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Influence of tyrosine phosphorylation on protein interaction with FcγRIIa

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Abstract

The cytoplasmic tail of FcγRIIa present on human neutrophils shares with other antigen receptors a common amino acid sequence called ITAM (Immunoreceptor Tyrosine-based Activation Motif). After receptor ligation, the tyrosine residues within this motif become phosphorylated. We prepared a recombinant fusion protein of the cytoplasmic tail of FcγRIIa (containing the ITAM) with glutathione–S-Transferase (GST-CT) to characterize the phosphorylation of FcγRIIa and its ability to interact with other proteins involved in signal transduction. The GST-CT became phosphorylated in the presence of Lyn, Hck and Syk (immunoprecipitated from human neutrophils), but not in the presence of Fgr. Of the active kinases, only Lyn (mainly present in the membrane fraction) was found to associate with the GST-CT in the absence of ATP. This association was also observed in immunoprecipitates of FcγRIIa from resting neutrophils, suggesting that Lyn might be the kinase responsible for the initial FcγRIIa phosphorylation. Moreover, we observed specific association of Syk and the p85 subunit of PI 3-kinase after incubation of the GST-CT with neutrophil cytosol. This interaction was dependent on tyrosine phosphorylation of the GST-CT. Substitution of 269Tyr by Phe almost completely abolished tyrosine phosphorylation of the fusion protein. Substitution of either 253Tyr or 269Tyr eliminated Syk binding, but only 253Tyr appeared to be essential for p85 binding. We hypothesize that, upon activation, the membrane-associated Lyn is responsible for the initial tyrosine phosphorylation of FcγRIIa, thus creating a docking site for Syk and PI 3-kinase. © 1997 Elsevier Science B.V.

Keywords: FcγRIIa; Antigen receptor; Immunoreceptor tyrosine-based activation motif; Tyrosine phosphorylation; Neutrophils

1. Introduction

Neutrophils play an important role in host defense, exerting various responses, such as phagocytosis, superoxide production and release of hydrolytic enzymes, to kill engulfed micro-organisms. Most of those responses are initiated through binding of the Fc domain of an IgG-antigen complex to an Fcγ

Abbreviations: FcγR, the receptors for IgG; PI-3 kinase, phosphatidylinositide-3-OH kinase; SH2, Src-homology type 2; ITAM, immunoreceptor tyrosine-based activation motif.

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receptor on the cells [1]. Two FcγR are present on the neutrophil membrane, FcγRIIa and FcγRIIIb. Although both receptors are able to induce neutrophil activation upon crosslinking, heterotypic clustering of these receptors results in a synergistic effect [2]. To understand the mechanism of this synergism, detailed knowledge on protein interactions with these receptors is mandatory. Unlike other Fcγ receptors such as FcγRI and IIIA, which are known to be associated with another protein [3], FcγRIIa and FcRIIIb on human neutrophils do not seem to have such an associated subunit (Masuda and Roos, unpublished observations). However, the cytoplasmic tail of FcγRIIa itself contains, along with the γ-chain and other subunits of antigen receptors, an amino acid sequence called ITAM (Immunoreceptor Tyrosine-based Activation Motif), which has been shown to be involved in signal transduction [4]. This motif consists, in FcγRIIa, of two YXXL sequences separated by 12 residues. When the tyrosines become phosphorylated, these YXXL motives become docking sites for proteins containing SH2 domains [5]. One of the earliest events following FcγRII cross-linking is tyrosine phosphorylation of multiple proteins including the receptor itself [6]. Because FcγRIIa has no intrinsic tyrosine kinase activity, other protein tyrosine kinases must be involved.

There are, up to now, nine known members of the Src family of non-receptor protein tyrosine kinases (PTK) which have been shown to be involved in the phosphorylation of cell-surface receptors. All share some common features: a carboxy-terminal kinase domain, an SH2 domain able to interact with phosphotyrosine residues, an SH3 domain able to recognize proline-rich sequences and a 70 amino acid amino-terminal sequence characteristic for each kinase, the unique region. Moreover, all can be myristoylated, important for the anchoring to the inner leaflet of the plasma membrane [7]. These kinases have been shown to be physically associated with B- and T-cell receptors and play an important role in the signal transduction of these receptors. In case of FcγRIIa, an association with Fgr has been reported in human neutrophils [8], and with Hck and Lyn in monocytic cells [9,10].

A second group of cytosolic protein tyrosine kinases comprises ZAP70 and Syk. These PTKs have two tandem SH2 domains which allow them to bind preferentially to the two phosphorylated tyrosines present in the same ITAM. This ITAM binding increases the activity of these kinases [11,12]. Syk has been shown to be phosphorylated after FcγRII cross-linking in HL-60 cells [13] and to be physically associated with phosphorylated FcγRII in platelets [14,15].

PI 3-kinase has been shown to be involved in a wide variety of cellular responses [16–18]. p85, the regulatory subunit of PI 3-kinase, has two SH2 domains enabling it to bind to tyrosine phosphorylated receptors such as CD19 in activated B cells [19]. A role for PI 3-kinase in FcγR cross-linking was first suggested by Ninomiya et al. [20] on the basis of experiments with U937 cells. We observed a rapid translocation of PI 3-kinase to the membrane after FcγR cross-linking in human neutrophils, even prior to the generation of $InsP_3$ and elevation of cytosolic free Ca^{2+} [21]. Moreover, two different inhibitors of PI 3-kinase block these early signal transduction events [21].

In the current model for signal transduction through ITAM-containing proteins [22], receptor cross-linking leads to transphosphorylation by receptor-associated Src-family kinases of opposing receptor ITAMs. This causes first a change in mechanism of the kinase–receptor association: from a binding via the kinase unique region to an SH2-(pY)ITAM interaction. But subsequently, other SH2-containing proteins such as Syk, Shc, and PI 3-kinase, can effectively compete for the double phosphorylated ITAM and can displace the original kinase [22]. In the study described here, we investigated the role of the tyrosine residues in the FcγRIIa cytoplasmic tail in its phosphorylation and the binding of SH2-containing proteins.

2. Experimental procedures

2.1. Reagents and chemicals

Rabbit antibodies against Hck, Lyn, and Syk were purchased from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A.). Antiserum against Fgr [23] was kindly donated by Dr. G. Berton (University of Verona, Italy). Mouse monoclonal antiphosphotyrosine (4G10) was obtained from UBI (Lake Placid,

New York, U.S.A.). The anti-human FcγRIII mAb CLBFcgran1 (mIgG2a) [24] and the anti-human FcγRII mAb IV.3 (mIgG2b) [25] were purified from hybridoma culture supernatant by precipitation with 50% saturated ammonium sulfate and subsequent protein A chromatography. A polyclonal rabbit antiserum directed against the cytoplasmic tail of FcγRIIa was generated in our laboratory by coupling of a synthetic peptide (CYLTLPPNDHVNSNN) to keyhole limpet hemocyanin (KLH). The last 14 residues in this peptide correspond to the FcγRIIa carboxy-terminal end, the cysteine being added to the N-terminus to allow coupling. After 3 immunizations in complete Freud's adjuvant, serum (designated CT10) was collected and IgG was purified on a protein A-Sepharose column (Pharmacia, Uppsala, Sweden). A 1000-fold dilution of purified CT10-antibodies recognized on a Western blot a 40-kD band in a neutrophil lysate or in an immunoprecipitate obtained with mAb IV.3 directed against FcγRII [25]. Moreover, the antibody was only positive in FACS analysis only after digitonin permeabilization of the neutrophils. All these were blocked in the presence of the synthetic peptide depicted above. Monoclonal anti-GST antibody was a kind gift of Dr. M. Gebbink (Netherlands Cancer Institute, Amsterdam, The Netherlands). Anti-p85 antibodies were raised in rabbits against purified recombinant p85 as described previously [26].The proteinase inhibitors DFP (diisopropylfluorophosphate), TPCK (L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone), pepstatin and leupeptin were from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.), Pefablock from Merck (Darmstadt, Germany).

2.2. GST-CT production

The coding sequence of the cytoplasmic tail (CT) of FcγRIIa (aa 206-282) was amplified from monocytes cDNA by PCR, then cloned into bacterial expression vector pGEX-2T (Pharmacia, Uppsala, Sweden) to produce a fusion protein of CT with glutathione–S-transferase (GST-CT). In addition, a plasmid encoding GST fused to Syk(5-278) (containing the two SH2 domains of Syk) was generously provided by Dr. Jürgen Wienands (Max-Planck-Institut für Immunbiologie, Freiburg, Germany) and processed as described below. Bacterial cultures were induced with 0.2 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) and grown overnight at 28°C. The cells were lysed in NP40 lysis buffer (see above) supplemented with lysozyme (250 μg/ml) by incubation for 30 min at 4°C. The lysate was centrifuged ($14\,000 \times g$, 10 min) and the fusion proteins were affinity purified on gluthatione–agarose (Sigma). Protein concentrations were estimated with the BCA method (Pierce, Rockfort, Illinois, U.S.A.) following the manufacturer's instructions. The fusion protein containing the two SH2 domains of Syk was cut from the GST by thrombin cleavage [27]. Standard PCR techniques were used to construct point mutants of the GST-CT fusion protein, in which each Tyr of the cytoplasmic tail was changed into Phe (Y246F = Y1F; Y253F = Y2F; Y269F = Y3F). The accuracy of the DNA products was checked by Taq-Track DNA sequencing (Promega).

2.3. Isolation and incubation of neutrophils

Neutrophils were isolated from blood obtained from healthy donors by Percoll density gradient centrifugation as described before [28]. After 2 washes in PBS, neutrophils (10^7/ml) were resuspended in incubation medium (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM KP_i, 5.5 mM glucose, 0.5% (w/v) human serum albumin, pH 7.4) and incubated at 37°C for 5 min with 5 μg/ml of biotinylated Fab fragments of mAb IV.3 and mAb CLBFcgran1 [2]. The cells were centrifuged and resuspended in fresh incubation medium at 37°C. Cross-linking was performed by adding 1.5 μg/ml of streptavidin (Sigma) with continuous stirring, resting cells were not exposed to streptavidin. Stimulation was stopped by addition of a ten-fold excess of ice-cold PBS.

2.4. Neutrophil fractionation

The cells were resuspended (10^8/ml) in ice-cold sonication buffer (10 mM Hepes, 150 mM NaCl, 170 mM sucrose, 1 mM EGTA, 1 mM sodium vanadate, pH 7.4) with proteinase inhibitors added (1 mM Pefablock, 50 μM TPCK, 50 μM leupeptin, 1 μM pepstatin) and treated with DFP (1 mM) and phenylarsine oxide (PAO, 25 μM) for 10 min at 4°C. After centrifugation, the cells were resuspended in the same

buffer without DFP and PAO and disrupted by sonication (3 × 15 s, 8-μm amplitude). After centrifugation (10 min, 800 × *g*), 1.5 ml of postnuclear supernatant were layered on a discontinuous sucrose gradient consisting of layers of 15% (w/v), 40%, and 52% sucrose and centrifuged (30 min, 100 000 × *g*) at 4°C. The top phase (1.0 ml of cytosol) and the 15/40% interphase (0.4 ml of plasma membranes) were collected and frozen.

2.5. Immunoprecipitation and in vitro kinase assay

Plasma membranes (adjusted to 0.2 mg of protein/ml) were first solubilized in lysis buffer (10 mM Hepes, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM sodium vanadate, pH 7.4 plus the proteinase inhibitors indicated above). For immunoprecipitation of FcγRIIa, 60 mM octyl-D-glucoside was used instead of NP-40. After incubation for 30 min on ice, the preparation was cleared by centrifugation (14.000 × *g*, 10 min). The solubilized membranes and cytosol sample were precleared by incubation with protein G-Sepharose beads for 1 h at 4°C and subsequently incubated with an appropriated amount of antibodies bound to protein G-Sepharose. After 90-min incubation at 4°C, the protein G-Sepharose beads were washed four times with ice-cold lysis buffer. For measurement of tyrosine kinase activity in the immunoprecipitate, the beads were washed once in kinase buffer (20 mM Tris-HCl, 150 mM NaCl, 50 μM ATP, 5 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM sodium vanadate, pH 7.4), then resuspended in kinase buffer in a final volume of 100 μl in the presence or absence of 10 μg of GST-CT fusion protein as substrate. The samples were incubated at 25°C for 15 min with continuous shaking. The reaction was stopped by adding 50 mM EDTA. The beads were spun down and resuspended in non-reducing Laemmli buffer [29]. Adsorbed proteins were separated by SDS-PAGE (10% acrylamide) and analyzed by Western blot.

2.6. GST-CT binding studies

To measure binding to unphosphorylated GST-CT, glutathione beads having bound GST-CT (10 μg/ml) were incubated with neutrophil membranes or cytosol (derived from 50×10^6 neutrophils, diluted in 1 ml of lysis buffer) for 2 h at 4°C. After washing, the beads were resuspended in 100 μl kinase buffer and subjected to an in vitro kinase assay as described above. To measure binding to phosphorylated fusion protein, 10 μg of either recombinant GST, GST-CT and the different GST-CT mutant proteins (adsorbed on glutathione–agarose) were incubated with solubilized neutrophil membrane fraction (200 μl, diluted into 500 μl of kinase buffer) for 2 h at 4°C. The beads were washed four times, resuspended in 100 μl of kinase buffer and incubated for 10 min at 37°C to obtain a more complete phosphorylation by the associated kinase (see text). After one additional wash, the beads were incubated with cytosol (1 mg protein/ml) for 120 min at 4°C in 1.0 ml of lysis buffer. The beads were washed four times in ice-cold lysis buffer and finally resuspended in reducing Laemmli buffer. Adsorbed proteins were separated by SDS-PAGE (10% acrylamide) and analyzed by Western blot.

2.7. Western blot

After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes. Unoccupied sites were blocked with 5% skimmed milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0) and the blot was probed with antibodies diluted in 2.5% skimmed milk in TBST for 1 h. Thereafter, the blot was washed and incubated with the secondary, HRP-conjugated antibody (CLB, Amsterdam, The Netherlands). Finally, bound antibodies were detected by enhanced chemiluminescence (Boehringer Mannheim, Germany). When necessary, the blots were stripped by incubation in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 100 mM β-mercaptoethanol at 50°C for 30 min prior to washing in TBST and reprobing.

3. Results

3.1. FcγRIIa phosphorylation by various tyrosine kinases

To investigate protein interactions with FcγRIIa, the cytoplasmic tail (CT) of this receptor was expressed as a fusion protein with glutathione–S-trans-

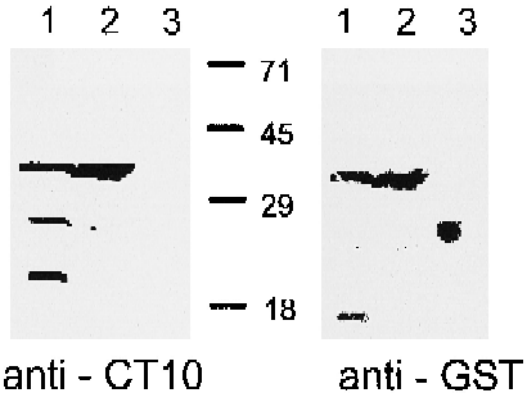

Fig. 1. Characterization of the GST-CT fusion protein. Bacteria containing a pGEX plasmid encoding GST coupled to the cytoplasmic tail of FcγRIIa were induced with IPTG and lysed as described in Section 2. Subsequently, the GST-CT fusion protein was isolated with glutathione–agarose beads. The figure shows the immunoblot of bacterial lysate (lane 1), purified GST-CT (lane 2) and purified GST (lane 3) incubated with rabbit antibodies recognizing the cytoplasmic tail (anti-CT10, left panel) and a monoclonal antibody directed against GST (right panel).

ferase (GST) and coupled to glutathione–agarose beads. Fig. 1 shows the immunoblot with an antiserum raised against a synthetic peptide derived from the C-terminus of the tail, which recognized a protein with an apparent molecular mass of 35 kD. A protein of the same size was recognized with an anti-GST antibody (Fig. 1).

We next investigated a possible specific role of Src-like tyrosine kinases in FcγRIIa phosphorylation by using the purified GST-CT fusion protein as a substrate for various kinases. Fgr, Hck and Lyn are the most prominent Src-like kinases expressed in neutrophils [30]. There are several isoforms of these proteins with different intracellular distributions. Hck is present in both the membrane and cytosol fraction of neutrophils, whereas Fgr and Lyn are mainly membrane-associated (data not shown). On the other hand, Syk, another tyrosine kinase, is completely cytosolic (data not shown). Thus, we immunoprecipitated Fgr and Lyn from the membrane fraction and Hck and Syk from the cytosol fraction of resting neutrophils and mixed these immunoprecipitates with the GST-CT fusion protein. Hck, Lyn and Syk immunoprecipitates were all able to tyrosine phosphorylate the GST-CT fusion protein, but the Fgr immunoprecipitate was clearly not (Fig. 2).

3.2. Lyn association with FcγRIIa

We next tested whether the cytoplasmic domain of FcγRIIa is able to interact with the Src-like kinases in resting conditions. The unphosphorylated GST-CT fusion protein was used as a probe to bind proteins from cytosol and NP40-solubilized membranes. The adsorbed proteins were subjected to an in vitro kinase assay, separated by SDS-PAGE and blotted with anti-phosphotyrosine antibodies (Fig. 3, left panel). Tyrosine phosphorylation of the GST-CT fusion protein due to adsorbed membrane proteins was much

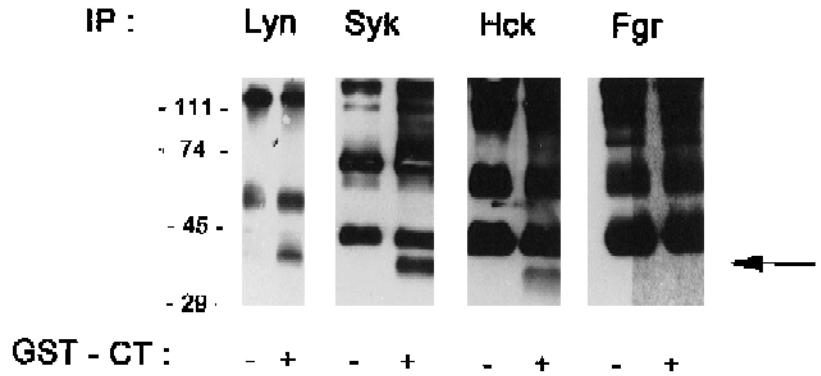

Fig. 2. Phosphorylation of GST-CT by various kinases. Membranes (in the case of Fgr and Lyn) and cytosol (for Hck and Syk) from $30 \times 10]^6$ neutrophils were used to immunoprecipitate tyrosine kinases with the appropiate antibodies as described in Section 2. The protein G beads were subjected to an in vitro kinase assay for 15 min at 25°C in the absence (−) or presence (+) of the GST-CT fusion protein. Tyrosine phosphorylation was assessed with anti-phosphotyrosine antibodies. The position of the GST-CT fusion protein is marked with an arrow. Lyn (lanes 1 and 2); Hck (lanes 3 and 4); Fgr (lanes 5 and 6); Syk (lanes 7 and 8). Results are representative of at least two experiments.

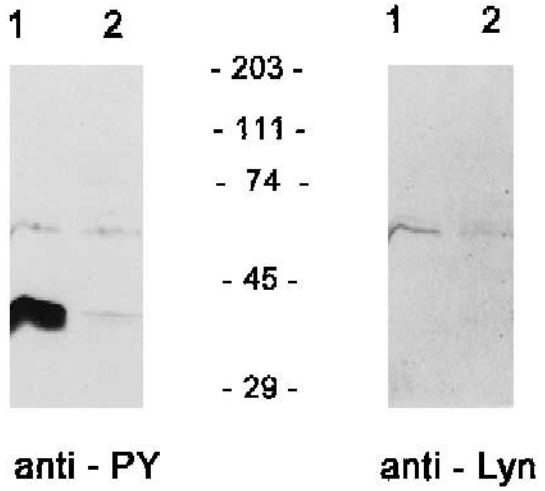

Fig. 3. Tyrosine kinase activity binding to the GST-CT fusion protein. GST-CT (10 μg, bound to glutathione–agarose) was incubated with membranes or cytosol (isolated from 50×10^6 neutrophils) in lysis buffer for 120 min at 4°C. After washing, the glutathione–agarose beads were subjected to an in vitro kinase assay. (A) Western blot with anti-phosphotyrosine antibodies to assess tyrosine phosphorylation. (B) Reprobing of the same blot with anti-Lyn antibodies. Results are representative of at least three experiments.

higher than with adsorbed cytosolic proteins. Two associated proteins of about 60 kD were also tyrosine phosphorylated. After reblotting with specific antibodies, these two bands were identified as the two Lyn isoforms (Fig. 3, right panel), suggesting a specific association with unphosphorylated FcγRIIa. Neither Fgr nor Hck were detectable in these samples (data not shown).

An association of Lyn with FcγRIIa was also suggested by immunoblot analysis of FcγRIIa immunoprecipitated from human neutrophils (Fig. 4). Both in resting neutrophils and in neutrophils activated via FcγR cross-linking, co-immunoprecipitation of Lyn with FcγRIIa was observed. Comparison of the specific Lyn signal obtained with a graded amount of neutrophil membranes indicated that about 5% of the FcγRIIa receptors were associated with Lyn (data not shown).

3.3. Interaction of cytosolic proteins with phosphorylated GST-CT

To further investigate the involvement of cytosolic proteins in the FcγRIIa signal transduction pathway, the phosphorylated GST-CT was used to affinity-purify proteins from neutrophil cytosol. The fusion protein was first incubated with neutrophil membranes in the presence of ATP to allow tyrosine phosphorylation prior to washing and incubation with cytosol from resting neutrophils. Using specific antibodies we identified Syk (Fig. 5A) and p85, the regulatory subunit of PI-3 kinase (Fig. 5B) as two of the proteins that had bound to phosphorylated GST-CT. These interactions were not observed in the presence of GST alone and were dependent on GST-

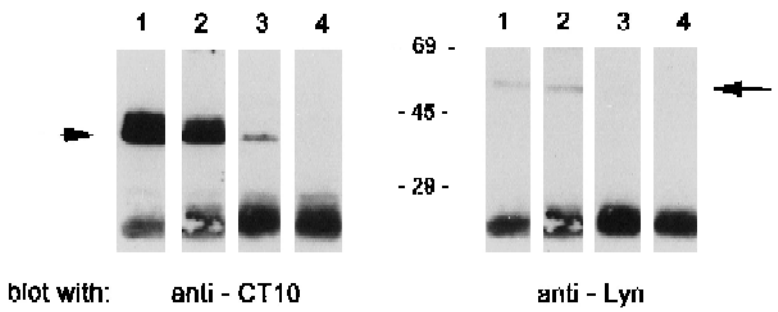

Fig. 4. Association of Lyn with FcγRIIa in human neutrophils. Neutrophils were incubated under resting conditions (lanes 1 and 3) or stimulated by cross-linking of FcγRIIa and FcγRIIIb (lanes 2 and 4) as described in Section 2. After 15 s of stimulation, neutrophils were fractionated and the membrane fraction derived from 50×10^6 neutrophils was used to immunoprecipitate FcγRIIa with mAb IV.3 (lanes 1 and 2). As control, the various membrane fractions were treated with mIgG2b coupled to protein G-Sepharose (lanes 3 and 4). The immunoprecipitates were checked by immunoblot analysis for the presence of FcγRIIa (A) with anti-CT10 antiserum or Lyn (B) with anti-Lyn antibodies.

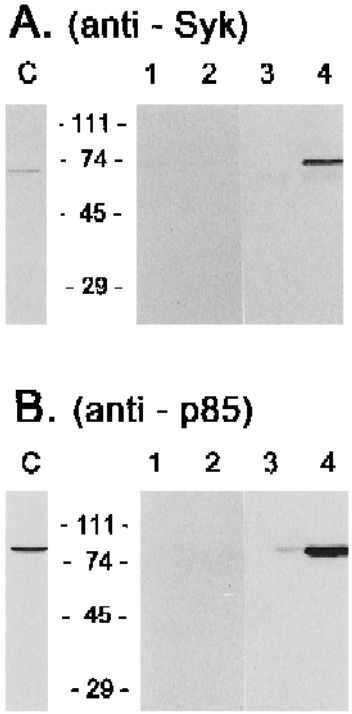

Fig. 5. Specific association of cytosolic proteins with phosphorylated GST-CT. GST (10 μg, lanes 1 and 3) or GST-CT fusion protein (10 μg, lanes 2 and 4) bound to glutathione–agarose were incubated with neutrophil membranes in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of ATP as described in Section 2. After washing, the glutathione–agarose beads were incubated (1.0 ml) with neutrophil cytosol (1 mg/ml) for 120 min at 4°C. After washing, adsorbed proteins were resolved by SDS-PAGE, transferred to nitrocellulose and probed with (A) anti-Syk and (B) anti-p85 antibodies. In the left lane, neutrophil cytosol (C) was analyzed as a positive control for the immunoblot.

CT phosphorylation, because binding was not observed in the absence of ATP. Both proteins were absent from the GST-CT beads prior to the incubation with cytosol (data not shown).

The binding of Syk to the GST-CT fusion protein was blocked in the presence of the tandem SH2 domains of Syk (Fig. 6), showing the specific role of these domains in the binding to phosphorylated GST-CT. In accordance with data obtained with other ITAMs able to bind Syk [11,31], autophosphorylation of Syk increased when the GST-CT was added to the immunoprecipitated kinase (Fig. 7).

3.4. Differential roles of tyrosine residues in FcγRIIa

To investigate the role of the three different tyrosines present in the cytoplasmic tail of FcγRIIa, we generated point mutated GST-CT fusion proteins in which each of these tyrosine residues was changed into a phenylalanine (Y1F, Y2F, Y3F). The 2nd and 3rd tyrosine residue are contained in the ITAM motif, whereas the most N-terminal tyrosine in the tail (Y1) has been shown to moderately influence phagocytosis in COS transfectants [32]. Substitution of Y3 severely compromised total tyrosine phosphorylation of the GST-CT fusion protein, while the Y2F substitution had a lower and the Y1F substitution had almost no effect (Fig. 8A). Substitution of Y1 decreased Syk binding to the fusion protein, whereas substituting either Y2 or Y3 inhibited this binding completely (Fig. 8B). The binding of p85 was completely blocked

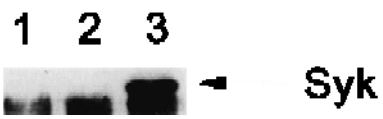

Fig. 6. Effect of Syk(5-278) on Syk binding to phoshorylated GST-CT. GST (10 μg, lane 1) or GST-CT fusion protein (10 μg, lanes 2 and 3) bound to glutathione–agarose were phosphorylated and incubated as described in the legend to Fig. 5. In the incubation with neutrophil cytosol loaded in lane 2, Syk(5-278) (50 μg) was added as well. Adsorbed proteins were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-Syk antibodies. The arrow indicates the position of the normal Syk protein.

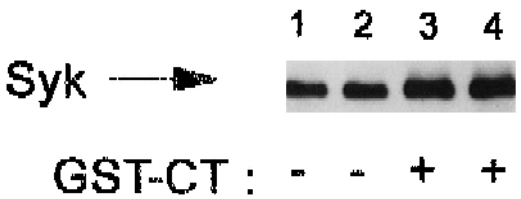

Fig. 7. Effect of GST-CT on Syk autophosphorylation. A Syk immunoprecipitate of neutrophil cytosol was obtained as described in Fig. 1, and incubated in the absence (−) or presence (+) of GST-CT fusion protein. Subsequently, samples were analyzed by immunoblotting with anti-phosphotyrosine antibodies. Only the part, containing the Syk protein (indicated by the arrow) is shown.

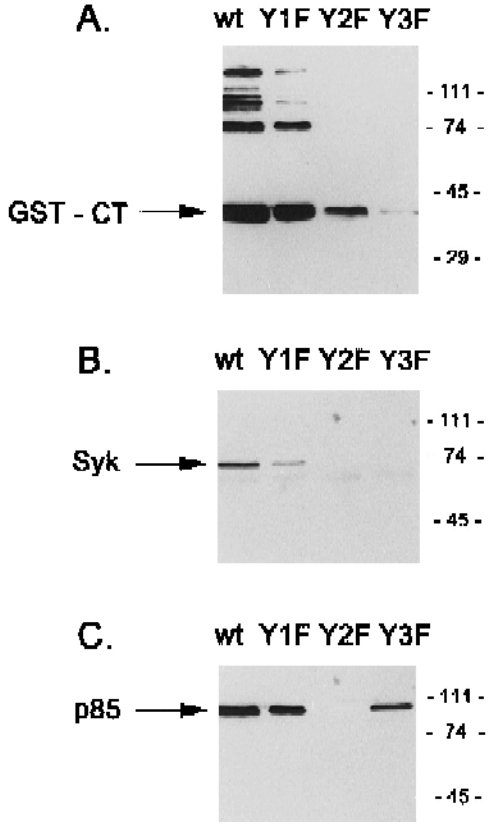

Fig. 8. Effect of mutations in the GST-CT fusion protein on tyrosine phosphorylation, Syk binding and p85 binding. Different mutant proteins of GST-CT were prepared as described in Section 2, in which each tyrosine of the cytoplasmic tail (Y1, Y2 and Y3) was replaced by a phenylalanine (F). The wild-type GST-CT fusion protein (wt) and the mutant proteins (Y1F, Y2F, Y3F) bound to glutathione–agarose were incubated with neutrophil membranes in the presence of ATP, washed and subsequently incubated with neutrophil cytosol in the absence of ATP. (A) Measurement of tyrosine phosphorylation on adsorbed proteins with anti-phosphotyrosine monoclonal antibody; (B) Western blot with anti-Syk antibodies; (C) Western blot with anti-p85 antibodies. Results shown are representative of three experiments.

in the Y2F mutant protein, whereas the substitutions in Y1 and Y3 had a much smaller effect (Fig. 8C).

4. Discussion

In this study we investigated early events after FcγR cross-linking in neutrophils by analysis of protein interactions with the cytoplasmic tail of FcγRIIa. The role of tyrosine phosphorylation and of the Src-family of tyrosine kinases has clearly been established in the signal transduction of immunological receptors, such as the B-cell receptor (involving Lyn, Fyn, Blk, Lck, and Fgr) [33–35], the T-cell receptor (involving Fyn [36] and Lck [37]), the FceRI (involving Lyn [38]) and FcγRIIIA (involving Lck [39]). A role for Lyn and Hck in FcγRII-mediated signal transduction in the monocytic cell line THP1 has also been suggested [9,40]. We investigated the possible role in the tyrosine phosphorylation of FcγRIIa in neutrophils of any of the three Src-family tyrosine kinases predominantly expressed in neutrophils: Fgr, Hck and Lyn [30]. We observed that both Hck and Lyn immunoprecipitates, but not Fgr immunoprecipitates phosphorylated the cytoplasmic tail of FcγRIIa coupled to GST. Previously, phosphorylation of FcγRIIa by Src and by Fyn has been reported [41,42]. Thus, it seems that the cytoplasmic tail of FcγRIIa can be a substrate for a wide variety of PTK except for Fgr. Surprisingly, Fgr is the only Src-like PTK which has been previously reported to be associated with FcγRIIa in neutrophils [8]. Possibly, there is a role for Fgr other than tyrosine phosphorylation of the receptor itself.

In the course of our studies, we observed co-immunoprecipitation of FcγRIIa when antibodies against membrane-associated kinases (Fgr and Hck) were used for precipitation, but not vice-versa. A similar observation in THP-1 cells has been reported for Hck [9]. This association was probably due to the interaction of the Fc part of the precipitating antibodies with the Fc receptor, because the co-immunoprecipitation was abolished in the presence of IV.3 Fab fragments (a blocking FcγRII antibody). These results warrant caution in the interpretation of co-immunoprecipitation experiments. However, when an in vitro kinase assay was carried out with that non-specifically bound FcγRIIa, we observed, in accordance with the results discussed above, that Hck could phosphorylate the receptor, but Fgr could not (data not shown).

Our results indicate that only Lyn associates with the unphosphorylated tail of FcγRIIa. We found association of Lyn with the nonphosphorylated GST-CT fusion protein in vitro (Fig. 3) as well as with FcγRIIa derived from resting neutrophils (Fig. 4). In both cases, tyrosine phoshorylation of FcγRIIa occurred upon addition of ATP (data not shown). Probably, the physiological activation mechanism is transphosphorylation in a dimerized complex [43],

which is then mimicked on the beads used for binding of FcγRIIa or the GST-CT fusion protein. It is known that GST fusion proteins can form dimers and thereby activate tyrosine kinases [44].

An association of Lyn with FcγRIIa in resting THP-1 cells can also be derived from the data published by Ghazizadeh et al. [9], although these authors emphasized the clear increase in this association upon FcγRIIa crosslinking. We did not observe such an increase, although an increase in autophosphorylation of Lyn was observed after activation (data not shown). Clark et al. [45] have shown that Lyn and Fyn can specifically bind to the nonphosphorylated tail of Ig-α via a sequence between the conserved ITAM tyrosines. The kinase unique region seems to be involved in that interaction [46]. Lyn is also activated by a wide variety of neutrophil agonists including immune complexes [47]. Taken together, these data strongly suggest that Lyn plays an important role in the initial tyrosine phosphorylation of FcγRIIa.

It is clear that the phosphorylated ITAM functions as a scaffold to recruit and organize effector molecules following receptor ligation [22]. Syk seems to play a major role in phagocytosis, because chimeric transmembrane proteins containing only the Syk catalytic domain are capable of triggering phagocytosis in COS cells [48]. Recently, the importance of Syk for the signal transduction by FcγRIIa has recently been revealed by anti-sense experiments in cultured monocytes [49]. On the other hand, Syk coexpression dramatically enhanced phagocytosis in FcγRI/γ and III/γ cotransfected COS cells, but had only a small effect in FcγRII-transfected cells, perhaps due to the original high phagocytic index of FcγRII-transfected cells without Syk. No other coexpressed PTK had any effect [50]. In our experiments, we demonstrated specific association of Syk to the phosphorylated GST-CT. We have shown that Syk binding was achieved through its tandem SH2 domains to both C-terminal phosphotyrosines, because substitution of Y2 or Y3 totally abolished that binding (Fig. 8B). The specific sequence for Syk binding has been established as YXXI/L [51], which is present in both Y2 and Y3. Moreover, autophosphorylation of Syk, a generally accepted measurement of activity, was found to be increased when the GST-CT was added to the immunoprecipitated kinase (Fig. 6). A similar conclusion was drawn recently by Chacko et al. [52], who showed that only double phosphorylated peptides spanning the ITAM of FcγRIIa were able to bind with high affinity to Syk.

We have also shown binding of p85, the regulatory subunit of PI 3-kinase, to the phosphorylated GST-CT. PI-3 kinase has been implicated previously in FcγR signalling [21,53,54]. Our results with respect to p85 binding differ in one aspect from those recently published by Chacko et al. [52]. These authors only observed p85 binding to phosphopeptides spanning the FcγRIIa-ITAM with lysates of activated platelets, indicating the requirement for an activated intermediate. We observed binding with cytosol of resting neutrophils, which was even detectable in the Y3F mutant of the GST-CT fusion protein that was only poorly phosphorylated (Fig. 8). It is noteworthy, that the consensus sequence of p85 binding to phosphorylated targets (pYM/VXM [55]) is absent from the cytoplasmic tail of FcγRIIa. However, binding of p85 independent of phosphorylation has previously been observed with Ig-α and Ig-β subunits [45]. Clearly, the precise requirements of p85 binding to the cytoplasmic tail of FcγRIIa remain to be established. The poor phosphorylation of the Y3F mutant might point to an important role of ^{269}Tyr in increasing Lyn binding via its SH2 domain, a mechanism suggested to be of importance for Src-like kinases [56].

In conclusion, the results of our study suggest that in human neutrophils Lyn, due to its association with FcγRIIa, is responsible for the initial phosphorylation of FcγRIIa. When the 1st tyrosine in the ITAM of FcγRIIa becomes phosphorylated, binding of p85 can take place, whereas for binding of Syk both tyrosines of the ITAM need to be phosphorylated.

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