Tyrosine phosphorylation and association of Syk with FcγRII in monocytic THP-1 cells

Soosan GHAZIZADEH,* Joseph B. BOLEN† and Howard B. FLEIT*‡

*Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY 11794, U.S.A., and †Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543, U.S.A.

Although the cytoplasmic portion of the low-affinity receptor for immunoglobulin G, FcγRII, does not contain a kinase domain, rapid tyrosine phosphorylation of intracellular substrates occurs in response to aggregation of the receptor. The use of specific tyrosine kinase inhibitors has suggested that these phosphorylations are required for subsequent cellular responses. We previously demonstrated the coprecipitation of a tyrosine kinase activity with FcγRII, suggesting that non-receptor tyrosine kinases might associate with the cytoplasmic domain of FcγRII. Anti-receptor immune complex kinase assays revealed the coprecipitation of several phosphoproteins, most notably p56/53^{lyn}, an Src-family protein tyrosine kinase (PTK), and a 72 kDa phosphoprotein. Here we identify the 72 kDa FcγRII-associated protein as p72^{syk} (Syk), a member of a newly described family of non-receptor PTKs. A rapid and transient tyrosine phosphorylation of Syk was observed following FcγRII activation. Syk was also tyrosyl-phosphorylated following aggregation of the high-affinity Fcγ receptor, FcγRI. The FcγRI activation did not result in association of Syk with FcγRII, implying that distinct pools of Syk are activated upon aggregation of each receptor in a localized manner. These results demonstrate a physical association between Syk and FcγRII and suggest that the molecules involved in FcγRII signalling are very similar to the ones utilized by multichain immune recognition receptors such as the B-cell antigen receptor and the high-affinity IgE receptor.

INTRODUCTION

Receptors for the constant domain of IgG (FcγRs) transmit activating signals upon aggregation by immune complexes or anti-receptor antibodies. In phagocytic leukocytes, FcγR aggregation results in a variety of immunological functions including phagocytosis, superoxide generation, and production and release of inflammatory mediators. Three distinct classes of human FcγRs have been identified: FcγRI (high affinity), FcγRII and FcγRIII (low affinities). All FcγRs are members of the immunoglobulin gene superfamily (for review, see [1,2]). Human FcγRI and hFcγRIII exist as oligomeric complexes associated with γ or ζ chains which also form part of the high-affinity IgE receptor (FcεRI) and T-cell receptor (TCR)–CD3 complex [3–5]. These accessory subunits are thought to play a role in the signalling events mediated by their associated receptors [6]. FcγRII has the broadest distribution and is expressed on a variety of hematopoietic cells. Three genes (A, B and C) have been identified for this receptor which gave rise to at least six products [7]. At least two hFcγRII isoforms (hFcγRIIA and C) can be expressed and deliver signals without the requirement for myeloid cell-specific accessory molecules [8,9]. A tyrosine-containing sequence motif containing two YXXL/I sequences separated by 6–7 residues, which has been designated as the antigen recognition activation motif (ARAM) [10,11], has been identified in the cytoplasmic tail of members of ζ gene family [12]. A similar but not identical domain is also present in the cytoplasmic domain of FcγRIIA and C [7,13]. The tyrosine and leucines of ARAM are required for the functional activity of the motif [14]. In addition, the YXXL/I motifs have been suggested to be the potential binding site for the SH2 domains of Src-related protein tyrosine kinase (PTK) and Syk family of kinases [15,16].

Recently, it has been shown that cross-linking of human FcγRII on monocytic cells with immune complexes or specific anti-receptor antibodies results in stimulation of signal-transducing events, including inositol phosphate metabolism [17], increase in cytoplasmic calcium concentration [18,19] and tyrosine phosphorylation of several cellular proteins including phospholipase C (PLC)-γ1 [17] and FcγRII itself [21]. Furthermore, inhibition of FcγRII-mediated signalling events by specific inhibitors of PTKs [17–21] suggested the involvement of cytoplasmic PTKs in initiating FcγRII-mediated signal transduction. As FcγRII has no intrinsic PTK activity, non-receptor PTKs were implicated in participating in FcγRII-mediated signalling pathway(s). Physical and functional association of FcγRII with Hck and Lyn in monocytic THP-1 cells and with Fgr in neutrophils has recently been demonstrated [22,23].

Our previous studies demonstrated that a PTK activity coprecipitated with FcγRII [22]. While *lyn* gene products were among the receptor-associated proteins, the most notable tyrosyl phosphorylated protein in these complexes was a 72 kDa protein. This phosphoprotein was also one of the most prominent substrates for FcγRII-mediated tyrosine phosphorylation [21]. Upon cross-linking of FcγRI and B-cell antigen receptor (BCR) a protein of similar size was also phosphorylated and has been

Abbreviations used: ARAM, antigen receptor activation motif; BCR, B-cell receptor; FcεRI, high-affinity receptor for IgE; FcγR, receptor for Fc domain of IgG; GST, glutathione *S*-transferase; h, human; mAb, monoclonal antibody; PAA, phosphoamino acid analysis; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; PMSF, phenylmethanesulphonyl fluoride; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride; TCR, T-cell receptor.

‡ To whom correspondence should be addressed.

shown to be PTK72^{syk} (Syk, [24,25]). In addition, Syk was tyrosine phosphorylated following FcγRI or FcγRII cross-linking in the HL-60 cell line [26]. In the present studies we identified the 72 kDa protein as Syk and further demonstrated that Syk is stably associated with FcγRII in a monocytic cell line.

EXPERIMENTAL PROCEDURES

Cells and antibodies

THP-1 cells were maintained in RPMI-1640 (GIBCO, Grand Island, NY, U.S.A.) with 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 i.u./ml penicillin. The hybridoma producing monoclonal antibody (mAb) IV.3, an IgG2b anti-FcγRII [27] was obtained from the American Type Culture Collection (ATCC; Bethesda, MD, U.S.A.). Fab fragments were generated using papain. Polyclonal antiserum directed against Syk amino acids 260–370 was produced using glutathione *S*-transferase (GST)–antigen fusion protein (Pharmacia LKB Biotechnology Inc.) as the immunogen. mAb 4G10 directed against phosphotyrosine was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Human IgG1 (hIgG1) myeloma protein (Bac) has been described previously [21]. $F(ab')_2$ fragments of goat anti-(mouse IgG) were used for cross-linking mouse IgG (Cappel, Organon Teknika, Corp., West Chester, PA, U.S.A.) and $F(ab')_2$ fragments of goat anti-(human IgG) were used for cross-linking of monomeric hIgG1 (E. Y. Labs Inc. San Mateo, CA, U.S.A.).

Immunoprecipitation and Western blotting

Cells [(1–2) × 10^7 per ml] were stimulated with mAb IV.3 Fab (5 μg/ml) or mAb IV.3 Fab and goat anti-mouse $F(ab')_2$ (50 μg/ml) at 37 °C for the indicated times. Cells were solubilized in lysis buffer [1% v/v Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 0.5 mM Na_3VO_4, 5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF), 0.27 i.u./ml aprotinin and 10 μg/ml soybean trypsin inhibitor]. Postnuclear lysates were precleared by incubation with protein G–agarose (GIBCO) for 1 h and then subjected to immunoprecipitation with optimized amounts of various mAbs or polyclonal antisera for 2–4 h followed by 20 μl of 50% (v/v) protein G–agarose for 1 h at 4 °C. The immunoprecipitates were washed four times in lysis buffer. Samples were eluted in sample buffer (125 mM Tris, pH 6.8/4% SDS/10% 2-mercaptoethanol/20% glycerol/0.02% Bromophenol Blue), boiled and resolved by SDS/7.5% (w/v) PAGE under reducing conditions. The proteins were electrotransferred to Immobilon P membranes [poly(vinylidene difluoride), Millipore Bedford, MA, U.S.A.] and then blotted with indicated antibodies. The primary antibodies were detected with goat anti-mouse or goat anti-rabbit antibodies conjugated with alkaline phosphatase or horseradish peroxidase and were detected by colorimetric methods (GIBCO) or an enhanced chemiluminescence (ECL) detection system (Amersham International, U.K.) respectively.

In vitro kinase assay and reimmunoprecipitation analysis

For *in vitro* kinase assay, cells were solubilized in lysis buffer containing 0.5% Triton X-100 and immune complexes were formed as described above. The immune complexes were washed four times in lysis buffer and four times in kinase buffer (50 mM Tris, pH 7.4, 10 mM $MnCl_2$), pellets were resuspended in 30 μl of kinase buffer including 1 μM cold ATP and 5 μCi (1 μCi = 37 GBq) [γ-^{32}P] ATP (specific radioactivity > 4500 Ci/mmol; ICN, Irvine, CA, U.S.A.) for 2 min at 25 °C. The reaction was stopped by addition of 1 ml of cold lysis buffer followed by rapid centrifugation. Samples were eluted in 2 × sample buffer under reducing condition. Alternatively, following the kinase assay, the samples were boiled in 2% (w/v) SDS for 10 min, and then diluted 10-fold with lysis buffer. After removal of any remaining antibody with protein G–agarose, samples were subjected to a second round of immunoprecipitation with indicated antibodies. Samples were separated by SDS/7.5% PAGE under reducing condition and detected by autoradiography.

Phosphoamino acid analysis (PAA)

Phosphoproteins were excised from Immobilon P membranes and hydrolysed in 6 M HCl at 100 °C for 90 min. The individual phosphorylated amino acids were separated on t.l.c. plates (EM Science, Gibbstown, NJ, U.S.A.) by electrophoresis (2000 V, 25 min) at pH 3.5 [28]. Non-radioactive phosphoamino acid standards were detected with ninhydrin, and radiolabelled phosphorylated amino acids were visualized by autoradiography.

RESULTS

Tyrosine phosphorylation of p72^{syk} following FcγRII aggregation

One of the major substrates for PTK activity observed after FcγRII aggregation was a 72 kDa protein [21]. A protein of similar electrophoretic mobility was also detected in FcγRII-coprecipitated complexes [22]. To examine the identity of this 72 kDa protein, THP-1 cells were stimulated with a saturating concentration of mAb IV.3 Fab followed by goat anti-mouse $F(ab')_2$ for 5 min. At this time tyrosine phosphorylation of the 72 kDa band is maximal [21]. Cell lysates from unstimulated or stimulated cells were subjected to immunoprecipitation with anti-phosphotyrosine or anti-Syk antibodies. Tyrosine phosphorylated proteins were detected in an anti-phosphotyrosine blot. As shown in Figure 1(a), cross-linking of FcγRII induced a rapid increase in tyrosine phosphorylation of several proteins including a 72 kDa polypeptide (lane 2). Similarly, immunoprecipitation of total cellular Syk assessed by an anti-phosphotyrosine blot indicated that a 72 kDa tyrosyl phosphorylated protein was only detectable after stimulation (lane 4). No detectable ZAP-70 was observed in THP-1 cells (data not shown). Heavy chain of IgG was visualized because of the reactivity of secondary antibody with antibodies used during immunoprecipitations (Figure 1a). To examine further the identity of the 72 kDa protein with Syk, a similar experiment was performed but proteins were detected in an immunoblot using anti-Syk antibody. As shown in Figure 1(b), tyrosine phosphorylation of Syk was only detected in FcγRII-activated cells (lanes 4). No cross-reactivity of anti-Syk antibody was observed in controls (lanes 1 and 2). A time course of Syk tyrosine phosphorylation after FcγRII stimulation revealed rapid and transient kinetics. Although basal tyrosine phosphorylation of Syk was undetectable, within seconds following FcγRII stimulation, phosphorylation of Syk was observed. Maximal tyrosine phosphorylation of Syk was reached within 5 min and Syk was rapidly dephosphorylated afterwards (Figure 1c, upper panel). These observations are consistent with a recent report demonstrating tyrosine phosphorylation of Syk following FcγRI or FcγRII engagement in another myelomonocytic cell line, HL-60 [26].

FcγRII has been shown to be a substrate for tyrosine kinase activity induced following cross-linking of the receptor [13,21]. Tyrosyl phosphorylated receptor migrates as a broad band with a molecular mass of 37–40 kDa (Figure 1a, lane 2; see also [21]). Interestingly, the tyrosyl-phosphorylated receptor was also detected in complexes coprecipitated with anti-Syk antibody

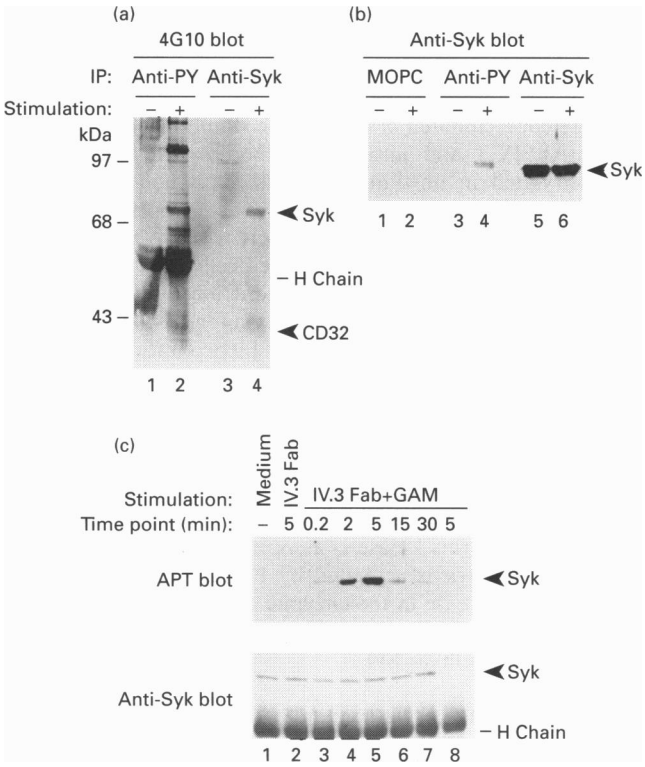

Figure 1 Syk is tyrosyl phosphorylated after FcγRII aggregation

(**a**) Triton X-100 (1%) lysates of cells before (−) and after (+) stimulation with 5 μg/ml IV.3 Fab followed by 50 μg/ml goat anti-mouse $F(ab')_2$ for 5 min were subjected to immunoprecipitation with anti-phosphotyrosine (anti-Py) (lanes 1 and 2) or anti-Syk (lanes 3 and 4) antibodies. Phosphotyrosine-containing proteins were detected by anti-phosphotyrosine blot using mAb 4G10. The position of the tyrosine-phosphorylated FcγRII (CD32) is indicated on the right-hand side of the Figure. The electrophoretic mobility of molecular-mass standards are indicated on the left-hand side of the figure. (**b**) Cells were prepared as described above. Lysates were immunoprecipitated with a control mouse IgG, MOPC 141 (lanes 1 and 2), anti-phosphotyrosine (lanes 3 and 4) or anti-Syk (lanes 5 and 6) antibodies. Proteins were detected by an immunoblot using anti-Syk antibody. The data are representative of several experiments. (**c**) Time course of Syk tyrosine phosphorylation following FcγRII stimulation. Syk was precipitated from unstimulated cells (lane 1), cells incubated with 5 μg/ml IV.3 Fab alone (lane 2), or cells stimulated with IV.3 Fab followed by goat anti-mouse $F(ab')_2$ as described for the indicated period of time (lanes 3–7). As a control, lysates from cells stimulated for 5 min were subjected to immunoprecipitation with normal rabbit serum (lane 8). Proteins were analysed by immunoblotting with either anti-phosphotyrosine (upper panel) or anti-Syk antibody (lower panel) to detect the amount of protein present during the time course. H Chain represents the heavy chain of IgG used during immunoprecipitation.

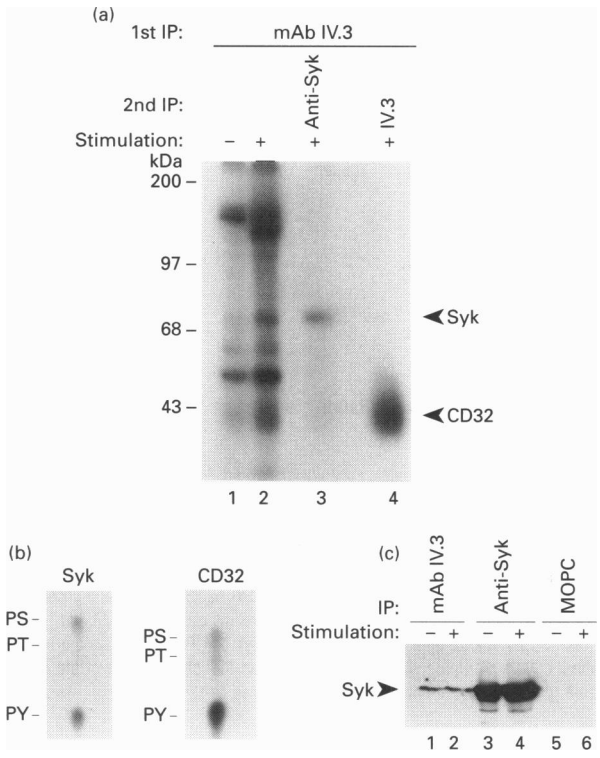

Figure 2 Association of FcγRII with Syk

(**a**) Coprecipitation of PTK activity with FcγRII in unstimulated cells (lane 1) or cells stimulated with IV.3 IgG followed by goat anti-mouse for 5 min (lane 2). Following kinase assay, FcγRII-associated complexes from stimulated cells were subjected to reprecipitation analysis using anti-Syk (lane 3) or mAb IV.3 (lane 4). The positions of Syk and FcγRII (CD32) are marked. Proteins were separated by SDS/7.5% (w/v) PAGE and visualized by autoradiography, using an exposure time of 48 h. Molecular-mass standards are indicated on the left-hand side of the Figure. IP, immunoprecipitation. (**b**) PAA of Syk and FcγRII (CD32) following an anti-receptor immune complex kinase assay. Proteins were separated and transferred to Immunobilon-P. The 40 and 72 kDa bands were excised and subjected to PAA analysis as described in the Experimental procedures section. The locations of phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY) are indicated. (**c**) Detergent lysates of unstimulated or FcγRII-cross-linked cells were subjected to immunoprecipitation with mAb IV.3 (lanes 1 and 2), anti-Syk (lanes 3 and 4) or an isotype matched mouse IgG, MOPC141 (IgG2b; lanes 5 and 6). Proteins were detected in an anti-Syk blot. The position of Syk is indicated by an arrowhead. A representative experiment is shown.

(lane 4), suggesting that a direct physical association may exist between Syk and FcγRII in stimulated cells.

Physical association of FcγRII and Syk

We have recently reported that a PTK activity was coprecipitated with FcγRII. This activity, as assessed in an *in vitro* kinase assay, increased significantly (an average of 10-fold) following FcγRII-activation. Several phosphoproteins with molecular masses of 160, 150, 75, 72, 58, 55 and 40 kDa were detected (Figure 2a, lanes 1 and 2; [22]. The increased PTK activity was, at least partially, attributable to the presence of $p56^{lyn}$ in these complexes [22]. However, a 72 kDa phosphoprotein was notably also constitutively coprecipitated with the receptor (lanes 1, 2). To examine the identity of this protein, FcγRII was immunoprecipitated from activated cells and proteins were labelled in an *in vitro* kinase reaction. Reimmunoprecipitation analysis of FcγRII coprecipitated complexes identified the 72 kDa protein as Syk (Figure 2a, lane 3). PAA indicated that both Syk and FcγRII (CD32) were mainly phosphorylated on tyrosine residues (Figure 2b). The presence of Syk in FcγRII precipitated complexes was further assessed in an anti-Syk blot. A small fraction of Syk was constitutively associated with FcγRII (Figure 2c, lanes 1 and 2). Densitometric analysis of three separate experiments indicated that only an average of 1–5% of the total cellular Syk was associated with the receptor (compare lanes 1 and 2 with 3 and 4). Because of the poor affinity of anti-FcγRII antibodies examined by Western blotting, the only means of detection of FcγRII on a blot was by an anti-phosphotyrosine blot, which detects tyrosyl phosphorylated receptor (Figure 1a, lanes 2 and 4). Therefore, we could not examine the stoichiometry of association of FcγRII with Syk.

The presence of FcγRII in Syk coprecipitated complexes was further examined using an *in vitro* kinase assay (Figure 3). Syk is ready proteolysed to a 36–40 kDa catalytically active enzyme [29]. Upon SDS/PAGE, FcγRII shares an identical migration

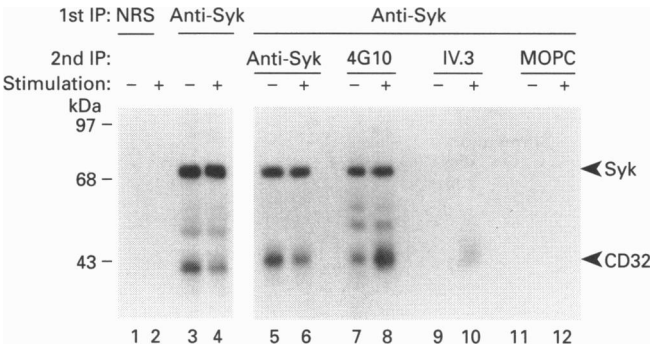

Figure 3 Reimmunoprecipitation analysis of anti-Syk precipitated complexes

0.5% Triton X-100 lysates of cells from unstimulated (−) or stimulated (+) cells were subjected to immunoprecipitation with normal rabbit serum (lanes 1 and 2) or anti-Syk antibody (lanes 3 and 4). The kinase activity of the precipitated proteins was assessed in an immune complex kinase assay as described in the Experimental procedures section. Proteins labelled in anti-Syk immune complex kinase assay were denatured in 2% (w/v) SDS and subjected to a second round of immunoprecipitation (IP) using anti-Syk (lanes 5 and 6), anti-phosphotyrosine (lanes 7 and 8), anti-FcγRII (lanes 9 and 10) or a control mouse IgG (lanes 11 and 12). Proteins were detected by autoradiography. The position of Syk and FcγRII (CD32) are indicated by arrowheads. Molecular-mass standards are shown on the left-hand side of the Figure. The exposure time for lanes 1–4 was 30 min and for lanes 5–12 was 16 h.

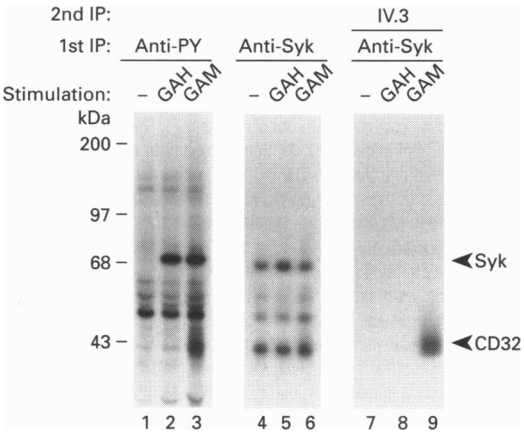

Figure 4 Specificity of the association of Syk with FcγRII

Cells were incubated with 5 μg/ml IV.3 Fab and 50 μg/ml hIgG1 and stimulated with goat anti-human (GAH) $F(ab')_2$ to cross-link FcγRI (lanes 2, 5 and 8) or goat anti-mouse (GAM) $F(ab')_2$ to cross-link FcγRII (lanes 3, 6 and 9) or remained unstimulated (lanes 1, 4 and 7). Immune complexes were formed using anti-phosphotyrosine (lanes 1–3) or anti-Syk (lanes 4–9) antibodies. Proteins were labelled in an immune complex kinase reaction for 2 min. A small fraction of anti-Syk complexes (1/20) was loaded as 1st immunoprecipitates (lanes 4–6) and the remainder of the samples were boiled in SDS and subjected to a second round of immunoprecipitation (IP) using mAb IV.3 to precipitate FcγRII (lanes 7–9).

pattern with this catalytically active fragment. To distinguish between the two proteins, anti-Syk precipitated complexes were labelled using the kinase assay and each protein was re-immunoprecipitated following denaturation. Whereas anti-Syk antibody precipitated both forms of Syk (Figure 3, lanes 5 and 6), the phosphorylated receptor could only be detected in FcγRII-activated cells (lane 10). All tyrosine phosphorylated proteins were identified by anti-phosphotyrosine immunoprecipitation under denaturing condition (lanes 7 and 8), and no phosphoprotein was detected using a control mouse antibody (lanes 11 and 12). The absence of FcγRII in Syk-coprecipitated complexes from unstimulated cells suggested that either the receptor was not present in these precipitates or Syk could not phosphorylate the receptor in unstimulated cells. However, when FcγRII and Syk were coprecipitated *in vitro* by the simultaneous addition of both mAb IV.3 and anti-Syk antibodies, the receptor was phosphorylated in unstimulated cells (data not shown). This observation suggests that FcγRII serves as a proper substrate for Syk *in vitro* and it is likely that FcγRII was not present in Syk precipitated complexes in unstimulated cells.

As shown in Figure 3, no significant increase in the kinase activity of Syk was detected following cross-linking of the receptor under the conditions used. Incorporation of labelled phosphate was linear at the time that the kinase activity was measured. The enzymic activity of Syk was also evaluated in a time course following FcγRII activation and in the presence of an exogenous substrate to maximize the sensitivity of the assay (data not shown). However, there was still no significant increase in the kinase activity of Syk. Our data demonstrated that only a small fraction of this kinase is associated with FcγRII. If only this fraction of Syk is activated by FcγRII cross-linking, it is likely that an increase in the enzymic activity of this small pool of Syk could remain undetected because of the high enzymic activity of total cellular Syk.

The specificity of the association of Syk with FcγRII

We have previously reported that FcγRI activation resulted in an increase in PTK activity very similar to FcγRII activation with the exception of FcγRII tyrosine phosphorylation [21,22]. Syk was also tyrosine phosphorylated following FcγRI activation in these cells (data not shown). Therefore, we examined the specificity of Syk and FcγRII interactions by comparing this association in FcγRI- and FcγRII-activated cells. Cells were incubated with a mixture of mAb IV.3 Fab fragment and monomeric hIgG1 (which binds only to FcγRI) at 4 °C for 45 min. Cells were transferred to 37 °C, and stimulated for 5 min with goat anti-mouse or goat anti-human $F(ab')_2$ fragments, to cross-link FcγRII or FcγRI respectively, or remained unstimulated. Cell lysates were subjected to immunoprecipitation using anti-phosphotyrosine (Figure 4, lanes 1–3) or anti-Syk (lanes 4–9) antibodies and proteins were labelled with [γ-^{32}P]ATP *in vitro*. The anti-Syk precipitated proteins were denatured by boiling in 2% (w/v) SDS and subjected to a second round of immunoprecipitation with mAb IV.3 to isolate FcγRII away from the 40 kDa fragment of Syk (lanes 7–9). As shown in Figure 4, monomeric human IgG or univalent IV.3 Fab did not induce tyrosine phosphorylation of Syk (lane 1); however, cross-linking of either receptor resulted in tyrosine phosphorylation of Syk (lanes 2 and 3). Interestingly, phosphorylated FcγRII was detected only in FcγRII-activated cells (lanes 3, 6 and 9) indicating that the FcγRII–Syk association was specific for FcγRII aggregation. The absence of Syk–FcγRII association in FcγRI-activated cells suggests that a distinct pool of Syk is activated following cross-linking of each receptor.

DISCUSSION

One of the earliest events associated with FcγRII-mediated signal transduction is the rapid tyrosine phosphorylation of cellular proteins. Several of these substrates including PLC-γ1 [17], PLC-γ2, GTPase activating protein, Vav [30], Shc [31], Syk [26] and the receptor itself [13,21] have been identified. As FcγRII has no intrinsic enzymic activity, the cytoplasmic PTKs have been implicated in FcγRII signal transduction. A physical and functional association of FcγRII with Src-related PTKs has

recently been demonstrated [22,23]. Immunoprecipitation of FcγRII assessed in an immune complex kinase assay indicated coprecipitation of PTK activity with the receptor in monocytic THP-1 cells. Several proteins, including Lyn and a 72 kDa phosphoprotein, were among the coprecipitated proteins [22] (Figure 2). The present study identified the 72 kDa protein as Syk, a member of a newly described Syk family of PTKs [29,32]. Syk is also one of the components of the heavily tyrosyl phosphorylated 72 kDa band observed upon FcγRII activation. The kinetics of Syk tyrosine phosphorylation paralleled the early appearance of tyrosyl phosphorylated proteins observed following FcγRII activation in THP-1 cells [21]. The rapid and transient kinetics of Syk tyrosine phosphorylation suggested that autophosphorylation and activation of Syk was likely. Although the possibility of cross-phosphorylation of Syk by Src-PTKs could not be excluded in our study, recent evidences utilizing the receptor–kinase chimeras suggest that the Syk family kinases act more directly in the signalling cascades triggered in T cells [33].

Human monocytic cells express two classes of Fcγ receptors, hFcγRI and hFcγRII. Aggregation of either receptors on phagocytic leukocytes triggers similar cellular effector responses [2]. In addition, the patterns of tyrosine phosphorylation following cross-linking of these receptors are comparable [20,21,26]. Interestingly, whereas Syk is phosphorylated following aggregation of either receptor [26] (Figure 4), tyrosine phosphorylation of FcγRII was unique to FcγRII activation [21] (Figure 4). In contrast to our observations, Kiener et al. [30] recently reported tyrosine phosphorylation and association of FcγRII in FcγRI-stimulated cells. However, in these studies, IV.3 IgG, not Fab, was used for stimulation of cells. This antibody can bind to protein G(A)–agarose during immunoprecipitation resulting in subsequent phosphorylation of FcγRII by Syk or Src-PTKs *in vitro*. Our data demonstrate that FcγRII is a substrate for Syk *in vitro*. If FcγRII also serves as a substrate for Syk *in vivo*, one explanation for the absence of FcγRII tyrosine phosphorylation in FcγRI-activated cells would be the focal activation of Syk. Tyrosine phosphorylation of only a small fraction of total cellular Syk following cross-linking of each receptor is supportive of this hypothesis.

Recently, the tyrosine phosphorylation of FcγRI-associated γ chain and association of Syk with this subunit in monocytic U937 cells was demonstrated [34]. Therefore, it is likely that aggregation of each class of FcγR promotes focal activation of the associated Syk with the subsequent tyrosine phosphorylation of the receptor (FcγRII) [21] or the receptor components (γ chain of FcγRI) [33]. This, in turn, would permit docking and recruitment of the potential substrates, Src-PTK and their substrates. The overlapping pattern of tyrosine-phosphorylated cellular substrates and biological responses mediated by these receptors in myeloid cells [2] suggests that Syk provides a potential convergence point for the different FcγR signalling pathways.

Both members of the Syk family kinases, ZAP-70 and Syk, have been shown to associate with the intracellular domains of associated subunits of multichain immune receptors. Association of ZAP-70 with the ζ chain of TCR–CD3 complex is transient and requires coexpression of Lck or Fyn [32,33]. Phosphorylation of ζ chain by Src-PTKs following TCR activation mediated the interaction of both SH2 domains of ZAP-70 with both phosphorylated tyrosine residues within each ARAM motif of the CD3-ζ [35]. Furthermore, the coaggregation of chimeras bearing ZAP-70 and Fyn was required for the initiation of biological responses in T cells [33]. These data suggest that ZAP-70 activation is distal from and dependent on the activation of members of Src-PTKs. However, Syk association with components of BCR or FcεRI has been shown to be stable [24,25]. In addition, aggregation of chimeras containing Syk kinase was sufficient to allow induction of specific functional responses [33], suggesting that the models of activation of Syk and ZAP-70 might be different.

Our data demonstrated a stable and specific association between FcγRII and Syk. Association of Syk with FcγRII was constitutive when anti-receptor antibody was utilized to precipitate the complex. In contrast, when these complexes were precipitated by anti-Syk antibody, FcγRII was associated with Syk only after stimulation. Recently, a similar observation was made for association of FcγRIIIA γ chain with Syk in macrophages [36]. The reason for this discrepancy is unclear; however, one possibility is that Syk and FcγRII interact with low affinity in unstimulated cells and anti-Syk antibody could disturb their interaction. However, phosphorylation of FcγRII by either Syk or Src-PTKs might mediate the ability of Syk SH2 domains to interact with the tyrosine-containing motifs present in the cytoplasmic domain of FcγRII, resulting in a tight interaction of these proteins that cannot be disrupted by anti-Syk antibody. Another possibility is that FcγRII is present in anti-Syk precipitated complexes from unstimulated cells, but cannot be phosphorylated. Tyrosine phosphorylation of FcγRII by Syk observed following simultaneous coprecipitation of both proteins from lysates of unstimulated cells argues against this possibility (data not shown). Because our findings demonstrated that FcγRII is an excellent substrate for both Src-PTKs and Syk *in vitro* [22] (Figures 3 and 4), it is not yet clear which kinase actually phosphorylates FcγRII *in vivo*. Genetic approaches will be taken to address this question. Nevertheless, the tyrosine residues within the cytoplasmic domain of FcγRIIA (C) are essential for subsequent activation of cellular effector programs [8].

Studies presented here, together with our previous studies and results obtained from other laboratories, enforce the concept that the signalling molecules employed by FcγRII are very similar to those utilized by the B-cell receptor and FcεRI. These receptors are expressed as multipolypeptide complexes composed of at least one homo- or hetero-dimer homologous to the ζ chain family [37]. These associated chains contain at least one copy of the ARAM [12] which carries sufficient structural information to activate signalling pathways [38,39]. Studies with chimeras indicated that ARAM-containing subunits interact with distinct sets of effector molecules and differ in their capacity to activate specific cellular functions [40–42]. Furthermore, expression of all subunits is required for full functional capacity of the receptors [42,43]. Jouvin et al. [42] recently reported a differential association of FcεRI subunits with PTKs [42]. Whereas the β subunit associated with Lyn, the γ chain recruited Syk and coexpression of both chains was required for full signalling capacity of FcεRI. In B cells, Ig-α and Ig-β activate different signalling pathways and interact with distinct sets of effector molecules [40,41]. In addition, genetic approaches utilizing Syk^- and Lyn^- B cell lines demonstrated that whereas Syk and Lyn mediate distinct functions in BCR signal transduction, both are required for coupling of BCR to full PTK activity [43]. Even though Syk was activated by receptor aggregation to some extent in Lyn^- cells, Lyn-dependent phosphorylation increased the autophosphorylation activity of Syk through BCR stimulation, suggesting regulation of Syk activity by Src-PTKs [44].

In contrast to BCR and FcεRI, FcγRII is a single-chain molecule that contains one copy of a tyrosine-containing motif similar but not identical to ARAM [7,8]. Therefore this receptor provides a model to examine how a single ARAM can interact with multiple families of PTKs and mediate a complete spectrum of cellular effector functions.

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