P, whereas [32P]PtdInsP3 and [32P]PtdIns(3,4)P2 were formed rapidly (Fig. 3A), a pattern similar to the “pre-integrin” accumulation of 3-OH-phosphorylated phosphoinositides in stimulated platelets (18). Furthermore, although much immunoprecipitable HsC2-PI3K activity was present in Triton or RIPA lysates of CHRF-288 cells, no increased activity was observed when CHRF-288 were activated with SFLLRN + FIB or PMA + FIB (Fig. 3B). In contrast, lysates of CHRF-288 incubated in the presence of Ca2+ showed a time-dependent increase in immunoprecipitable HsC2-PI3K activity (2.3 ± 0.2-fold in 10 min, 3.6 ± 0.1-fold in 20 min, and 4.2 ± 0.4-fold in 60 min), which was prevented by omitting Ca2+ or including calpain inhibitor. This indicated that HsC2-PI3K of CHRF-288 cells could be activated in an apparently calpain-dependent manner if the requirement for activated integrin were bypassed. Thus, failure to activate α1β1α2β3 is associated in intact CHRF-288 with failure to activate HsC2-PI3K and stimulate accumulation of PtdIns3P.

Western blotting of CSK and Triton-soluble (TS) fractions of platelets (Fig. 4) with antibody to HsC2-PI3K revealed a protein band at about 200 kDa in both fractions. This band in the Triton-soluble fraction could be decreased by immunoprecipitation with HsC2-PI3K antibody (PS), and a corresponding band was found for the Western blot of the immunoprecipitate (IP). The migration of the band did not shift detectably for blot from activated platelets (SFLLRN) or from platelet lysates incubated with Cu2+. In conclusion, our data indicate that HsC2-PI3K is activated in platelets in a manner dependent upon the reorganization of integrin α1β1α2β3, FIB binding and aggregation, and, most probably, calpain I activation. Given its strong preference for PtdIns as a substrate, its susceptibility to wortmannin, and its activation under the same conditions required for the accumulation of PtdIns3P in intact cells, HsC2-PI3K is the most reasonable choice for the enzyme responsible for generating PtdIns3P in stimulated platelets. It is possible that HsC2-PI3K is a substrate for calpain I and thereby activated by it, but, at this point, other mechanisms involving other calpain targets are equally likely to contribute to the stimulation observed in vivo. Inasmuch as the antibody used for immunoprecipitation and identification of resting and activated HsC2-PI3K is directed to the N-terminal 350-amino acid region of HsC2-PI3K, and the catalytic domain lies approximately between amino acids 1037 and 1320 (14), any activating cleavage target for calpain would have to be C-terminal to these regions. If the cleavage site were in the C2 domain of HsC2-PI3K, for example, a decrease in size might not be easily detectable under our conditions. Further studies with tagged, expressed HsC2-PI3K and purified calpain I may be needed to identify possible activating cleavage sites for HsC2-PI3K in stimulated platelets.

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