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Phosphoinositide 3-Kinase and p72^{syk} Noncovalently Associate with the Low Affinity Fcγ Receptor on Human Platelets through an Immunoreceptor Tyrosine-based Activation Motif

RECONSTITUTION WITH SYNTHETIC PHOSPHOPEPTIDES*

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Previously, we have demonstrated that the cytoplasmic tyrosine kinase p72^{syk} is coupled to the platelet Fc receptor for IgG (FcγRIIA) (Chacko, G. W., Duchemin, A. M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1994) *J. Biol. Chem.* 269, 32435–32440). Further analysis of the platelet activation by FcγRIIA demonstrated that FcγRIIA is also inducibly coupled to the serine/threonine and lipid kinase, phosphoinositide 3-kinase (PI 3-K). Activation of platelets with anti-FcγRIIA antibodies resulted in the noncovalent association of PI 3-K with FcγRIIA as well as an increase in FcγRIIA-associated PI 3-K activity. Binding of both p72^{syk} and PI 3-K to FcγRIIA was reconstituted with synthetic phosphopeptides corresponding to the sequence of the atypical immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain of FcγRIIA. Our findings demonstrate that coupling of both p72^{syk} and PI 3-K activities to FcγRIIA is regulated by tyrosine phosphorylation of the ITAM, and we speculate that p72^{syk} might act as an adapter to recruit PI 3-K to activated FcγRIIA.

Human platelets express a single Fc receptor for IgG (FcγR),[1] the low affinity receptor encoded by the FcγRIIA gene (1–4). Clustering of FcγRIIA with ligand or anti-FcγRIIA antibodies induces platelet activation characterized by a dramatic increase in tyrosine phosphorylation of a number of platelet proteins, by size and shape changes in platelets, by secretion of intracellular granule contents, by increased adhesion to platelet-specific ligands, and by platelet aggregation (5–8).

The intracytoplasmic domain of FcγRIIA contains a single copy of an activation motif termed immunoreceptor tyrosine-based activation motif (ITAM) that is loosely defined by the consensus sequence $YX_{(2)}LX_{(6-8)}YX_{(2)}L$, where *X* is any amino acid (9). ITAM sequences have also been identified in signaling subunits of the T and B cell antigen receptors, the high affinity Fc receptor for IgE (FcϵR), and other FcγR (10–14). Clustering of ITAM-containing receptors by cognate ligand or specific antibodies has been shown to result in phosphorylation of two critical tyrosine residues in the ITAM which converts the ITAM into a high affinity binding site for members of the Syk/ZAP-70 family of tyrosine kinases that interact with this motif through tandem *src* homology region 2 (SH2) domains. Phosphorylation of ITAMs appears to be mediated by members of the Src family of tyrosine kinases, and the importance of Src kinases in ITAM phosphorylation and Syk/ZAP-70 recruitment has been well documented (15–17). Thus, ITAM phosphorylation initiated by receptor clustering and the subsequent recruitment of SH2 domain-containing proteins provides one inducible mechanism by which receptors lacking endogenous catalytic activity can be coupled to effector molecules that are catalytically active.

The ITAM integral to FcγRIIA is unusual in that it contains an extended spacer of 12 amino acids (18) between the two *YXX*L pairs, while other members of the ITAM family contain spacer regions of 6–8 amino acids. Additionally, while ITAM-containing receptors exist as multisubunit complexes comprising an extracellular ligand binding unit that is noncovalently associated with intracellular ITAM-containing signaling subunits (10), FcγRIIA is a single-chain transmembrane polypeptide with an ITAM integral to its primary structure. Mutational analysis of the tyrosine residues of the FcγRIIA-ITAM has shown that they are required for receptor tyrosine phosphorylation and for phagocytosis mediated by FcγRIIA (19–21), suggesting that the FcγRIIA-ITAM remains functional despite its divergence from the consensus sequence. Results from another study suggest that the ITAM of FcγRIIA transduces a qualitatively different signal from the ITAM of the γ-chain subunit of the high affinity FcγR, implying that there are unique aspects to the FcγRIIA-ITAM despite the common functional features it shares with other members of the group (22). Analysis of the mechanism by which FcγRIIA in platelets transduces signal therefore provides insight into the understanding of ITAM signaling from the perspective of this simple yet unique model.

Previous work from our laboratory, and from others, has revealed that clustering of FcγRIIA on platelets induces receptor tyrosine phosphorylation and noncovalent association of the tyrosine kinase p72^{syk} with tyrosine-phosphorylated FcγRIIA (2, 7). These observations lend support to a model of p72^{syk} coupling to FcγRIIA induced by tyrosine phosphorylation of the ITAM (1, 5) presumably by one or more of the five Src kinases expressed in platelets (23). p72^{syk} is a member of the Syk/ZAP70 family of tyrosine kinases and has been shown to be

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[1] The abbreviations used are: FcγR, Fc receptor for IgG; ITAM, immunoreceptor tyrosine-based activation motif; PDGF, platelet-derived growth factor; SOS, Son of Sevenless; Grb2, growth factor receptor binding protein 2; PI 3-K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; SH2, *src* homology region 2; SH3, *src* homology region 3.

involved in signal generation by the ITAM-containing receptors, such as the B cell antigen receptor), and the high affinity Fc receptors for IgG and IgE (10).

Activation of $p72^{syk}$ in platelets is induced by several platelet agonists including thrombin, collagen, and platelet activation factor (24–26). Engagement of the fibrinogen receptor (the integrin $\alpha_{IIb}\beta_3$) by fibrinogen in thrombin-activated platelets further up-regulates $p72^{syk}$ activity (8, 23, 27). These observations suggest that $p72^{syk}$ plays a central role in platelet activation and that regulation of its activity is complex. Additionally, targeted gene disruption of the $p72^{syk}$ gene in the mouse has been shown to result in perinatal death due to massive hemorrhages (28, 29), further suggesting a critical role for $p72^{syk}$ in platelet function.

Experiments with ITAM-containing receptors have shown that phosphoinositide 3-kinase (PI 3-K) is often activated in response to receptor clustering (30, 31). PI 3-K is a serine/threonine and lipid kinase that exists as a heterodimer comprising an 85-kDa regulatory subunit containing two SH2 domains and one SH3 domain and a 110-kDa catalytic subunit (32, 33). The presence of the SH2 and SH3 domains in PI 3-K implies that noncovalent interactions with target proteins take place, and considerable experimental evidence exists to support this contention (31, 32). PI 3-K is also rapidly activated and translocated to the cytoskeleton when platelets are stimulated with the potent agonist thrombin (34, 35). More recently, it has been shown that the translocation of PI 3-K to the cytoskeleton is integrin-dependent and that platelet aggregation induced by thrombin through its G-protein-coupled receptor can be reversed by inhibitors of PI 3-K activity (36, 37) suggesting that PI 3-K, like $p72^{syk}$, plays an important role in platelet activation.

PI 3-K has been implicated in FcγR function in neutrophils, the monocytic cell line U937, and natural killer (NK) cells suggesting a conserved role in FcγR-mediated signaling (38–40). Fc receptor ligation results in an increase in the specific activity of PI 3-K as well as an increase in phosphotyrosine-associated PI 3-K activity in response to FcγR stimulation, although the specific mechanism of activation was not determined. The understanding of how PI 3-K is activated on phagocytes and NK cells is further complicated by the different isoforms of FcγR expressed on these cell types. Analysis of FcγRIIA activation in platelets which express only a single FcγR, provides specific information on the mechanism by which the low affinity FcγRIIA transduces signal and contributes to the general paradigm describing how FcγR activates PI 3-K.

In this study we demonstrate that, upon platelet activation by FcγRIIA clustering, PI 3-K is coupled-associated with FcγRIIA. We show that noncovalent association of $p72^{syk}$ and PI 3-K can be reconstituted using synthetic phosphopeptides that correspond to the sequence of the FcγRIIA-ITAM. Interestingly, while $p72^{syk}$ association with the phosphorylated FcγRIIA-ITAM appears independent of the activation status of platelets, the binding of PI 3-K to FcγRIIA requires platelet activation. These data showing that the presence of a phosphorylated ITAM is sufficient to induce the binding of $p72^{syk}$ but not PI 3-K to FcγRIIA suggest that additional modifications are required to promote association of the activated FcγRIIA complex with molecules that are downstream of the receptor complex in the signaling cascade. We propose that PI 3-K binding to FcγRIIA may require an adapter molecule(s) that binds both FcγRIIA and PI 3-K and speculate that $p72^{syk}$ is an attractive candidate for this function.

EXPERIMENTAL PROCEDURES

Materials—Neutravidin was obtained from Pierce. HP-TLC plates were from E. Merck, Darmstadt, Germany. Wortmannin was purchased from Sigma.

Antibodies—Monoclonal antibody IV.3 (IgG2b) against human FcγRII was obtained from Medarex, Annandale, NJ (41). Monoclonal antibody CIKM5 (IgG1) against human FcγRII was used as ascites (42). The monoclonal antibody against the N-terminal SH2 domain of the p85 subunit of PI 3-K and monoclonal antibody 4G10 against phosphotyrosine were obtained from UBI, Lake Placid, NY. Polyclonal goat anti-mouse IgG (GAM) was obtained from Pierce. The anti-$p72^{syk}$ polyclonal antibody was the kind gift of Drs. Couture and Mustelin (43). MOPC141 (IgG2b), a myeloma protein of irrelevant specificity, was purified as described (2).

Platelets and Activation Procedures—Human platelets were obtained by venipuncture from healthy humans with informed consent, activated by FcγRIIA cross-linking using anti-FcγRIIA monoclonal antibody IV.3 and GAM as a secondary clustering reagent, and lysed in Triton X-100 lysis buffer (1% Triton X-100, 25 mM Hepes-KOH, pH 7.4, 145 mM NaCl, 10 mM EDTA) as described (2). Immunoadsorptions, SDS-PAGE, transfer to nitrocellulose, immunoblotting, and detection by chemiluminescent procedures were also performed as described (2).

Platelet Aggregation Assays—Human platelets were isolated as described above. Platelets were maintained at room temperature throughout isolation procedures. For aggregation studies, platelets were washed in platelet wash buffer (145 mM NaCl, 5.0 mM KCl, 5.5 mM D-glucose, 1.0 mM $MgCl_2$, 15 mM Hepes-KOH, pH 7.4), and incubated at room temperature with concentrations of wortmannin ranging from 1 to 100 nM for 5 min or incubated with platelet wash buffer alone. Platelets were stimulated by first equilibrating them at 37 °C for 3 min in aggregation buffer (platelet wash buffer containing 1 mM $CaCl_2$) with stirring and then adding agonists. Aggregation responses were recorded on a Chronolog Dual Channel LumiAggregometer (Model 560). Platelet responses to thrombin, monoclonal antibody IV.3 in combination with GAM, anti-FcγRIIA monoclonal antibody CIKM5 (IgG1), and GAM alone were tested in three independent experiments with platelets from different donors. Doses of agonists used were as follows: thrombin, 2 units/ml; IV.3, 2.8 μg/ml; CIKM5, 1:25 dilution of ascites, and GAM, 100 μg/ml.

PI 3-K Activity Assay—Phosphatidylinositol liposomes were prepared by resuspending vacuum-dried phosphatidylinositol in assay buffer (30 mM Hepes-KOH, pH 7.4) at 10 mg/ml and sonicating on ice for 10 min at 50 MHz. Immunoadsorbates were washed four times in Triton X-100 lysis buffer and three times in 10 mM Hepes-KOH, pH 7.4. Washed immunoadsorbates were then resuspended in 40 μl of PI 3-K assay buffer to which 10 μCi of [γ-^{32}P] ATP, of specific activity 6000 Ci/mmol, in assay buffer was then added to the immune complexes at a final concentration of 1 μCi/μl followed by the addition of 20 μl of liposomes. Reactions were allowed to proceed for 30 min at room temperature or 15 min at 30 °C and were terminated by the addition of 100 μl of 1 N HCl. Lipids were extracted by adding 300 μl of $CHCl_3$:CH_3OH (1:1), removing the organic phase which was then dried under vacuum, redissolved in 50 μl of $CHCl_3$:CH_3OH (1:1), and spotted on Silica G-60, HP-TLC plates. Lipid products were developed by thin layer chromatography (TLC) in a chloroform:pyridine:boric acid:formic acid:water solvent system as described (44) and visualized by autoradiography. R_f values were estimated for putative PI 3-P and PI 4-P products. PI 3-K products were identified by their sensitivity to 100 nM wortmannin (45) and 0.5% Triton X-100 (39, 46).

Synthetic Phosphopeptides—Peptides corresponding to the ITAM sequence of FcγRIIA plus flanking residues (Fig. 4) were synthesized by the Peptide Synthesis Facility, Ohio State University, and Quality Controlled Biochemicals, Hopkinton, MA. Peptides were purified by reverse-phase and size exclusion chromatography to greater than 95% purity. Peptides were evaluated by mass spectrometry and ^{31}P NMR for correct molecular weight and incorporation of phosphotyrosine residues, respectively. Peptides were biotinylated at the N-terminal glutamic acid residue to allow coupling to an avidin-coated solid phase (Neutravidin).

Peptide Binding Assay—Peptides were diluted in 100 mM Hepes-KOH, pH 7.4, buffered Hanks' balanced salt solution supplemented with 5% bovine serum albumin to which was added 100 μl of Neutravidin bead slurry. The suspension was placed on a rocking platform for 1 h, washed five times in Hanks' balanced salt solution, and then incubated for 1 h or overnight with platelet lysates. Peptide adsorbates were washed extensively in lysis buffer, separated by reducing SDS-polyacrylamide gel electrophoresis, transferred to nitro-

cellulose, and immunoblotted to detect phosphotyrosine or candidate molecules.

RESULTS

FcγRIIA-associated PI 3-K Activity Is Rapidly and Transiently Increased after Platelet Activation by FcγRIIA Stimulation—Previous work has demonstrated that clustering of FcγRIIA on platelets and other cells induces tyrosine phosphorylation of FcγRIIA, noncovalent association, and activation of the tyrosine kinase $p72^{syk}$ with FcγRIIA (2, 7, 47, 48). Since several tyrosine-phosphorylated proteins including $p72^{syk}$ co-immunoadsorb with activated FcγRIIA, we sought to determine whether other catalytically active molecules were also noncovalently complexed with activated FcγRIIA. Of special interest was the lipid and serine/threonine kinase, PI 3-K, since it has been reported to play a role in platelet activation by thrombin (2, 35, 49) as well as to be activated by tyrosine kinase pathways (31). Consequently, PI 3-K activity co-immunoadsorbing with FcγRIIA was detected by assaying for the ability of FcγRIIA immunoadsorbates to phosphorylate phosphatidylinositol at the D-3 position of the inositol ring (see "Experimental Procedures"). Since commonly used solvent systems do not resolve PI 3-K products from those of other phosphoinositide kinases such as PI 4-K, phospholipids were separated by TLC using a solvent system containing boric acid. The ability of boric acid to bind *cis*-diols and thereby to allow distinction between phosphatidylinositol phosphorylated at the D-3 or D-4 positions of the inositol ring has been documented (44). The R_f value of phosphatidylinositol 3-phosphate was in good correspondence with published values (44), and addition of the PI 3-K inhibitors wortmannin and Triton X-100 to PI 3-K immunoadsorbates (39, 46) abolished any detectable PI 3-K activity (Fig. 1, *top panel*, *lanes 2* and *3*) showing that PI 3-K activity was specifically detected and resolved in this assay. Within 30 s of clustering FcγRIIA on platelets, there is a rapid and transient increase in FcγRIIA-associated PI 3-K activity (Fig. 1, *bottom panel*) that returns to near resting levels by 1 min. A secondary peak of PI 3-K activity was also observed 3–5 min after FcγRIIA clustering (data not shown) consistent with reports of two waves of PI 3-K activity in thrombin-stimulated platelets (37). Therefore, FcγRIIA-mediated platelet activation results in transiently elevated levels of FcγRIIA-associated PI 3-K activity.

PI 3-K Rapidly Associates with FcγRIIA in Platelets Activated by Clustering of FcγRIIA—To determine whether the PI 3-K activity co-immunoadsorbing with activated FcγRIIA was due to the binding of PI 3-K to the activated receptor complex, FcγRIIA was immunoadsorbed from detergent lysates of resting and activated platelets with monoclonal antibody IV.3, and associated PI 3-K was detected by immunoblotting with a monoclonal antibody against the p85 subunit of PI 3-K. Noncovalent association of PI 3-K with FcγRIIA was detected approximately 30 s after clustering (Fig. 2, *lane 4*) consistent with our observations on FcγRIIA-associated PI 3-K activity (Fig. 1). Although the amount of PI 3-K that coimmunoadsorbs with FcγRIIA appears to decrease to near resting levels (Fig. 2, *lane 4*) by a 1-min or greater interval after clustering (Fig. 2, *lane 3*), it is still readily detected in detergent-soluble fractions of activated platelets by immunoadsorption and immunoblotting with anti-p85 antibodies (Fig. 2, *lanes 1* and *2*) suggesting that depletion of PI 3-K from supernatants of detergent lysates of activated platelets is unlikely to be the cause of reduced association with FcγRIIA. Therefore, activation of platelets by anti-FcγRIIA antibodies along with secondary cross-linking antibodies results in the phosphorylation of FcγRIIA on tyrosine residues and a rapid and apparently transient association of PI 3-K with the activated (tyrosine-phosphorylated) FcγRIIA.

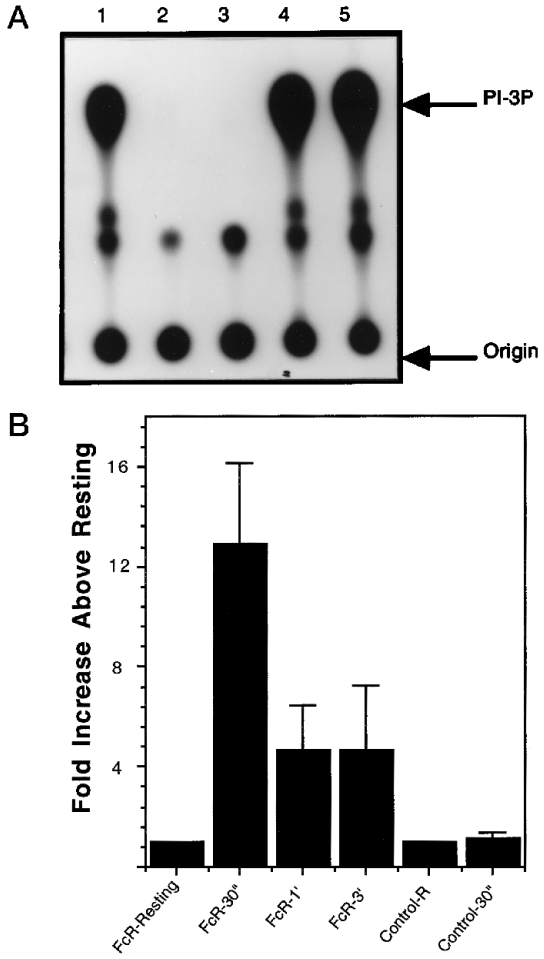

FIG. 1. **Clustering of FcγRIIA on platelets induces a rapid and transient increase in FcγRIIA-associated PI 3-K activity.** Platelets were activated by FcγRIIA clustering and lysed in 1% Triton X-100, and immune complex kinase assays to detect PI 3-K activity were performed on PI 3-K or FcγRIIA immunoadsorbates (see "Experimental Procedures"). PI 3-K products were separated by TLC and visualized by autoradiography. ^{32}P incorporated in PI 3-K products was quantified by scraping TLC plates and counting by liquid scintillation. *Top panel*, PI 3-K immunoadsorbates from platelets were subjected to the immune kinase complex assay for PI 3-K, and lipid products were separated by TLC. Addition, *in vitro*, of 100 nM wortmannin (*lane 2*) or of 0.1% Triton X-100 (*lane 3*) completely inhibits detectable PI 3-K activity (*arrow*) compared to untreated samples (*lanes 1* and *5*) or platelets pretreated with 1 μM genistein (*lane 4*). Other labeled lipid products are insensitive to both Triton X-100 and wortmannin and are likely the result of low levels of contaminating phospholipases and phosphoinositide kinases. *Bottom panel*, FcγRIIA immunoadsorbates from resting and activated platelets show a rapid increase in associated PI 3-K activity within 30 s of receptor clustering. Absolute values of ^{32}P (as disintegrations/min) incorporated due to PI 3-K activity are expressed as fold increase above PI 3-K activity associated with FcγRIIA immunoadsorbates of unstimulated platelets which were normalized to an arbitrary value of 1. Control immunoadsorptions to ascertain nonspecific binding of PI 3-K were performed with MOPC 141 (IgG2b) a monoclonal antibody of irrelevant specificity from both resting and activated platelet lysates. These results are derived from three independent experiments. *Error bars* indicate standard deviation from the mean.

PI 3-K Activity Is Required for FcγRIIA-mediated Platelet Aggregation—Clustering of FcγRIIA on platelets induces platelet activation resulting in platelet aggregation which is conveniently measured *in vitro*. To determine whether the coupling of PI 3-K to FcγRIIA was required for platelet aggregation, wortmannin, an inhibitor of PI 3-K, was used in platelet aggregation assays. Addition of wortmannin at nanomolar concentrations inhibited FcγRIIA-induced platelet aggregation in

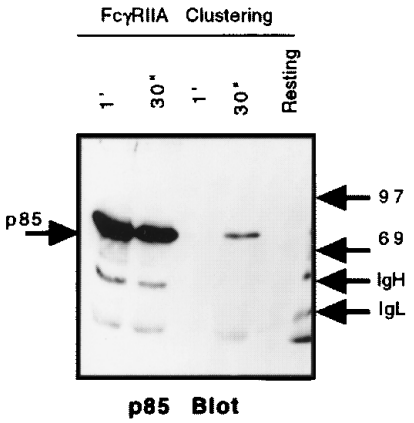

FIG. 2. **Clustering of FcγRIIA on platelets induces a rapid and transient association of PI 3-K with activated FcγRIIA.** Platelets were activated by FcγRIIA clustering and lysed in 1% Triton X-100 as described in Fig. 1. Immunoadsorbates with anti-FcγRIIA (*lanes 3*, *4*, and *5*) or anti-PI 3-K antibodies (*lanes 1* and *2*) were separated by reducing SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted to detect the p85 subunit of PI 3-K. An 85-kDa band is detected in FcγRIIA immunoadsorbates from lysates of platelets activated for 30 s by FcγRIIA clustering (*lane 4*), is not detected in FcγRIIA immunoadsorbates from resting platelets (*lane 5*) or in FcγRIIA immunoadsorbates from lysates of platelets activated for 1 min or greater by FcγRIIA clustering (*lane 3*). Control immunoadsorptions with anti-p85 antibodies demonstrate the presence of PI 3-K in activated platelet lysates (*lanes 1* and *2*) and the position to which it migrates on the gel.

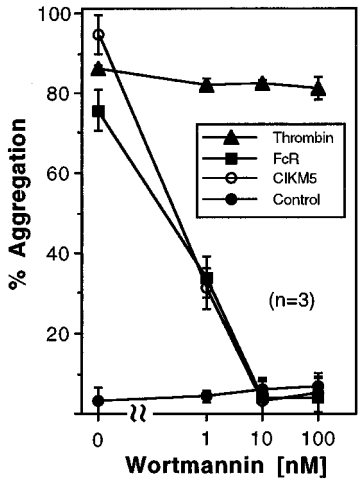

FIG. 3. **Inhibition of FcγRIIA-mediated platelet aggregation by wortmannin.** Human platelets were isolated and prepared for aggregometry as described (see "Experimental Procedures"). Platelets were incubated with varying doses of wortmannin and then stimulated with thrombin (2 units/ml), anti-FcγRII monoclonal antibody IV.3 (IgG2b), and goat anti-mouse (GAM), anti-FcγRIIA monoclonal antibody CIKM5 (IgG1), or GAM alone. CIKM5 is an anti-FcγRII monoclonal antibody of the murine IgG1 subclass that clusters FcγRII via a two-point interaction through its Fab and Fc portions and does not require clustering with a secondary antibody (31). Maximal platelet aggregation was measured on a Chronolog Dual Channel Lumi-Aggregometer (Model 560). Results shown are the average of three independent experiments with platelets from different donors. *Error bars* indicate standard deviation from the mean.

a dose-dependent manner (Fig. 3). Wortmannin pretreated platelets were still able to respond to thrombin at the maximal aggregating dose of 2 units/ml (Fig. 3, *solid triangles*) indicating that at the nanomolar concentrations used in this assay, wortmannin did not totally inhibit the ability of platelets to be activated and to aggregate. At doses of thrombin lower than 2 units/ml (data not shown), platelet aggregation was similarly inhibited by wortmannin implying a similar requirement for PI 3-K activation in both FcγRIIA and thrombin signaling pathways. Therefore, inhibition of PI 3-K activity in platelets by wortmannin results in the inhibition of platelet aggregation mediated by FcγRIIA.

P1	Biotin-ETADGG **Y**	MTLNPRAPTDDDKNI **Y** LTLG
P2	Biotin-ETADGG **pY**	MTLNPRAPTDDDKNI **Y** LTLG
P3	Biotin-ETADGG **Y**	MTLNPRAPTDDDKNI **pY** LTLG
P4	Biotin-ETADGG **pY**	MTLNPRAPTDDDKNI **pY** LTLG

FIG. 4. **Analysis of FcγRIIA function with synthetic phosphopeptides.** Peptides (*P1* through *P4*) were synthesized that overlap the sequence of the ITAM present in the cytoplasmic tail of FcγRIIA. Phosphotyrosine residues were incorporated to allow representation of all four phosphorylation states of the two tyrosine residues of the ITAM. Biotinylation of the N-terminal glutamic acid residue facilitated coupling to a solid support.

Reconstitution of Proximal Events of FcγRIIA Signaling with Synthetic Phosphopeptides—The observations documenting noncovalent association of $p72^{syk}$ and PI 3-K with activated FcγRIIA are consistent with the presence of an ITAM in the cytoplasmic tail of FcγRIIA. To determine whether this noncovalent association of $p72^{syk}$ and PI 3-K with activated FcγRIIA could be reconstituted with the FcγRIIA-ITAM alone, synthetic peptides were generated that corresponded to the sequence of the FcγRIIA-ITAM and included appropriate flanking residues (Fig. 4). Phosphotyrosine residues were incorporated at tyrosine positions to create four peptides corresponding to all possible tyrosine phosphorylation states of the FcγRIIA-ITAM. Peptides were biotinylated at the N terminus to facilitate binding to avidin coupled to a solid support. Peptides bound to avidin-coated plastic beads (see "Experimental Procedures") were incubated with detergent lysates of Raji cells which express $p72^{syk}$.[2] $p72^{syk}$ binding to the doubly phosphorylated peptide (P4) but not to the unphosphorylated peptide (P1) or to the avidin-coated beads alone (Fig. 5) was detected at nanomolar concentrations of peptides. In other experiments binding of $p72^{syk}$ to single phosphorylated FcγRIIA-ITAM peptides (P2, P3) was also detected by immunoblotting although at apparently lower affinity (data not shown). The binding of $p72^{syk}$ to the four phosphorylation states of the FcγRIIA-ITAM is presently being detailed by using recombinant $p72^{syk}$ and quantitative surface plasmon resonance methods in our laboratory.[3] Consistent with other reports on the modular nature of SH2 domains and their phosphotyrosine targets (12, 13, 15), the association of $p72^{syk}$ with the atypical FcγRIIA-ITAM can also be reconstituted using ITAM sequences independently of the parent receptor.

$p72^{syk}$ from Both Activated and Resting Platelets Binds the Doubly Phosphorylated FcγRIIA-ITAM—Activation of platelets by FcγRIIA clustering as well as by other agonists has been shown to result in dramatic increases in tyrosine phosphorylation of a number of proteins including $p72^{syk}$, as well as platelet aggregation. To determine whether the activation of $p72^{syk}$ influenced its association with the FcγRIIA-ITAM, both unphosphorylated (P1) and doubly phosphorylated (P4) peptides were incubated with detergent lysates of platelets that were either in the resting state or had been activated by FcγRIIA clustering. Equivalent binding of $p72^{syk}$ to the doubly phosphorylated peptide was observed regardless of whether

[2] Immunoblotting experiments determined that the expression $p72^{syk}$ from Raji cells was reactive with the anti-$p72^{syk}$ antibody under our conditions of immunoprecipitation and immunoblotting. Therefore, Raji cells were used a source of $p72^{syk}$ in some binding experiments.

[3] D. M. Maresco, A. C. Chan, and C. L. Anderson, manuscript in preparation.

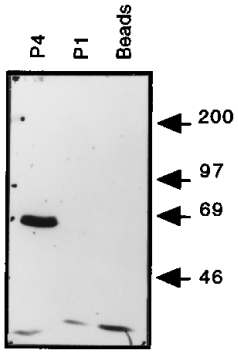

FIG. 5. **p72^{syk} binds the doubly phosphorylated FcγRIIA-ITAM.** Synthetic peptides of sequence corresponding to the FcγRIIA-ITAM were coupled to beads and incubated with lysates of Raji cells (see "Experimental Procedures"). Peptides coupled to beads were separated by centrifugation and washed extensively with Triton X-100 lysis buffer. Peptide-associated proteins were analyzed by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and associated p72^{syk} was detected by immunoblotting with a specific anti-p72^{syk} antibody and visualized by enhanced chemiluminescence. A 72-kDa band reactive in immunoblots with anti-p72^{syk} antibody is seen in adsorbates from Raji cell lysates of the doubly phosphorylated peptide (*P4*) but not in adsorbates of the unphosphorylated peptide (*P1*) or avidin-coated beads alone (*Beads*). These observations were consistent for six independent experiments.

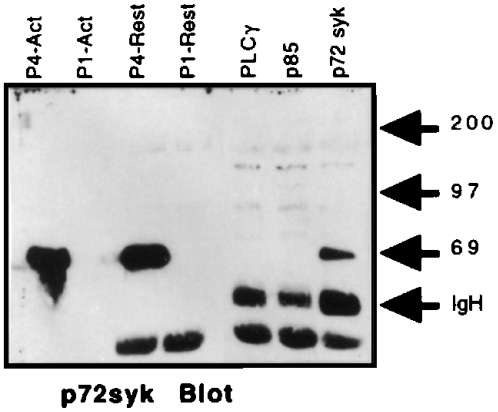

FIG. 6. **p72^{syk} from both activated and resting platelet lysates binds the doubly phosphorylated FcγRIIA-ITAM.** Lysates of resting and activated platelets were incubated with FcγRIIA-ITAM peptides and processed to detect p72^{syk} association as described (Fig. 4). p72^{syk} is detected in P4 adsorbates of both resting and activated platelets (*lanes 1* and *3*) but is not detected in P1 adsorbates of either resting or activated platelets. The p72^{syk} band detected comigrates with p72^{syk} immunoadorbed with anti-p72^{syk} antibody (*lane 7*). p72^{syk} was not detected in control immunoadsorptions with anti-PLCγ and anti-p85 antibodies (*lanes 5* and *6*).

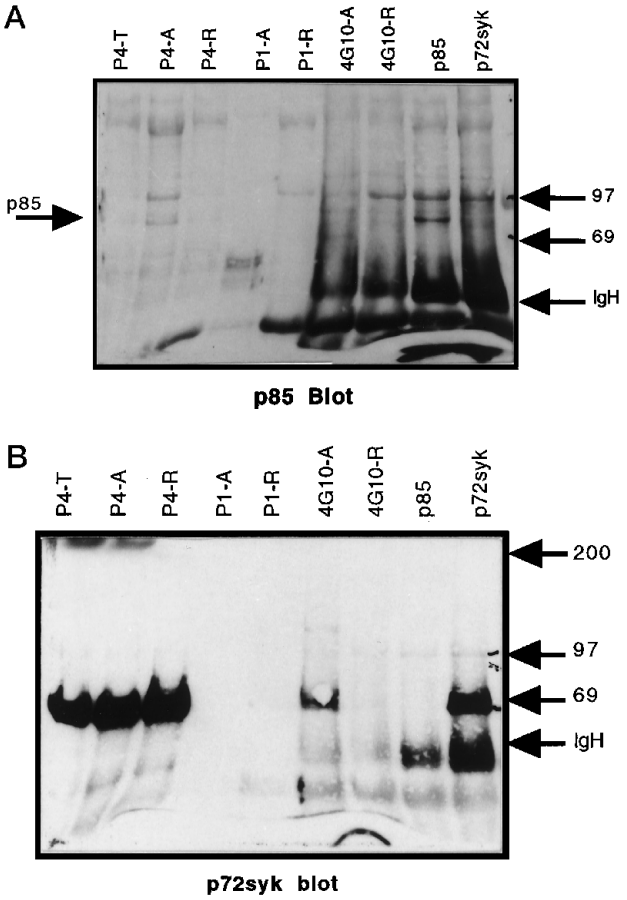

FIG. 7. **PI 3-K from activated but not resting platelet lysates binds the doubly phosphorylated FcγRIIA-ITAM.** *Top panel*, platelets were activated by FcγRIIA clustering (*lanes 2*, *4*, and *6*) or by thrombin stimulation (*lane 1*). Lysates of resting and activated platelets were incubated with FcγRIIA-ITAM peptides (P4 and P1), anti-phosphotyrosine antibody 4G10 (*lanes 6* and *7*), anti-p85 antibody (*lane 8*), or p72^{syk} antibody (*lane 9*) and processed to detect p72^{syk} association as described (Fig. 5). *A*, activated; *R*, resting; *T*, thrombin-activated. PI 3-K is detected in P4 adsorbates from activated (*lane 2*) platelets but not from resting (*lane 3*) platelets. There is no detectable PI 3-K in P1 adsorbates (*lanes 4* and *5*), 4G10, or p72^{syk} adsorbates (*lanes 6*, *7*, and *9*). *Bottom panel*, stripping and reprobing the filter with anti-p72^{syk} antibodies indicates the presence of p72^{syk} in P4 adsorbates from both resting (*lane 3*) and activated platelet lysates (*lane 1*, thrombin; *lane 2*, FcγRIIA) but not in P1 adsorbates from either resting or activated platelets (*lanes 4* and *5*). p72^{syk} is detected in anti-phosphotyrosine immunoadsorbates from platelets activated by FcγRIIA clustering (*lane 4*) but not from resting platelets. p72^{syk} detected in association with peptide P4 comigrates with p72^{syk} immunoadsorbed with anti-p72^{syk} antibodies from platelets.

p72^{syk} was derived from resting or activated platelets (Fig. 6, *lanes 1* and *3*). In contrast, there was no detectable binding of p72^{syk} to the unphosphorylated peptide (Fig. 6, *lanes 2* and *4*). These data suggest strongly that activation of p72^{syk} is not required for it to bind the phosphorylated FcγRIIA-ITAM.

PI 3-K from Activated but Not Resting Platelets Binds the Doubly Phosphorylated ITAM—Unlike members of the Syk/ZAP-70 family of tyrosine kinases which bind phosphorylated Y*XX*L sequences, PI 3-K exhibits specificity for the sequence YM*X*M or Y*XX*M through the SH2 domains of its p85 subunit (50). Interestingly, the PI 3-K binding sequence is not present in the cytoplasmic domain of FcγRIIA suggesting that additional molecules may be required to recruit PI 3-K to FcγRIIA. Upon incubation of peptides P4 (doubly phosphorylated) and P1 (unphosphorylated) with platelet lysates, PI 3-K was detectable only when peptide P4 was incubated with activated lysates although apparently at low stoichiometry (Fig. 7, *top panel*, *lane 2*). Stripping and reprobing the immunoblot with anti-p72^{syk} antibody demonstrated the presence of p72^{syk} in P4 adsorbates from resting platelets or platelets activated by thrombin or FcgRIIA clustering (Fig. 7, *bottom panel*, *lanes 1*, *2*, and *3*). This finding is interpreted by us to suggest that PI 3-K may be indirectly associated with the FcγRIIA-ITAM. The primary structure of p72^{syk} contains three Y*XX*M sites although it has not been demonstrated whether tyrosine phosphorylation occurs at these residues when p72^{syk} is activated by FcγRIIA in platelets, although we and others have demonstrated the phosphorylation of p72^{syk} on tyrosine residues after clustering of FcγRIIA on platelets and other cell types (2, 7, 47, 48, 51). In addition, it has been reported that p72^{syk} and PI 3-K are complexed in platelets treated with thrombin (52). At this time, it is not possible to exclude the presence of other proteins

within the activated FcγRIIA complex. Experiments are ongoing to further dissect the components of the activated FcγRIIA complex.

DISCUSSION

Previously, we demonstrated functional coupling of the low affinity receptor for IgG on platelets to the tyrosine kinase $p72^{syk}$. In this report we show that FcγRIIA is functionally coupled as well to a serine/threonine and lipid kinase. Others have described $p72^{syk}$ activation during platelet activation by several agonists including thrombin which stimulates a G-protein coupled receptor. Thrombin as well activates PI 3-K, although the precise mechanisms by which these events take place are not clearly understood. However, as our experiments show, in platelets activated by the ITAM containing FcγRIIA, the recruitment of $p72^{syk}$ and PI 3-K to the phosphorylated ITAM occurs concurrently with increased catalytic activity of both $p72^{syk}$ and PI 3-K. Therefore, it would appear that the activation of $p72^{syk}$ and PI 3-K may be a conserved phenomenon in platelet activation induced by stimulation of membrane receptors.

The kinetics of association with FcγRIIA of both $p72^{syk}$ and PI 3-K are rapid, occurring within seconds of FcγRIIA clustering and are consistent with reports of tyrosine phosphorylation and PI 3-K activation in platelets treated with thrombin. In thrombin-treated platelets, the activation of PI 3-K, resulting in multiple phosphatidylinositol products, is manifest over time by two major peaks of PI 3-K activity, the first due to the generation of PtdIns-3,4,5-P_3, and the second due to the generation of PtdIns-4,5-P_2 (24, 25). We, as well, observed two temporal peaks of platelet PI 3-K activity after FcγRIIA clustering, although we were not able to characterize the actual products of PI 3-K activity since we used an *in vitro* assay. Although there is correspondence between PI 3-K activation by FcγRIIA clustering and that induced by thrombin a valid comparison of our results with those of other workers awaits a more detailed study.

Recently, it has been proposed that the PtdIns-3,4,5-P_3, a catalytic product of PI 3-K, may serve to dissociate SH2 domains from their phosphotyrosine targets (53). The transient nature of the association between PI 3-K and FcγRIIA that we observed is very consistent with this hypothesis. It is conceivable, therefore, that phosphorylation of the FcγRIIA-ITAM results in PI 3-K recruitment and activation which causes dissociation of PI 3-K from the ITAM complex by the products of PI 3-K. Subsequently, PI 3-K may undergo translocation to the cytoskeleton as reported.

Although the precise nature of the molecular interaction between FcγRIIA and PI 3-K is not clear, we speculate that an adapter molecule could mediate the association of PI 3-K with the phosphotyrosines of the ITAM in the FcγRIIA molecule. Significantly, the consensus Y*XX*M-described binding site for the SH2 domain of PI 3-K is not present in the cytoplasmic domain of FcγRIIA but it is represented three times in the sequence of $p72^{syk}$ (54–56), although none of these sites has yet been shown to be phosphorylated. Our observation that PI 3-K associates with FcγRIIA when both FcγRIIA and $p72^{syk}$ are tyrosine-phosphorylated but not when only FcγRIIA is phosphorylated would suggest that PI 3-K could bind phosphorylated $p72^{syk}$ which in turn could bind phosphorylated FcγRIIA. While PI 3-K and $p72^{syk}$ are reported to be associated in thrombin-activated platelets (52), conditions under which FcγRIIA is not phosphorylated, it is not clear whether PI 3-K binding to $p72^{syk}$ is enhanced by the simultaneous association of $p72^{syk}$ with phosphorylated FcγRIIA. It is likely that phosphotyrosine-mediated interactions are the primary binding determinant in the molecular interactions between FcγRIIA and PI 3-K. While the role of other interactions such as those mediated by SH3 domains and proline-rich regions may indeed contribute to the association of FcγRIIA and PI 3-K, such interactions have been shown to be of considerable lower affinity and are unlikely to have been maintained under the relatively harsh conditions of detergent lysis and washes employed in this study (see "Experimental Procedures"). Therefore, the contribution, if any, of the SH3 domain of PI 3-K to the association with FcγRIIA and PI 3-K has yet to be clarified.

Our reconstitution experiments with synthetic peptides unequivocally demonstrate that $p72^{syk}$ binds the doubly phosphorylated FcγRIIA-ITAM with high affinity relative to the affinity for unphosphorylated FcγRIIA-ITAM. This observation is consistent with the general paradigm of ITAM-SH2 interactions despite, in the case of FcγRIIA, the variance from the consensus ITAM sequence. We have observed in preliminary experiments that single phosphorylated ITAMs bind $p72^{syk}$ with intermediate affinity suggesting that engagement of both phosphorylated tyrosines by the SH2 domains of $p72^{syk}$ confers maximal binding affinity but that binding occurs even when only one tyrosine residue is phosphorylated. It is not clear whether both or only one of the SH2 domains of $p72^{syk}$ is involved in the interaction with the FcγRIIA-ITAM. Having identified the structural elements in FcγRIIA that allow $p72^{syk}$ association, we are now in a position to identify the specific structural elements in $p72^{syk}$ that interact with the phosphorylated FcγRIIA-ITAM.

In this study, we have focused on the proximal effects of FcγRIIA clustering and the catalytic molecules that are recruited to its cytoplasmic domain. It is not clear whether the binding of PI 3-K to FcγRIIA is direct or indirect and experiments with purified recombinant PI 3-K are ongoing. Nevertheless, our data lend support to a speculative model of FcγRIIA activation in which receptor clustering initiates a concatenation of events that begins with tyrosine phosphorylation of the ITAM. The phosphorylated ITAM would then be bound by $p72^{syk}$ which would in turn be phosphorylated at Y*XX*M sites to then induce PI 3-K binding. We observe that the stoichiometry of association of PI 3-K with FcγRIIA, apparently low, is consistent with a model of multiple interactions where the extent of association of downstream molecules is proportional to the level of tyrosine phosphorylation on specific sites of the adapter molecules. Precedence for such models exists. In the case of the T cell antigen receptor, it has been proposed (57) that recruitment of the tyrosine kinase ZAP-70 to a phosphorylated ITAM results phosphorylation of ZAP-70 at multiple tyrosine residues providing multiple docking sites for other SH2-containing proteins. The observation that the protein-tyrosine phosphatase Syp acts as an adapter to link the G62-SOS complex to the PDGF receptor is another instance where a cytoplasmic enzyme binds to an activated receptor and also serves to recruit another element of the signaling cascade (58).

To test our model, it is necessary to map the sites at which $p72^{syk}$ is tyrosine-phosphorylated and to determine whether PI 3-K binds such sites in the context of the anchoring of $p72^{syk}$ to tyrosine-phosphorylated FcγRIIA or whether additional molecules are required for PI 3-K recruitment and binding to the activated FcγRIIA complex. Identification of the kinases that are responsible for phosphorylating the tyrosine residues of the FcγRIIA-ITAM would then present us with an initial sketch of the proximal effects of FcγRIIA clustering in platelets.

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Addendum—While this manuscript was in preparation, Yanaga *et al.* (59) also demonstrated binding of $p72^{syk}$ to a synthetic phosphopeptide corresponding to the sequence of the FcγRIIA-ITAM.

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