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

RECONSTITUTION WITH SYNTHETIC PHOSPHOPEPTIDES\textsuperscript{*}

(Received for publication, December 4, 1995, and in revised form, February 14, 1996)

George W. Chacko\textsuperscript{§}, John T. Brandt\textsuperscript{¶}, K. Mark Coggeshall\textsuperscript{¶}, and Clark L. Anderson\textsuperscript{‡**}

From the Departments of Internal Medicine, Pathology, and Microbiology, The Ohio State University, Columbus, Ohio 43210

Previously, we have demonstrated that the cytoplasmic tyrosine kinase p72\textsuperscript{syk} is coupled to the platelet Fc receptor for IgG (Fc\textgamma R IIA) (Chacko, G. W., Duchemin, A. M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1994) J. Biol. Chem. 269, 32385–32400). Further analysis of the platelet activation by Fc\textgamma R IIA demonstrated that Fc\textgamma R IIA is also inducibly coupled to the serine/threonine and lipid kinase, phosphoinositide 3-kinase (PI 3-K). Activation of platelets with anti-Fc\textgamma R IIA antibodies resulted in the noncovalent association of PI 3-K with Fc\textgamma R IIA as well as an increase in Fc\textgamma R IIA-associated PI 3-K activity. Binding of both p72\textsuperscript{syk} and PI 3-K to Fc\textgamma R IIA was reconstituted with synthetic phosphopeptides corresponding to the sequence of the atypical immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain of Fc\textgamma R IIA. Our findings demonstrate that coupling of both p72\textsuperscript{syk} and PI 3-K activities to Fc\textgamma R IIA is regulated by tyrosine phosphorylation of the ITAM, and we speculate that p72\textsuperscript{syk} might act as an adapter to recruit PI 3-K to activated Fc\textgamma R IIA.

Human platelets express a single Fc receptor for IgG (Fc\textgamma R).\textsuperscript{1} The low affinity receptor encoded by the Fc\textgamma R IIA gene (1–4). Clustering of Fc\textgamma R IIA with ligand or anti-Fc\textgamma R IIA 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 Fc\textgamma R IIA contains a single copy of an activation motif termed immunoreceptor tyrosine-based activation motif (ITAM) that is loosely defined by the consensus sequence $YX_LX_N[L,K]X_LY_{(6–8)}$, 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 (Fc\textepsilon R), and other Fc\textgamma R (10–14). Clustering of Fc\textgamma R IIA-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 Fc\textgamma R IIA is unusual in that it contains an extended spacer of 12 amino acids (18) between the two YXXL 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), Fc\textgamma R IIA is a single-chain transmembrane polypeptide with an ITAM integral to its primary structure. Mutaional analysis of the tyrosine residues of the Fc\textgamma R IIA-ITAM has shown that they are required for receptor tyrosine phosphorylation and for phagocytosis mediated by Fc\textgamma R IIA (19–21), suggesting that the Fc\textgamma R IIA-ITAM remains functional despite its divergence from the consensus sequence. Results from another study suggest that the ITAM of Fc\textgamma R IIA transduces a qualitatively different signal from the ITAM of the γ-chain subunit of the high affinity Fc\textepsilon R, implying that there are unique aspects to the Fc\textgamma R IIA-ITAM despite the common functional features it shares with other members of the group (22). Analysis of the mechanism by which Fc\textgamma R IIA 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 Fc\textgamma R IIA on platelets induces receptor tyrosine phosphorylation and noncovalent association of the tyrosine kinase p72\textsuperscript{syk} with tyrosine-phosphorylated Fc\textgamma R IIA (2, 7). These observations lend support to a model of p72\textsuperscript{syk} coupling to Fc\textgamma R IIA induced by tyrosine phosphorylation of the ITAM (1, 5) presumably by one or more of the five Src kinases expressed in platelets (23). p72\textsuperscript{syk} is a member of the Syk/ZAP70 family of tyrosine kinases and has been shown to be...
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\textsuperscript{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 \(\alpha_\text{IIb}\beta_3\)) by fibrinogen in thrombin-activated platelets further up-regulates p72\textsuperscript{syk} activity (8, 23, 27). These observations suggest that p72\textsuperscript{syk} plays a central role in platelet activation and that regulation of its activity is complex. Additionally, targeted gene disruption of the p72\textsuperscript{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\textsuperscript{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\textsuperscript{syk}, plays an important role in platelet activation.

PI 3-K has been implicated in Fc\text{y}R function in neutrophils, the monocyte cell line U937, and natural killer (NK) cells suggesting a conserved role in Fc\text{y}R-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 phosphoryrasine-associated PI 3-K activity in response to Fc\text{y}R 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 Fc\text{y}R expressed on these cell types. Activation of Fc\text{y}RIIA activation in platelets which express only a single Fc\text{y}R, provides specific information on the mechanism by which the low affinity Fc\text{y}RIIA transduces signal and contributes to the general paradigm describing how Fc\text{y}R activates PI 3-K.

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

FcyRIIA-associated PI 3-K Activity Is Rapidly and Transiently Increased after Platelet Activation by FcyRIIA Stimulation—Previous work has demonstrated that clustering of FcyRIIA on platelets and other cells induces tyrosine phosphorylation of FcyRIIA, noncovalent association, and activation of the tyrosine kinase p72shc with FcyRIIA (2, 7, 47, 48). Since several tyrosine-phosphorylated proteins including p72shc co-immunoadsorb with activated FcyRIIA, we sought to determine whether other catalytically active molecules were also noncovalently complexed with activated FcyRIIA. 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 FcyRIIA was detected by assaying for the ability of FcyRIIA 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 Rf 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 FcyRIIA on platelets, there is a rapid and transient increase in FcyRIIA-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 observed 3–5 min after FcyRIIA clustering (data not shown) consistent with reports of two waves of PI 3-K activity in thrombin-stimulated platelets (37). Therefore, FcyRIIA-mediated platelet activation results in transiently elevated levels of FcyRIIA-associated PI 3-K activity.

PI 3-K Rapidly Associates with FcyRIIA in Platelets Activated by Clustering of FcyRIIA—To determine whether the PI 3-K activity co-immunoadsorbed with activated FcyRIIA was due to the binding of PI 3-K to the activated receptor complex, FcyRIIA was immunoadsorbed from detergent lysates of resting and activated platelets with monoconal antibody IV.3, and associated PI 3-K was detected by immunoblotting to detect phosphotyrosine or candidate molecules.

with the activated (tyrosine-phosphorylated) FcyRIIA.

PI 3-K Activity Is Required for FcyRIIA-mediated Platelet Aggregation—Clustering of FcyRIIA 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 FcyRIIA 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 FcyRIIA-induced platelet aggregation in
FcγRIIA Clustering

![Image of FcγRIIA Clustering](image)

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 antibodies (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 immunoadsorbents 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.

![Image of Inhibition of FcγRIIA-mediated platelet aggregation by wortmannin](image)

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 C1KR5 (IgG1), or GAM alone. C1KR5 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.

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.

Reconstitution of Proximal Events of FcγRIIA Signaling with Synthetic Phosphopeptides—The observations documenting noncovalent association of p72SYK 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 p72SYK 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 p72SYK (2) and p72SYK 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 p72SYK to single phosphorylated FcγRIIA-ITAM peptides (P2, P3) was also detected by immunoblotting although at apparently lower affinity (data not shown). The binding of p72SYK to the four phosphorylation states of the FcγRIIA-ITAM is presently being detailed by using recombinant p72SYK and quantitative surface plasmon resonance methods in our laboratory.2 Consistent with other reports on the modular nature of SH2 domains and their phosphotyrosine targets (12, 13, 15), the association of p72SYK with the atypical FcγRIIA-ITAM can also be reconstituted using ITAM sequences independently of the parent receptor.

p72SYK 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 p72SYK, as well as platelet aggregation. To determine whether the activation of p72SYK 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 p72SYK to the doubly phosphorylated peptide was observed regardless of whether

---

2 Immunoblotting experiments determined that the expression p72SYK from Raji cells was reactive with the anti-p72SYK antibody under our conditions of immunoprecipitation and immunoblotting. Therefore, Raji cells were used as a source of p72SYK in some binding experiments.

3 D. M. Marasco, A. C. Chan, and C. L. Anderson, manuscript in preparation.
These data suggest strongly that activation of p72syk and P1 binds the doubly phosphorylated FcγRIIA-ITAM and functional molecules may be required to recruit PI3-K to FcγRIIA clustering and activated platelets were incubated with FcγRIIA-ITAM and activated platelets were incubated with FcγRIIA-ITAM peptides with anti-p72syk antibody and visualized by enhanced chemiluminescence. A 72-kDa band reactive in immunoblots with anti-p72syk 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.

p72syk was derived from resting or activated platelets (Fig. 6, lanes 1 and 3). In contrast, there was no detectable binding of p72syk to the unphosphorylated peptide (Fig. 6, lanes 2 and 4). These data suggest strongly that activation of p72syk 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 YXXL sequences, PI 3-K exhibits specificity for the sequence YMMX or YXXM 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-p72syk antibody demonstrated the presence of p72syk in P4 adsorbates from resting platelets or platelets activated by thrombin or FcγRIIA 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 p72syk contains three YXXL sites although it has not been demonstrated whether tyrosine phosphorylation occurs at these residues when p72syk is activated by FcγRIIA in platelets, although we and others have demonstrated the phosphorylation of p72syk 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 p72syk 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 FcRIIA complex. Experiments are ongoing to further dissect the components of the activated FcRIIA complex.

**DISCUSSION**

Previously, we demonstrated functional coupling of the low affinity receptor for IgG on platelets to the tyrosine kinase p72<sup>syk</sup>. In this report we show that FcRIIA is functionally coupled as well to a serine/threonine and lipid kinase. Others have described p72<sup>syk</sup> 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 FcRIIA, the recruitment of p72<sup>syk</sup> and PI 3-K to the phosphorylated ITAM occurs concurrently with increased catalytic activity of both p72<sup>syk</sup> and PI 3-K. Therefore, it would appear that the activation of p72<sup>syk</sup> and PI 3-K may be a conserved phenomenon in platelet activation induced by stimulation of membrane receptors.

The kinetics of association with FcRIIA of both p72<sup>syk</sup> and PI 3-K are rapid, occurring within seconds of FcRIIA 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<sub>3</sub>, and the second due to the generation of PtdIns-4,5-P<sub>2</sub> (24, 25). We, as well, observed two temporal peaks of platelet PI 3-K activity after FcRIIA 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 FcRIIA 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<sub>3</sub>, 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 FcRIIA that we observed is very consistent with this hypothesis. It is conceivable, therefore, that phosphorylation of the FcRIIA-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 FcRIIA 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 FcRIIA molecule. Significantly, the consensus YYXM-described binding site for the SH2 domain of PI 3-K is not present in the cytoplasmic domain of FcRIIA but it is represented three times in the sequence of p72<sup>syk</sup> (54–56), although none of these sites has yet been shown to be phosphorylated. Our observation that PI 3-K associates with FcRIIA when both FcRIIA and p72<sup>syk</sup> are tyrosine-phosphorylated but not when only FcRIIA is phosphorylated would suggest that PI 3-K could bind phosphorylated p72<sup>syk</sup> which in turn could bind phosphorylated FcRIIA. While PI 3-K and p72<sup>syk</sup> are reported to be associated in thrombin-activated platelets (52), conditions under which FcRIIA is not phosphorylated, it is not clear whether PI 3-K binding to p72<sup>syk</sup> is enhanced by the simultaneous association of p72<sup>syk</sup> with phosphorylated FcRIIA. It is likely that phosphotyrosine-mediated interactions are the primary binding determinant in the molecular interactions between FcRIIA 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 FcRIIA 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 FcRIIA and PI 3-K has yet to be clarified.

Our reconstitution experiments with synthetic peptides unequivocally demonstrate that p72<sup>syk</sup> binds the doubly phosphorylated FcRIIA-ITAM with high affinity relative to the affinity for unphosphorylated FcRIIA-ITAM. This observation is consistent with the general paradigm of ITAM-SH2 interactions despite, in the case of FcRIIA, the variance from the consensus ITAM sequence. We have observed in preliminary experiments that single phosphorylated ITAMs bind p72<sup>syk</sup> with intermediate affinity suggesting that engagement of both phosphorylated tyrosines by the SH2 domains of p72<sup>syk</sup> 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<sup>syk</sup> is involved in the interaction with the FcRIIA-ITAM. Having identified the structural elements in FcRIIA that allow p72<sup>syk</sup> association, we are now in a position to identify the specific structural elements in p72<sup>syk</sup> that interact with the phosphorylated FcRIIA-ITAM.

In this study, we have focused on the proximal effects of FcRIIA clustering and the catalytic molecules that are recruited to its cytoplasmic domain. It is not clear whether the binding of PI 3-K to FcRIIA is direct or indirect and experiments with purified recombinant PI 3-K are ongoing. Nevertheless, our data lend support to a speculative model of FcRIIA 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<sup>syk</sup> which would in turn be phosphorylated at YYXM sites to then induce PI 3-K binding. We observe that the stoichiometry of association of PI 3-K with FcRIIA, 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<sup>syk</sup> is tyrosine-phosphorylated and to determine whether PI 3-K binds such sites in the context of the anchoring of p72<sup>syk</sup> to tyrosine-phosphorylated FcRIIA or whether additional molecules are required for PI 3-K recruitment and binding to the activated FcRIIA complex. Identification of the kinases that are responsible for phosphorylating the tyrosine residues of the FcRIIA-ITAM would then present us with an initial sketch of the proximal effects of FcRIIA clustering in platelets.

Acknowledgments—We thank Michelle Wilder for technical assistance with platelet aggregometry. Drs. C. Couture and T. Mustelin for anti-p72<sup>syk</sup> antibody, Dr. M. Wissel, Monica Ross, and the American Red Cross for providing human platelets. We also thank Dr. Robert Kimberly for a critical reading of this manuscript.
Addendum—While this manuscript was in preparation, Yanaga et al. (59) also demonstrated binding of p72

REFERENCES

1. Chacko, G. W., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1994) in Human Fc Receptors (van de Winkel, J. G. J., ed) R. G. Landes Co, Austin, TX, in press.

2. Chacko, G. W., Duchemin, A. M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1996) in Semin.

3. Cambier, J. C. (1995).

4. Clark, E. A., Shattil, S. J., and Brugge, J. S. (1994).

5. Chan, A. C., Desai, D. M., and Weiss, A. (1994).

6. Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, R., and Anderson, C. L. (1994) J. Biol. Chem. 269, 22732–22737.

7. Fifis, P., Bewärder, N., Weinrich, V., and Frey, J. (1994) Eur. J. Cell Biol. 64, 45–60.

8. Mitchell, M. A., Huang, M. M., Chien, P., Indik, Z. J., and Schreiber, A. D. (1994) J. Biol. Chem. 269, 1753–1759.

9. Kanakaraj, P., Duckworth, B., Azzoni, L., Kanik, M., Cantley, L. C., and Hartwig, J. H. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 10350–10354.

10. Weiss, A. and Littman, D. R. (1994) J. Biol. Chem. 269, 2277–2279.

11. Zhang, J., Fy, M. J., Waterfield, M. D., Jaken, S., Doud, D. E., and Hawes, C. R. (1995) Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 340, 337–344.

12. Pawson, T. (1995) Trends Biochem. Sci. 20, 464–469.

13. Mitchell, M. A., Huang, M. M., Chien, P., Indik, Z. J., and Schreiber, A. D. (1993) J. Biol. Chem. 268, 2243–2248.

14. Agarwal, A., Salem, P., and Robbins, K. C. (1993) J. Biol. Chem. 268, 15900–15905.

15. Zhang, J., Fy, M. J., Waterfield, M. D., Jaken, S., Doud, D. E., and Hawes, C. R. (1995) Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 340, 337–344.

16. Agarwal, A., Salem, P., and Robbins, K. C. (1993) J. Biol. Chem. 268, 15900–15905.

17. Mitchell, M. A., Huang, M. M., Chien, P., Indik, Z. J., and Schreiber, A. D. (1993) J. Biol. Chem. 268, 2243–2248.

18. Agarwal, A., Salem, P., and Robbins, K. C. (1993) J. Biol. Chem. 268, 15900–15905.
Phosphoinositide 3-Kinase and p72 Noncovalently Associate with the Low Affinity Fc Receptor on Human Platelets through an Immunoreceptor Tyrosine-based Activation Motif: RECONSTITUTION WITH SYNTHETIC PHOSPHOPEPTIDES
George W. Chacko, John T. Brandt, K. Mark Coggeshall and Clark L. Anderson

J. Biol. Chem. 1996, 271:10775-10781.
doi: 10.1074/jbc.271.18.10775

Access the most updated version of this article at http://www.jbc.org/content/271/18/10775

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 33 of which can be accessed free at http://www.jbc.org/content/271/18/10775.full.html#ref-list-1