A Role for Shc, Grb2, and Raf-1 in FcγRI Signal Relay*

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The activation of the serine/threonine kinase, Raf-1, serves to connect upstream protein tyrosine kinases to downstream signaling events. We previously reported that FcγRI stimulation of interferon γ-differentiated U937 cells (termed U937IF cells) induces a mobility shift in Erk2. Herein, we report that cross-linking of FcγRI receptor in U937IF cells induces a marked tyrosine phosphorylation of Raf-1 (10-fold increase). Tyrosine phosphorylation of Raf-1 is induced by FcγRI activation and not by PMA (1 μg/ml), N-formyl-Met-Leu-Phe (1 μM), calcium ionophore (1 μM), thrombin (0.05 unit/ml), FcγRII, or FcγRIII stimulation. The kinetics of Raf-1 tyrosine phosphorylation is rapid, reaching peak levels 1–2 min after FcγRI activation, and the tyrosine phosphorylation of Raf-1 precedes the activation of the respiratory burst. FcγRI cross-linking induces the tyrosine phosphorylation of Shc; tyrosine-phosphorylated Shc binds to Grb2 forming a Shc-Grb2 complex. The data provide evidence that the FcγRI receptor signals via the upstream activation of nonreceptor protein tyrosine kinases, which leads to the subsequent activation of Ras family GTPases and serine/threonine kinases, Raf-1 and mitogen-activated protein kinase.

Phosphorylation on tyrosine, serine, or threonine residues is a key regulatory mechanism in mammalian cells used to regulate the mitogenic or oncogenic potential of proteins by augmenting their enzymatic activity or modifying their association with other signal transducers (1). In this manner, Raf-1 activity also appears to be regulated by phosphorylation of key serine/threonine and tyrosine residues in that kinase activity correlates with the phosphorylation state of the Raf-1 protein (2). Raf-1 is rapidly phosphorylated and activated following stimulation with growth factors and mitogens (3). Kovacina et al. (4) demonstrated that treatment with phosphatase remarkably decreased the catalytic activity of Raf-1 in insulin-stimulated cells. While Raf-1 is exclusively phosphorylated on serine residue in resting and mitogen-stimulated cells, it has been demonstrated that phosphorylation of Raf-1 increased on threonine and tyrosine residues after IL-2 or TCR stimulation (5). However, the stoichiometry of tyrosine phosphorylation is low compared to the extent of serine/threonine phosphorylation. Ser35, Ser259, and Ser621 are known to be major in vivo phosphorylation sites in mammalian and Sf9 insect cells expressing human Raf-1 proteins. Phosphorylation of both Ser259 and Ser621 modify the catalytic activity of Raf-1 (6).

Although tyrosine phosphorylation of Raf-1 has been demonstrated in several systems, including IL-3 and granulocyte/macrophage colony-stimulating factor-stimulated murine myeloid cells (7), IL-2-treated murine T cells (8), platelet-derived growth factor-stimulated or v-src-transformed murine fibroblasts (2, 3), the biological relevance of this phosphorylation has not yet been clarified. Fabien et al. (9) recently suggested an importance for the tyrosine phosphorylation in regulating the biological activity of Raf-1 and identified Tyr340 and Tyr341 as major tyrosine phosphorylation sites of Raf-1. They demonstrated the phosphorylation of Tyr340 and Tyr341 by coexpressing Raf-1 with the activated tyrosine kinase, pp60csrc, in baculovirus Sf9 cells (9). More recently the data of Pulmiglia et al. (10) has defined in more detail the NH2 terminus of Raf-1, demonstrating that in certain systems the tyrosine phosphorylation of Raf-1 alone is sufficient to activate Raf-1 kinase activity. In these experiments, mutation of residues 53–156 required for Ras-Raf-1 binding abrogated activation by Ras but had no effect on activation of Raf-1 by activated Src in Sf9 cell system. These and other data suggest that several independent mechanisms may exist for the regulation of Raf-1. This is a paradigm which may play itself out more than once in mammalian signal relay.

Raf-1, the proto-oncogene product of the v-raf gene which is the cellular homologue of the murine transforming gene v-raf, is a 72–76-kDa phosphoprotein with intrinsic kinase activity for serine and threonine residues (2). Raf-1 is an effector of Ras and is one of the activators of mitogen-activated protein kinase (MEK) (11). Sequence analysis suggests that Raf family proteins have three unique conserved domains, named conserved region 1 (CR1), CR2, and CR3 (12). CR1 is a cysteine-rich residue having a putative zinc binding region. CR2 is a serine/threonine-rich region, and CR3 contains the protein kinase domain. Both CR1 and CR2 are located in the amino-terminal half of the Raf-1, which appears to regulate the catalytic activity of carboxyl-terminal kinase domain. The v-Raf protein of murine sarcoma virus 3611 is observed to have a deletion of the amino-terminal half of the protein. Deletion or mutation of the amino terminus activates the oncogenic trans...
forming potential of Raf-1 (13, 14). In protein kinase cascades, Raf-1 appears to be a central intermediate in the transmission of proliferative, developmental, and oncogenic signals by mediating signals from receptor or nonreceptor tyrosine kinases, from p21ras to serine/teherone kinases, including MAP kinase kinase, MEK, MAP kinase, or ribosomal S6 kinase (RSK) ultimately leading to activation of transcriptional factor, such as NF-κB/Rel, in the nucleus (15–17). The role of Raf-1 in postmitotic cells is less clear.

Proteins of the FcγR family have a number of conserved biological characteristics of multisubunit Ig supergene family (18, 19). FcγRs, receptors for the Fc portion of IgG, are composed of three groups including FcγRI (CD64), FcγRII (CD32), or FcγRIII (CD16) according to their binding affinity for the ligand. FcγRII, found in monocytes and macrophages, is a 74-kDa glycoprotein that binds monomeric IgG with high affinity (20, 21). The FcγRI receptor signaling via a conserved sequence of amino acids termed the immunoreceptor tyrosine-based motif (ITAM) (22). Signaling through the ITAM shares a number of conserved features among the Ig gene superfamily of multisubunit receptors (23). We and others have reported that the FcγRII receptor stimulation results in the sequential activation of FcγRIγ, Hck, Syk and MAP kinase (24). The FcγRII receptor is also linked to the cytoskeleton and is involved in a number of well characterized cell biologic signals (activation of respiratory burst, phagocytosis, or cell motility, etc.). Importantly, the myeloid respiratory burst is a well characterized response known to be regulated by small GTPases, Rac1, Rac2, and Rap1a (25). It is tempting to speculate that the respiratory burst may also be regulated by the known effectors of these GTPases (i.e. Raf-1 or PAK65, etc.) (26).

Cross-linking of FcγR induces activation events, including tyrosine phosphorylation of γ subunit (27) and activation of phospholipase C-γ1 and γ2 (28), increases phosphatidylinositol hydrolysis and calcium mobilization (29), production of cytokines (30), and generation of superoxide anions (31). Our laboratory uses the myeloid cell line U937 differentiated in IFNγ (termed U937IF cells) to study FcγRI signal transduction. We are interested in the mechanism by which signals are transmitted from FcγRI receptor to the respiratory burst. We previously reported that the stimulation of FcγRII receptor in U937IF cells results in tyrosine phosphorylation and activation of Syk (32). More recently we demonstrated that FcγRI cross-linking activated Src family kinase, Hck and a mobility shift of MAP kinase (33). These results lead us to hypothesize a role for Raf-1 in FcγRI signal relay. Herein, we demonstrate that Raf-1 is tyrosine-phosphorylated after FcγRII stimulation (10-fold increase). Both Hck and Syk are activated following FcγRII stimulation, making them good candidate kinases for the tyrosine phosphorylation of Raf-1. We also observe the tyrosine phosphorylation of Shc and the association of Shc and Grb2 in U937IF cells activated by FcγRII stimulation (not FcγRI or FcγRII stimulation). Our results suggest that Raf-1 is a major substrate for protein tyrosine kinases following FcγRII cross-linking, which results in the sequential activation of Shc, Grb2, Ras, Rap-1, and MAP kinases transmitting FcγRII signals that result in the assembly of an active respiratory burst complex.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The FcγR-specific antibodies were obtained from Medarex Inc. (West Lebanon, NH). The mAb 197 and mAb 32 are specific for the FcγRIα subunit, mAb 32.2 is a F(ab2)2 fragment of IgG, mAb 1V.3 is specific for FcγRIα subunit, and mAb 3G8 is specific for FcγRIIα subunit of Fc receptor for IgG. The cross-linking antibody was a rabbit anti-mouse F(ab2)2 fragment (RmAb) purchased from Organon Teknika Corp. (West Chester, PA). Anti-Raf-1 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine (anti-Tyr(P)) and anti-Shc antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-Grb2 antibody was obtained from Transduction Laboratories (Lexington, KY). The anti-MAP kinase antiserum (polyconal antibody 1913.2) against the peptide KEILFEETARQPGY, corresponding to the extreme COOH terminus of the Xenopus Erk2, was provided by Jonathan A. Cooper, Fred Hutchinson Cancer Center (Seattle, WA). This region of the Xenopus MAP kinase is 100% conserved with human Erk2 and 85% conserved in human Erk1 (34).

**RESULTS**

**Tyrosine Phosphorylation of Raf-1 upon FcγR Activation**—To evaluate the involvement of Raf-1 in FcγRII signaling, we examined whether Raf-1 is tyrosine-phosphorylated after FcγRII stimulation in U937IF cells. Lysates of 2 x 10⁶ U937IF cells were immunoblotted for Raf-1 after differentiation in IFNγ. IFNγ increased the expression of p74 Raf-1 (3-fold in-
Tyrosine phosphorylation of Raf-1 upon FcγRI activation in U937IF cells. A, anti-Raf-1 IPs from 2 x 10^6 U937IF cells were immunoblotted for phosphotyrosine (lanes 1-9). Lane 1 is a sham IP without lysate from anti-Raf-1 immunoprecipitation procedure. Lane 2 represents an IP with preimmune serum. Other lanes correspond to: resting U937IF cells (lane 3), U937IF cells incubated with anti-FcγRI antibody (mAb 197) alone for 1 min (lane 4), U937IF cells stimulated with RαM alone for 1 min (lane 5), and U937IF cells activated by anti-FcγRI cross-linking and RαM stimulation for 0.5, 1, or 5 min (lanes 6-8, respectively). Lane 9 is a whole cell lysate of FcγRI-stimulated U937IF cells. B, after probing with anti-Tyr(P), the same blot was stripped and reprobed for Raf-1. The tyrosine-phosphorylated p74 bands are superimposed on the Raf-1 band.

increase). Raf-1 expression directly correlated with the length of exposure to IFNγ and peaked at 4 days after differentiation (data not shown). We subsequently used U937IF cells differentiated for 4 days with IFNγ for all experiments. To determine whether FcγRI cross-linking can induce the tyrosine phosphorylation of Raf-1 in myeloid cells, 2 x 10^7 U937IF cells were first incubated with mAb 197, followed by stimulation with RαM. We immunoprecipitated Raf-1 from resting or FcγRI-stimulated U937IF cells with a rabbit anti-Raf-1 antibody and performed anti-Tyr(P) immunoblots (mAb 4G10) (Fig. 1). Marked tyrosine phosphorylation of Raf-1 was detected after cross-linking of FcγRI receptor (10-fold increase) (Fig. 1A, lanes 6-8). The tyrosine phosphorylation of Raf-1 is very rapid and reaches its maximum response 30 s to 1 min after stimulation (Fig. 1A, lanes 6 and 7). Raf-1 was not tyrosine-phosphorylated in resting cells (Fig. 1A, lane 3) or in U937IF cells stimulated with FcγRII alone or RαM alone (Fig. 1A, lanes 4 and 5). To confirm the identity of the 74-kDa protein, the membrane was stripped and reprobed for Raf-1 (Fig. 1B). All lanes except sham and preimmune immunoprecipitates brought down an equivalent amount of Raf-1 protein (Fig. 1B, lanes 3-8). The p74 immunoreactive bands of anti-Raf-1 immunoblot is superimposed on tyrosine-phosphorylated bands in anti-Raf-1 immunoprecipitates. The results show that Raf-1 immunoprecipitated from FcγRI activated cells is tyrosine-phosphorylated. In other experiments, separate immunoblots of rabbit anti-Raf-1 immunoprecipitates were probed with mouse anti-Tyr(P) or mouse anti-Raf-1 antibodies. The results were exactly the same as our findings previously shown in Fig. 1. Parallel blots of anti-Raf-1 immunoprecipitates, probed with secondary antibody alone, showed no Raf-1 band. Finally, the whole cell lysate confirmed the integrity of our Raf-1 immunoblots (Fig. 1, lane 9). We suggest that the observed difference in intensity of the p74 band in the whole cell lysate versus the anti-Raf-1 immunoprecipitates is due to the presence of more than one p72-74 phosphoprotein with similar electrophoretic mobility. Evidence to support this conclusion include the identification of Raf-1 and Syk kinases as components of the p72-74 phosphoprotein bands (32).

To confirm these results, we performed anti-Tyr(P) immunoprecipitation using agarose-conjugated anti-Tyr(P) antibodies on resting and FcγRI-stimulated U937IF cells. We probed these immunoprecipitates with rabbit anti-Raf-1 antibody (Fig. 2A). Only anti-Tyr(P) immunoprecipitate from FcγRI activated reacted specifically with anti-Raf-1 antibody (Fig. 2A, lane 3). FcγRIII activation did not induce tyrosine phosphorylation of Raf-1 (Fig. 2A, lane 4). When the membrane was stripped and reprobed for phosphotyrosine, the tyrosine-phosphorylated Raf-1 band was superimposed on the upper band of anti-Tyr(P) immunoblot indicated (Fig. 2B, lane 3). The whole cell lysate confirmed the integrity of anti-Tyr(P) and anti-Raf-1 immunoblots (Fig. 2B, lane 5). We observe a diminished number of tyrosine-phosphorylated proteins in our anti-Tyr(P) immunoprecipitates as compared to the whole cell lysates. This is likely due to decreased efficiency of immunoprecipitation by the agarose-conjugated anti-Tyr(P) antibody. The loss of membrane-bound proteins during stripping and washing may decrease the resolution of tyrosine-phosphorylated proteins in our anti-Tyr(P) immunoblot. These data confirm the results shown in Fig. 1 and reveal that Raf-1 is tyrosine-phosphorylated upon FcγRI activation.

Kinetics of Raf-1 Tyrosine Phosphorylation—We determined the kinetics of Raf-1 tyrosine phosphorylation upon FcγRI activation and its relation to respiratory burst, a signaling pathway in myeloid cells known to be modulated by GTPases Rac and Rap1a (25, 26). Tyrosine phosphorylation of Raf-1 occurs 20 s after FcγRI stimulation (Fig. 3A, lane 5) and reached a peak around 1-2 min (Fig. 3A, lanes 7 and 8) and disappeared 10 min after stimulation (Fig. 3A, lane 10). The respiratory burst begins 3-5 min after FcγRI activation (Fig. 3B). FcγRI cross-linking activated respiratory burst to produce 2.2 nm superoxide from 2 x 10^6 cells 10 min after stimulation and showed peak response 30 min after stimulation. PMA also stimulated the respiratory response to produce superoxide anion, but its maximum response was delayed compared to activation of respiratory burst through FcγRI stimulation. The respiratory burst is preceded by the tyrosine phosphorylation of Syk, Hck, MAP kinases, and Raf-1, suggesting that Raf-1 could function upstream in the FcγRI signal pathway leading to the activation of the respiratory burst response (32, 33).

Tyrosine Phosphorylation of Raf-1 Is Specific in FcγRI Activation Pathway—To determine whether the tyrosine phosphorylation of Raf-1 we observed is specific for FcγRI activation, we determined the effect of cross-linking of other Fcγ receptors, such as FcγRII and FcγRIII, as well as stimulation with other agonists, such as PMA (1 μg/ml), FMLP (1 μM), calcium ionophore (1 μM), or thrombin (0.05 unit/ml), on tyrosine phosphorylation of Raf-1 (Fig. 4). In these experiments, we immunoprecipitated an equivalent amount of Raf-1 (Fig. 4B, lanes 3-14). Only FcγRII stimulation of U937IF cells resulted in the tyrosine phosphorylation of Raf-1 (Fig. 4A, lanes 11 and 12). PMA, FMLP, calcium ionophore, and thrombin did not induce the tyrosine phosphorylation of Raf-1 (Fig. 4A, lanes 4-7). Similarly, FcγRII or FcγRIII cross-linking did not induce the tyrosine phosphorylation of Raf-1 (Fig. 4A, lanes 8, 9, 13, and 14). These results indicate that FcγRII stimulation specifically induces the tyrosine phosphorylation of Raf-1. Interestingly, FcγRII stimulation is not observed to induce the respiratory burst in U937IF cells, nor does it induce a mobility shift in MAP kinase, or the activation of Hck or Syk kinases (Fig. 6, lanes 11 and 12) (32, 33). Lane 15, a whole cell lysate of U937IF cells stimulated with FcγRI, shows the prominent 74-kDa tyrosine-phosphorylated Raf-1 specific band.

Shc Is Tyrosine-phosphorylated after FcγRI Activation—One
These results suggest that Shc is involved in Fc receptor (RI) signal transduction. Recent studies have showed that Raf-1 is tyrosine-phosphorylated in response to growth factors including platelet-derived growth factor, IL-2, IL-3, granulocyte/macrophage colony-stimulating factor, or insulin (4, 5, 8, 44). Platelet-derived growth factor treatment of NIH 3T3 or Chinese hamster ovary cells induces the tyrosine phosphorylation of Raf-1, which activates its serine/threonine kinase activity dominantly, and tyrosine residues in the NH2 terminus (9, 10).

**DISCUSSION**

The activity of Raf-1 appears to be regulated by multiple mechanisms in mammalian signaling. The presence of a cysteine-rich motif in CR1 suggests that certain modulatory lipids may function on the allosteric regulation of Raf-1 activity (37). The β isoform of the 14-3-3 family of proteins was also identified as a Raf-1 activator in NIH 3T3 cells (38). Several lines of evidence suggest that phosphorylation and/or alteration of the amino-terminal regulatory domain may be a mechanism for the regulation of Raf-1 activity. NH2-terminal truncation of the Raf-1 cDNA modifies the catalytic activity of Raf-1. Many growth factors stimulate Raf-1 phosphorylation on serine, predominantly, and tyrosine residues in the NH2 terminus (9, 39–42). Phosphorylation of Ser259 and/or Ser621 regulates the activity of Raf-1 (15). Morrison et al. have recently reported that tyrosine phosphorylation regulates the activity of Raf-1, since a mutant containing a tyrosine to phenyalanine mutation at Tyr341 and Tyr344 sites is not activated and since a truncated Raf-1 lacking tyrosine residues between positions 26 and 303 of amino terminus modifies the function of Raf-1 (43). However, the molecular basis by which tyrosine phosphorylation alters Raf-1 activity is unknown. In this study, we demonstrated that Raf-1 is tyrosine-phosphorylated upon FcγRI stimulation, suggesting that Raf-1 is involved in FcγRI signal transduction. Recent studies have showed that Raf-1 is tyrosine-phosphorylated in response to growth factors including platelet-derived growth factor, IL-2, IL-3, granulocyte/macrophage colony-stimulating factor, or insulin (4, 5, 8, 44). Platelet-derived growth factor treatment of NIH 3T3 cells induces the tyrosine phosphorylation of Raf-1, which activates its ser-
respectively.

After FcRI (Fig. 3B) has been cloned, including p47phox, p67phox, gp91 and p22, Rac1, Rac2, and Rap1a (45, 46). The respiratory burst response can be reconstituted in vitro using recombinant proteins or membrane preparations, making it an excellent model for study of mammalian signal relay (47) (Fig. 7). The respiratory burst in neutrophils, induced through stimulation with PMA or heterotrimeric G proteins, is regulated by serine phosphorylation and the conversion small GTPases Rac and Rap1a to their GTP-bound state (48). Gabig et al. (47) recently reported that the expression of dominant negative mutants of Rac and Rap1a blocks the FMLP-induced respiratory burst in HL-60 cells. Considerable similarity exists between effect of FMLP and Fc receptor signaling (49). Dusi et al. (50) have reported a potential role for MAP kinase in regulation of respiratory burst more recently. The respiratory burst of U937F cells occurring after FcRI stimulation is less well described but likely is regulated similarly (Fig. 3B) (51). The respiratory burst begins around 5 min and reaches maximal response around 30 min after FcRI stimulation. Interestingly, the FcRI-induced respiratory burst occurs subsequent to tyrosine phosphorylation of Hck, Syk, and Raf-1. PMA also activates the respiratory burst response, but its kinetics differs from the FcRI stimulation. Ongoing experiments seek to determine if the respiratory burst is regulated by the tyrosine phosphorylation and activation of these nonreceptor protein tyrosine kinases and Raf-1. To answer the Raf-1 question, we will overexpress the dominant inhibitory Raf-1 mutant containing tyrosine to phenylalanine mutation at Tyr340 and/or Tyr341 in association with a γ-chain homodimer (52), which contains a YXXL amino acid motif termed the ITAM (22). The γ-subunit of the FcRI and FcεRI, as well as the subunit of TCR/CD3, contain ITAM sequences (53). The FcRI, FcεRI, and FcγRII associate with the γ-chain for the stable transport and assembly of the receptors in the plasma membrane. The signaling pathway through the ITAM involves the activation of the Src family and the Syk/Zap70 family kinases (1, 33, 54). The γ-chain is rapidly phosphorylated upon FcRI activation on serine, threonine, and tyrosine residues and is associated with Syk (31, 55). In our study, the specificity of Raf-1 tyrosine phosphorylation upon FcRI activation in U937F cells was investigated by activating different Fcγ receptors (i.e. FcγRII or FcγRIII) and by stimu-
In other experiments, we observed Shc is physically associated with Grb2 upon FcγRI activation. A, lane 1 is a sham IP. Lane 2 is a preimmune serum IP. Shc IPs were probed with anti-Tyr(P) antibody. Other lanes are: resting U937IF cells (lane 3), U937IF cells stimulated with PMA (1 μg/ml) (lane 4), FcγRI alone (lane 5), FcγRIII alone (lane 6), RαM alone (lane 7), FcγRI activated for 1 min (lane 8) or 5 min (lane 9), FcγRIII activated for 1 min (lane 10), or FcγRIII activated for 1 min (lane 11). Lane 12, whole cell lysate of U937IF cells, shows position of Grb2 and Shc specific bands. The heavy chain of immunoglobulin G is indicated as H-chain of IgG. B, the lower part of same blot was immunoblotted for Grb2. The light chain of IgG is indicated as L-chain. C, the same part of A was stripped and reprobed for Shc. The tyrosine-phosphorylated Shc in A is superimposed on p52 isoform of Shc.

FIG. 5. Shc is phosphorylated and associates with Grb2 upon FcγRI activation. Anti-MAP kinase immunoblotting carried out on whole lysates of resting U937IF cells (NS, lane 1) stimulated with PMA (1 μg/ml, lane 2), FcγRI (mAb 197) alone (lane 3), FcγRII (mAb 32.3) alone (lane 4), FcγRIII (mAb 32.3) alone (lane 5), RαM alone (lane 6), FcγRI (mAb 197) plus RαM for 1 and 5 min (lanes 7 and 8, respectively), FcγRII (mAb 32.3) plus RαM for 1 min (lane 9) or 5 min (lane 10), or FcγRIII plus RαM for 1 min (lane 11) or 5 min (lane 12). Proteins were resolved by SDS-PAGE (10%) under reducing conditions, transferred to nitrocellulose, and immunoblotted with anti-MAP kinase (19132) as detailed under “Experimental Procedures.”

FIG. 6. A mobility shift of MAP kinase upon FcγRI activation. Anti-MAP kinase immunoblotting carried out on whole lysates of resting U937IF cells (NS, lane 1) stimulated with PMA (1 μg/ml, lane 2), FcγRI (mAb 197) alone (lane 3), FcγRII (mAb 32.3) alone (lane 4), FcγRIII (mAb 32.3) alone (lane 5), RαM alone (lane 6), FcγRI (mAb 197) plus RαM for 1 and 5 min (lanes 7 and 8, respectively), FcγRII (mAb 32.3) plus RαM for 1 min (lane 9) or 5 min (lane 10), or FcγRIII plus RαM for 1 min (lane 11) or 5 min (lane 12). Proteins were resolved by SDS-PAGE (10%) under reducing conditions, transferred to nitrocellulose, and immunoblotted with anti-MAP kinase (19132) as detailed under “Experimental Procedures.”

Shc, Grb2, and Raf-1 in FcγRII Signaling

BCR, signaling through the activation of Ras and Raf-1. Previously, Morrison et al. demonstrated that PMA enhances the serine/threonine activity, thereby increasing the catalytic activity of Raf-1 (57). It is interesting to speculate that the tyrosine phosphorylation of Raf-1 may be sequentially linked to signals mediated through phosphorylation of γ-chain, Hck, or Syk in this system. In this model, Raf-1 may be a substrate for Hck, Syk, or other tyrosine kinases. In support of such a model in preliminary experiments, we have observed a physical interaction between Hck and Raf-1.

Additional lines of evidence in this report support a potential role for Ras in FcγRII signal transduction. We have observed that FcγRII stimulation induced the tyrosine phosphorylation of Shc. A mobility shift occurs in MAP kinase after FcγRII activation. It is well known that tyrosine phosphorylation of Shc increases the binding of Grb2 to Shc in our system (Fig. 5A, lane 8). We found that Shc is phosphorylated and associated with Grb2 upon FcγRII stimulation (Fig. 5A, lanes 8 and 9). Tyrosine phosphorylation of Shc increased the binding of Grb2 to Shc in our system (Fig. 5B, lane 8). It is well known that tyrosine phosphorylation of Shc is linked to the activation of Ras. Recent studies have shown that the tyrosine phosphorylation of Shc induces its interaction with Grb2, which is essential for the binding of nucleotide exchange protein SOS and Ras activation (15, 61). In other experiments, we observed Shc is physically associated with γ-chain of FcγRII receptor in U937IF cells. Our data support the notion that tyrosine phosphorylation of Raf-1 connects the FcγRII signaling pathway sequentially through γ-chain, Shc, Grb2-SOS, and possibly Ras. Recent data suggest

FIG. 7. Schematic representation for FcγRI signaling to the respiratory burst. FcγRI signaling involves the binding of the FcγRIα and γ subunits to ligand, resulting in a conformation change in α or γ subunits. This change induces the activation of Hck kinase activity, which results in the tyrosine phosphorylation of the ARHI motif of FcγRI and Raf-1. Phosphorylation of FcγRII recruits and activation of Syk kinase. The nonreceptor kinases, Syk and Hck, may phosphorylate critical substrates including Raf-1, MAP kinase, Shc, etc. The activation of small GTPases in the cell is mediated through the action of nucleotide exchange proteins, the Shc-Grb2-SOS complex, which convert GDP->GTP, GTP-activates downstream cascades including Raf-1 and MAP kinase. Other GTPases, Rap1a and Rac2, regulate as yet undefined effectors of signal relay driving the assembly of the respiratory burst proteins, p47phox, p67phox, gp91phox, and p22phox, which results in production of superoxide anions.

Other agonists such as PMA, FMLP, calcium ionophore, or thrombin. We found that tyrosine phosphorylation of Raf-1 is specifically induced upon FcγRI cross-linking (Fig. 4). Importantly, we have generated similar data for Raf-1 tyrosine phosphorylation following FcγRII cross-linking in human bone marrow derived primary macrophage (data not shown). Our results are novel in that they represent the first evidence implicating Raf-1 in Fc receptor signaling. The data are consistent with other results reported by Gupta et al. (56) showing the activation of Raf-1 by other ITAM-linked multisubunit receptors, TCR and
a direct connection between Ras and Rac in several signaling pathways (62). These observations suggest a mechanism by which the conversion of GDP\textsuperscript{Ras} to GTP\textsuperscript{Ras} could lead to the formation of GTP\textsuperscript{Rac}, known to be required for the assembly of the respiratory burst response in myeloid cells. Hence we hypothesize that GTP\textsuperscript{Rac} needed to assemble the respiratory burst comes from the activation of Ras.

MAP kinases are located downstream of Rac1 kinase. MAP kinases have been implicated in signaling pathway of many hematopoietic receptors, such as TCR, BCR, FcRI, and FcRII receptors (63, 64). We show that FcRII cross-linking induces a mobility shift of MAP kinase, suggesting that MAP kinase is retarded mobility on SDS-PAGE. Experiments are ongoing to determine if a direct relationship exists between the tyrosine phosphorylation of FcRI- and activation of Raf-1 kinase and MAP kinase.

Our data also demonstrate that Raf-1 is tyrosine-phosphorylated upon FcRI stimulation. Tyrosine phosphorylation of Raf-1 is correlated with phosphorylation of Shc, which associates with pathways (62). These observations suggest a mechanism by which the conversion of GDP\textsuperscript{Ras} to GTP\textsuperscript{Ras} could lead to the formation of GTP\textsuperscript{Rac}, known to be required for the assembly of the respiratory burst response in myeloid cells. Hence we hypothesize that GTP\textsuperscript{Rac} needed to assemble the respiratory burst comes from the activation of Ras.

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