Physical and Functional Association of the High Affinity Immunoglobulin G Receptor (FcγRI) with the Kinases Hck and Lyn

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Summary

The high affinity immunoglobulin G (IgG) receptor FcγRI (CD64) is expressed constitutively on monocytes and macrophages, and is inducible on neutrophils. FcγRI has recently been shown to be associated with the signal transducing γ subunit of the high-affinity IgE receptor (FcεRIγ). Induction of cytoplasmic protein tyrosine phosphorylation by FcγRI cross-linking is known to be important in mediating FcγRI-coupled effector functions. Recently, syk has been implicated in this role. We now report that the src-type kinases hck and lyn are physically and functionally associated with FcγRI. Hck and lyn coimmunoprecipitated with FcγRI from detergent lysates of normal human monocytes and of the monocytic line THP-1. Hck and lyn showed rapidly increased phosphorylation and increased exogenous substrate kinase activity after cross-linking of FcγRI. These results demonstrate both physical and functional association of the FcγRI/FcεRIγ receptor complex with hck and lyn, and suggest a potential signal transducing role for these kinases in monocyte/macrophage activation.

FcγRI is a 72-kD glycoprotein constitutively expressed on monocytes and macrophages, and inducible on neutrophils. Its ectodomain is similar to that of the other IgG receptors except for the presence of a third extracellular Ig-like domain, lending it greater ligand affinity (1). Three FcγRI genes have been mapped to chromosome 1, which give rise to four transcripts (2). Only one transcript is full-length, and encodes the predicted transmembrane receptor with a 292-amino acid extracellular region, a 21-amino acid transmembrane domain, and a highly charged 61-amino acid intracytoplasmic domain.

Ligand binding to FcγRI initiates multiple immune activation events including phagocytosis, cytotoxicity against Ig-coated target cells, and expression of proinflammatory cytokine genes (for a review see reference 3). Engagement of FcγRI induces tyrosine phosphorylation of multiple cytoplasmic proteins that include phospholipase C (PLCγ-1) (4, 5). The protein tyrosine kinase (PTK) inhibitor herbimycin A inhibits FcγRI-triggered Ca^{2+} fluxes and TNF-α–mRNA accumulation (5), indicating an important role for PTK in distal activation events that follow FcγRI engagement.

As the intracytoplasmic region of FcγRI lacks a tyrosine kinase domain, signalling via FcγRI likely involves the activation of distinct tyrosine kinases that associate with the receptor. Activation of src-family kinases after ligation of surface receptors has been described in T and B cell antigen receptors (6, 7), CD4 and CD8 molecules (8, 9), the FcεRI receptor (10), and FcγRIII (11). A potential intermediate between FcγRI and PTKs is the FcεRIγ chain recently shown to be associated with FcγRI (12, 13), as well as FcγRII (14), FcγRIII (15), and the TCR in γ/δ T cells (16). FcεRIγ contains a tyrosine associative motif (17) that may provide the interface for signal transduction through an oligomeric FcγRI receptor complex.

Monocytes and macrophages express several PTKs, including fgr, fyn, hck, lyn, and src by mRNA analysis (18). Expression of hck is essentially limited to cells of the monocytic/macrophage and granulocyte lineages (19, 20), increases with precursor differentiation (19), and is induced by activation stimuli including IFN-γ, LPS, CSF-1, and GM-CSF (21, 22). Lyn has been found to be associated with signal transduction pathways in hematopoietic cells and its expression is also upregulated by LPS and IFN-γ (10, 23–25). Our data provide the first evidence for a physical and functional association of the kinases hck and lyn with FcγRI, and the first evidence for a specific role for hck.

Materials and Methods

Reagents and Antibodies. ^{14}C-methylated protein standards were from Amersham Corp. (Arlington Heights, IL). γ-[^{32}P]ATP and ^{125}I-protein G (15–25 μCi/μg) were from New England Nuclear (Boston, MA). Anti-FcγRI mAbs 197 and 32.2, and 32.2 F(ab')$_2$ fragments were obtained from Medarex, Inc. (Lebanon, NH). Polyclonal antipeptide antisera directed against human hck, lyn, fgr, and fyn, were obtained from rabbits immunized with KLH-conjugated synthetic peptides corresponding to amino acid sequences

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37-56, 6-25, 48-67, and 11-30, respectively (26). Anti-γ chain mAb 4D8 (27) was generously provided by Drs. D. H. Presky and J. P. Kochan (Hoffmann-La Roche, Inc., Nutley, NJ). Biotinylation of mAb 32.2 fragments was performed as previously described (5).

Cells and Cell Culture. The human monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection (Tumor Immunology Bank [TIB] #202, Rockville, MD). Cells were grown in RPMI 1640 medium with glutamine (JRH Biosciences, Lenexa, KS), supplemented with 10% low endotoxin FCS (HyClone Laboratories, Logan, UT), penicillin at 10 U/ml, and streptomycin at 100 μg/ml, and were kept at 37°C in 5% CO_2 and 95% humidified air. Viability and function were optimized by maintenance of the cells in the log phase of growth and monitoring for >95% cell exclusion of trypan blue dye. Monocytes were isolated from PBMC or from pheresis-derived leukocytes by Ficoll-Hypaque centrifugation, followed by adherence onto petri dishes for 1 h as previously described (28).

Receptor Engagement. Cells were preincubated at 10–50 × 10^8 cells/ml with 20 μg/ml mAb 32.2 F(ab')$_2$ for 30 min, followed by rapid pelleting, washing, resuspension in fresh cold HBSS, and cross-linking with F(ab')$_2$ goat anti-mouse Ig (GAMIg) 10 μg/ml (Cappel, West Chester, PA). Alternatively, cells were preincubated with biotinylated 32.2 F(ab')$_2$, followed by cross-linking with Streptavidin (Pierce, Rockford, IL). In some experiments FcγRI was cross-linked with intact mAb197 at 20 μg/ml, which effectively cross-links because of trivalent binding.

Immunoprecipitation. At various times after receptor cross-linking, cells were rapidly pelleted, lysed with 10 μl per 0.5-10^6 cells of ice-cold 0.5% Triton X-100 lysis buffer (150 mM NaCl, 30 mM NaF, 50 mM Hepes, pH 7.5, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml each of leupeptin, pepstatin A, chymostatin, and antipain [Sigma Chemical Co., St. Louis, MO]) on ice for 15 min. Nonsoluble material was pelleted by centrifugation at 16,000 *g* for 15 min. Supernatants were transferred to 25 μl protein G–Sepharose beads (Boehringer Mannheim, Mannheim, Germany or Pharmacia, Piscataway, NJ) which had been preincubated with antibody for 30 min (to overnight) and rinsed four times with lysis buffer. Beads and supernatants were gently rotated from 1 h to overnight at 4°C and again gently washed four times with cold lysis buffer. Proteins were eluted by adding Laemmli sample buffer and β-ME, heating to 95°C for 5 min before electrophoresis.

Western Blotting. Immunoblotting was performed as previously described with minor modifications (5). After transfer, membranes were blocked with 3% BSA. Blots were reacted with the antibody probe (1:2,000 dilution in Tris buffered saline-Tween[TBST]) for 1–2 h. Where indicated, reacted blots were stripped (62.5 mM Tris-HCL [pH 6.7], 100 mM β-ME, 2% SDS, and 50°C for 30 min), reblocked, and reprobed with a different antibody.

In Situ Phosphorylation. This was performed as described previously (5) with minor modifications. THP-1 cells were washed with cold phosphate–free MEM medium (GIBCO BRL, Gaithersburg, MD) twice, and incubated at 2.5–5 × 10^5 cells/ml in the same medium for 1–2 h at 37°C. Cells were then activated by FcγRI receptor cross-linking as described above. The cells were rapidly pelleted and resuspended in permeabilization buffer containing α-lysophosphatidylcholine (LPC). γ-[^{32}P]ATP was added and phoshorylation allowed to proceed for 15 min on ice. The cells were rapidly pelleted and solubilized in lysis buffer containing 1% NP-40. Lysates were precleared with normal rabbit serum as described elsewhere (28) and the various src-type PTKs were immunoprecipitated from the precleared lysates using kinase-specific antisera. Precipitated proteins were analyzed by SDS-PAGE alongside protein molecular weight standards. Gels were dried and exposed to film (Kodak XAR; Rochester, NY) for 4–72 h at −80°C.

In Vitro Immune Complex Kinase Assay. A peptide derived from the amino acid sequence surrounding the phosphorylation site in pp60^{src}, RR-SRC (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) (GIBCO/BRL), was utilized as substrate (29) for precipitated immune complexes. 10 μl of protein G–Sepharose immunoprecipitate was mixed with tyrosine kinase assay buffer (30 mM Hepes [pH 7.4], 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 20 μM EDTA, 25 μg/ml BSA, 0.15% [vol/vol] NP-40, 70 μM Na_3VO_4, 60 μM ATP, and 0.5 mM RR-SRC peptide) and 1 μCi [γ^{32}P]ATP. Reactions were incubated at 30°C for 30 min, stopped with 20 μl ice-cold 10% TCA, iced for 10 min, and microfuged for 10 min. 20 μl of the supernatant was spotted onto phosphocellulose discs, rinsed twice with 1% acetic acid and twice with water, and counts measured in a scintillation counter.

Results and Discussion

Putative physical association of FcγRI and the src family PTKs hck, lyn, fgr, and fyn was examined by Western blotting of FcγRI immunoprecipitates derived from the human monocytic cell line THP-1 (Fig. 1) and from PBMC (Fig. 2). FcγRI was immunoprecipitated from cell lysates utilizing mAb 197 and protein G–Sepharose. The IgG2a mAb 197 selectively engages FcγRI via both its F(ab')$_2$ and Fc portions (30). To further insure that the Fc portion of mAb 197 did not engage FcγRII, we used biotinylated F(ab')$_2$ mAb 32.2 and Streptavidin to confirm results obtained with mAb 197.

Proteins were eluted from anti-FcγRI precipitates, sized by SDS-PAGE, transferred to polyvinylidene membranes, and probed with anti-PTK polyclonal antisera. Fig. 1 shows that the anti-hck antiserum reacted in both anti-FcγRI immunoprecipitates with two bands at 56 and 59 kD. These two bands comigrated with the two bands recognized by anti-hck serum in hck immunoprecipitates from the same lysates. The mobilities of these two bands were consistent with those previously described for the two isoforms of hck (19, 20). Fig. 1 also shows that the anti-lyn antiserum revealed the presence in anti-FcγRI immunoprecipitates of a doublet composed of bands migrating at 53 and 56 kD. These two bands comigrated with the two bands recognized by the anti-lyn antiserum in anti-lyn immunoprecipitates (31). The previously described coprecipitation of FcγRI and FcεRIγ was confirmed by probing FcγRI immunoprecipitates with the anti-FcεRIγ mAb 4D8. This revealed a 12-kD band that comigrated with the FcεRIγ band recognized by mAb 4D8 in FcεRIγ immunoprecipitates (Fig. 1). We could not detect by Western blot analysis the presence of either fgr or fyn in mAb 197 anti-FcγRI immunoprecipitates. These data suggest that hck and lyn are physically associated with FcγRI in THP-1 cells.

It was important to demonstrate that the association of FcγRI with hck and lyn is not unique to the cell line THP-1. To this purpose we examined the presence of hck and lyn in anti-FcγRI immunoprecipitates from lysates of freshly isolated human monocytes. The results obtained are shown in Fig. 2 and parallel those obtained in THP-1 cells. Both hck

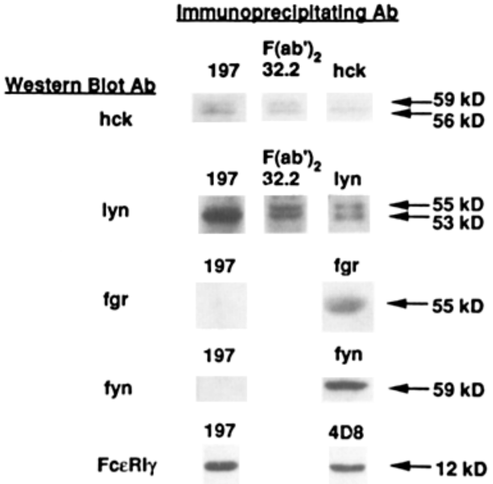

Figure 1. Physical association of FcγRI with hck and lyn in THP-1 cells. FcγRI complexes were immunoprecipitated from postnuclear supernatants of THP-1 cell lysates using protein G–Sepharose with intact mAb 197, or Streptavidin-agarose with biotinylated 32.2 F(ab')$_2$. Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting with anti-src sera or anti-FcγRIγ mAb 4D8 as indicated, as described in Materials and Methods. The right column shows the corresponding proteins immunoprecipitated by the immunoblotting reagents from THP-1 cell lysates. Results are representative of five experiments.

and lyn, but not fgr or fyn (data not shown) were detected in the mAb 197 anti-FcγRI immunoprecipitates, as well as F(ab')$_2$ anti-FcγRI immunoprecipitates. These results indicate that hck and lyn are associated with FcγRI in normal monocytes.

We next examined whether cross-linking of FcγRI results in the phosphorylation and activation of hck and lyn. FcγRI was cross-linked on THP-1 cells using intact anti-FcγRI mAb 197, or mAb 32.2 F(ab')$_2$ cross-linked by GAMIg. Cells were then permeabilized, incubated with [γ^{32}P]ATP, and PTK immunoprecipitates examined for intensity of phosphorylation. Fig. 3 shows a representative in situ phosphorylation experiment. Within 20 s after FcγRI cross-linking by mAb 197, there was evidence of increased phosphorylation of both isoforms of hck and lyn. Phosphorylation rapidly increased during the first minute after FcγRI engagement, and remained sustained for the 15-min duration of the experiment. Western blot analysis of anti-FcγRI–stimulated THP-1 cell lysates confirmed constant hck and lyn mass and equal sample loading over the time course examined (data not shown). The effect of anti-FcγRI mAb on hck and lyn was specific in that neither fgr nor fyn showed any change in phosphorylation with FcγRI cross-linking (data not shown).

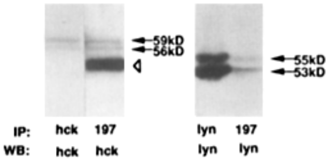

Figure 2. FcγRI associates with hck and lyn in normal human monocytes. Normal human monocyte lysates were prepared, and anti-FcγRI immunoprecipitates analyzed, as described for THP-1 cells in Fig. 1. Western blots of the electrophoresed immunoprecipitates were probed with anti-hck serum (*left*) and anti-lyn serum (*right*), followed by ^{125}I protein G and autoradiography. (◁ , *left*) migration of heavy chain. Left lane of each panel shows the corresponding proteins immunoprecipitated by the immunoblotting antisera from THP-1 cell lysates. Results are representative of three experiments.

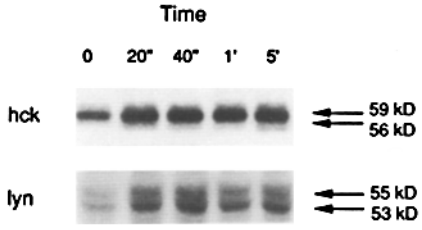

Figure 3. FcγRI cross-linking induces phosphorylation of hck and lyn. THP-1 cells were phosphate depleted in phosphate-free DMEM and incubated at 37°C for the indicated times with anti-FcγRI mAb 197 (2.5 μg/ml). The cells were permeabilized with α-LPC, incubated on ice with γ-[^{32}P]ATP (3,000 μCi/ml), and lysed in 1% NP-40 lysis buffer. Hck and lyn were then immunoprecipitated from thee postnuclear supernatants with protein G–Sepharose beads incubated with specific antisera as indicated. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Results are representative of five experiments.

In parallel with the in situ phosphorylation assays, we examined the tyrosine kinase activity of hck, lyn, fgr, and fyn in THP-1 cells stimulated with anti-FcγRI mAb using an immune complex kinase assay with RR-SRC peptide as substrate. Anti-FcγRI immunoprecipitates were incubated with γ-[^{32}P]ATP and RR-SRC substrate, and ^{32}P incorporation was measured. Fig. 4 shows representative experiments where FcγRI cross-linking resulted in increased phosphorylation of the exogenous src-peptide substrate by anti-hck and anti-lyn immunoprecipitates. Within 1 min after stimulation with anti-FcγRI mAb, both hck and lyn immunoprecipitates showed increased capacity to phosphorylate the RR-SRC peptide. Consistent with the detected in situ phosphorylation, lyn kinase activity peaked with a 2.4-fold increased activity at 5 min, declining to 1.7-fold of baseline by 15 min. Similarly, hck enzymatic activity increased after FcγRI cross-linking in parallel with the increased phosphorylation of hck. FcγRI-triggered activation of hck was relatively less than that of lyn, which may reflect its reproducibly higher baseline activity. Analysis of fyn and fgr immunoprecipitates for kinase activity revealed no detectable activation after FcγRI cross-linking (Fig. 4).

The present results, together with our previous finding that FcεRIγ associates with FcγRI, indicate that the oligomeric FcγRI receptor complex includes FcγRI, FcεRIγ, hck, and/or lyn. It is likely that hck and lyn play roles in protein tyrosine phosphorylation, Ca^{2+} flux, and other PTK-dependent events that follow FcγRI receptor cross-linking (5). An integral role for hck and/or lyn in FcγRI

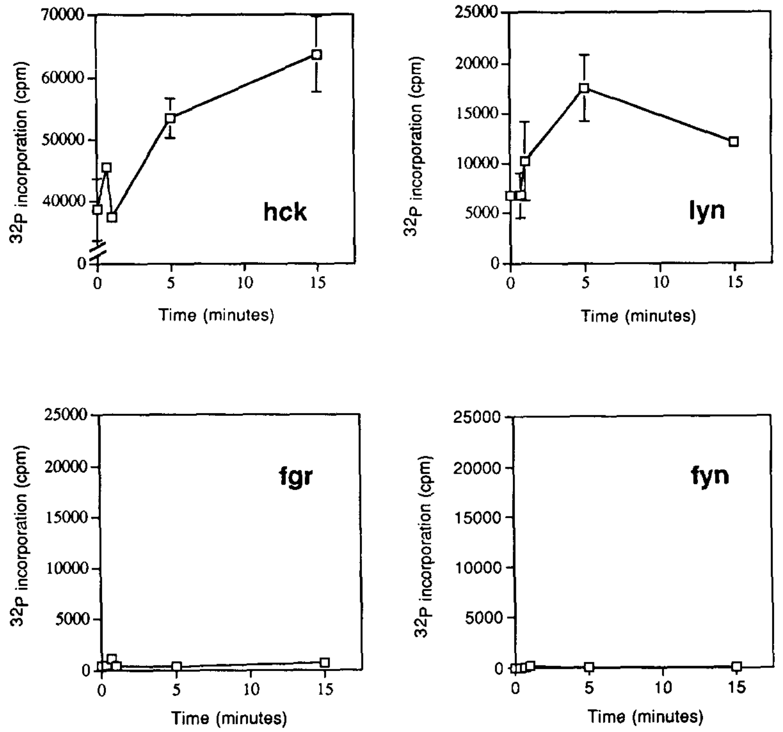

Figure 4. FcγRI cross-linking increases hck and lyn kinase activity. THP-1 cells (10^7/ml HBSS) were incubated at 37°C for the indicated times with anti-FcγRI mAb 197 (2.5 μg/ml), and lysed in 1% NP-40 lysis buffer, and subject to anti-hck and anti-lyn immunoprecipitation as before from the postnuclear supernatants. PTK assay was performed on the hck or lyn immunoprecipitates by adding src-peptide substrate RR-SRC and γ-[^{32}P]ATP. After incubation, samples were spotted onto cellulose discs. After washing, peptide incorporation ^{32}P was quantitated by scintillation counting. Values represent means (± SD) of duplicate determinations. Results are representative of three similar experiments.

signal transduction may explain the inability of Cos-1 cells transfected with cDNA encoding FcγRI (32), or FcγRI and FcεRIγ (13), to phagocytose IgG-coated erythrocytes.

Hck and lyn each have two isoforms, produced, respectively, from alternate translation start sites and alternate splicing. Sublocalization studies have found all isoforms of hck and lyn within the membrane fraction, but only the p59-kD isoform of hck in the cytosol (31, 33). Different lyn or hck isoforms may interact with different targets, and thus could potentially mediate unique distal cellular events. It has been shown, for example, that p59fyn-T induces IL-2 production via TCR stimulation by antigen whereas p59fyn-B does not (34). Analogously, lyn isoforms respond differentially upon stimulation of the B cell antigen receptor (35).

Among the known src-family PTK, hck and lyn share unique similarities which might explain their coincident association with FcγRI. Of all src PTK, hck and lyn share the greatest amino acid homology (71%), a relatively high identity (36%) in the "unique" NH_2-terminal region, and near identical peptide sequence within the lyn A isoform insert (^{33}YVRDPTS) and both hck isoforms (^{30}YVPDPTS) (36). NH_2-terminal sequences have previously been shown to be critical to the association of lck and fyn with CD4/CD8 and the TCR, respectively (37).

Recent work (38) indicates a role for p72^{syk} in FcγRI signal transduction, with increased tyrosine phosphorylation upon cross-linking of FcγRI or FcγRII. Studies with THP-1 cells suggest a preferential association of p72^{syk} with the FcγRII complex upon cross-linking either FcγRI or FcγRII (39). Aggregation of the high-affinity IgE receptor causes similar tyrosine phosphorylation of p72^{syk} and its association with activated FcεRIγ (40). A cooperative recruitment model has been delineated whereby FcεRIβ-associated lyn phosphorylation leads to phosphorylation of FcεRIβ and FcεRIγ, causing the subsequent association and activation of syk (41).

The details of the physical coupling of hck and fyn to the FcγRI receptor complex remain to be determined. Given the lack of sequences known to mediate the binding of src PTK in the cytoplasmic tail of FcγRI, the associated FcεRIγ chain may couple FcγRI to hck, lyn, and perhaps to other components via its Reth motif (17). The presence of a β-like chain adaptor is suggested from studies in FcεRI. Utilizing a fusion protein consisting of GST and the cytoplasmic tail of FcγRI, we have failed to reveal a direct association between FcγRI and either hck or lyn (Scholl, P. R., and R. S. Geha, unpublished observations). Experiments are currently underway to unravel the mechanisms of the association of the FcγRI receptor complex with the kinases hck and lyn.

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