Tyrosine phosphorylation induced by cross-linking of Fc γ-receptor type II in human neutrophils

Li LIANG and Chi-Kuang HUANG*
Department of Pathology, University of Connecticut Health Center, Farmington, CT 06030, U.S.A.

Neutrophils express several receptors for the Fc region of IgG molecules. Specific cross-linking of the type II receptor (FcγRII) can be achieved by treating neutrophils with the Fab fragment of a specific monoclonal antibody IV.3 against the receptor followed by goat anti-mouse IgG $F(ab')_2$ fragment. Such treatment initiates a number of neutrophil responses including the release of $O_2^{-\cdot}$ and increased protein tyrosine phosphorylation. The increase in tyrosine phosphorylation is rapid and transient and correlates with $O_2^{-\cdot}$ release. Both responses are inhibited by pretreatment of neutrophils with a protein tyrosine kinase inhibitor, genistein. The increase in protein tyrosine phosphorylation is not inhibited by pretreatment of neutrophils with pertussis toxin or an intracellular Ca^{2+} chelator, but is enhanced by a phosphoprotein phosphatase inhibitor, okadaic acid. The activity of a neutrophil Ca^{2+}/calmodulin-dependent protein kinase II (CAMPKII) is also stimulated by cross-linking FcγRII. The increase in CAMPKII activity is inhibited by pretreatment with either genistein or Ca^{2+} chelator. The results suggest that the increase in protein tyrosine phosphorylation induced by cross-linking of FcγRII requires neither pertussis-toxin-sensitive G-proteins nor a rise in intracellular Ca^{2+} but can be regulated by protein phosphatases. Furthermore, protein tyrosine phosphorylation may be an early signal functionally linked to FcγRII-mediated signal transduction leading to CAMPKII activation and $O_2^{-\cdot}$ release in human neutrophils.

INTRODUCTION

Human neutrophils take part in several cellular functions of host defence such as phagocytosis, antibody-dependent cell-mediated cytotoxity and release of $O_2^{-\cdot}$ and the contents of specific granules. Each of these functions can be triggered via receptors for the Fc region of IgG (FcγRs). Neutrophils express several FcγRs [1,2]. They constitutively express both FcγRII and FcγRIII, and expression of FcγRI can be induced by interferon γ. FcγRII has a membrane-spanning domain and cytoplasmic tail whereas FcγRIII is anchored to the cell surface via a phosphatidylinositol–glycan linkage. Neither receptor has a catalytic domain of protein kinase. Therefore they probably couple to signal-transduction pathways indirectly. The cytoplasmic tail of FcγRII has been shown to play an important role [3].

Protein tyrosine phosphorylation is thought to be important in the signal transduction of haematopoietic cells [4,5]. For example, in RBL-2H3 rat tumour mast cells, IgE induces tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1) by coupling the IgE receptor to a non-receptor protein tyrosine kinase. This is apparently the mechanism by which IgE receptor aggregation triggers PLC-γ1 activation and intracellular Ca^{2+} mobilization [6,7]. Cross-linking of FcγRs on human neutrophils by heat-aggregated IgG stimulates the phosphorylation of a number of proteins on tyrosine residues [8]. Genistein, a tyrosine kinase inhibitor, blocks the $O_2^{-\cdot}$ production induced by immune complexes [9]. These studies suggest a possible role of tyrosine phosphorylation in FcγR-mediated signal transduction. However, the mechanism by which each of the three FcγRs transduces its intracellular signals remains unknown.

Monoclonal antibodies have been used to induce specific receptor cross-linking [10]. for FcγRII, the use of mAb IV.3 against FcγRII has allowed an independent evaluation of the specific roles of FcγRII-mediated signal transduction [10]. It has been shown that cross-linking of FcγRII in human neutrophils causes increases in intracellular Ca^{2+} mobilization [10,11]. One potential target for the Ca^{2+} signal is Ca^{2+}/calmodulin-dependent protein kinase II (CAMPKII) [12]. CAMPKII is a multifunctional protein kinase involved in the signal transduction of the rise in $[Ca^{2+}]_i$. On activation by Ca^{2+}/calmodulin it autophosphorylates and becomes fully active even in the absence of Ca^{2+} [13].

In the present study, we have attempted to characterize the tyrosine phosphorylation induced by cross-linking of FcγRII, as well as the regulation of CAMPKII in human neutrophils.

MATERIALS AND METHODS

Materials

Hanks' balanced-salt solution (HBSS) and okadaic acid (OA) were purchased from Gibco (Gaithersburg, MD, U.S.A.). ^{125}I-labelled Protein A and [γ-^{32}P]ATP were from DuPont–New England Nuclear (Boston, MA, U.S.A.). Pertussis toxin (PT), peptide inhibitor of cyclic AMP-dependent protein kinase (P6062) and rabbit anti-mouse IgG were obtained from Sigma (St. Louis, MO, U.S.A.). Electrophoresis chemicals were supplied by Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Prestained molecular-mass standards were purchased from Bio-Rad (Richmond, CA, U.S.A.). Genistein and [bis-(*o*-aminophenoxy)-ethane-*NNN'N'*-tetra-acetic acid, tetra(acetoxymethyl) ester] (BAPTA/AM) were from Calbiochem (San Diego, CA, U.S.A.). Monoclonal antibody mAb IV.3 was purchased from Medarex (West Lebanon, NH, U.S.A.). Goat anti-mouse IgG $F(ab')_2$ and mAb MOPC 141 were supplied by Cappel (Durham, NC, U.S.A.). Autocamtide-2 (RRQETVDCLKK-amide) was from

Abbreviations used: FcγRII, Fc γ-receptor type II; HBSS, Hanks balanced-salt solution; mAb IV.3, Fab fragments of monoclonal antibody to FcγRII; PT, pertussis toxin; BAPTA/AM, [bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid, tetra(acetoxymethyl) ester]; OA, okadaic acid; PLC-γ1, phospholipase C-γ1; CAMPKII, Ca^{2+}/calmodulin-dependent protein kinase II; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

* To whom correspondence should be addressed.

Multiple Peptide System (San Diego, CA, U.S.A.). Monoclonal anti-phosphotyrosine antibody was obtained from Upstate Biotech (Lake Placid, NY, U.S.A.). Phosphocellulose paper (P81) was from Whatman (Clifton, NJ, U.S.A.).

Preparation of neutrophils

Neutrophils were isolated from whole human blood using Ficoll/Hypaque gradients as described initially by Boyum [14]. The remaining erythrocytes were lysed by hypotonic shock (45 s, 4 °C). Neutrophils represented at least 97% of the cells. Cell viability, as estimated by Trypan Blue exclusion, was 98%. The neutrophils were resuspended in HBSS containing 1 mM Ca^{2+} before various treatments.

Cross-linking of FcγRII on neutrophils

Isolated human neutrophils (2.4×10^7/ml) were exposed to 10 μg/ml mAb IV.3 [anti-FcγRII (CD32) IgG2b] or 10 μg/ml mAb MOPC 141, a monoclonal antibody of irrelevant specificity used as a control, for 10 min at room temperature. Pretreated cells were washed twice, then resuspended in HBSS containing 1 mM Ca^{2+} and adjusted to 2×10^7/ml for immunoblotting or 1×10^6/ml for $O_2^{-\cdot}$ assay.

$O_2^{-\cdot}$ production

$O_2^{-\cdot}$ production was determined at 37 °C by a kinetic microplate assay using a $Thermo_{max}$ microplate reader from Molecular Devices (Menlo Park, CA, U.S.A.) as described [15]. To each assay well were added mAb IV.3-pretreated neutrophils (1×10^6/ml, 250 μl) which were suspended in HBSS containing ferricytochrome *c* (75 μM). $O_2^{-\cdot}$ production was initiated by the addition of the indicated amounts of goat anti-mouse IgG $(Fab')_2$. The release of $O_2^{-\cdot}$ was determined as the change in A_{550} (1 nm bandwidth) from baseline. Generation of $O_2^{-\cdot}$ was calculated by subtracting the absorbance change in the presence of 2 mM superoxide dismutase from that in its absence and then dividing by $21.1 \times 10^3\ M^{-1} \cdot cm^{-1}$ (the molar absorption coefficient). A light path of 0.6 cm was used for wells containing 250 μl of reaction mixture [15].

Determination of protein tyrosine phosphorylation

Neutrophils pretreated with mAb IV.3 or mAb MOPC 141 (approx. 2×10^7 cells/ml) were incubated at 37 °C in HBSS containing 1 mM Ca^{2+}. Portions were removed and stimulated with goat anti-mouse IgG $F(ab')_2$ for a given period of time at a given concentration. The reaction was stopped by adding 0.5 sample vol. of stopping solution [9% (w/v) SDS, 6% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol]. The mixture was boiled and analysed by SDS/PAGE (7.5% gel) followed by immunoblotting with anti-phosphotyrosine antibody [16]. Each lane on the gel represented total cell protein from 1×10^6 cells. The blotted proteins were first probed with anti-phosphotyrosine monoclonal antibody. After washing with buffer A (0.15 M NaCl and 100 mM Hepes, pH 7.4), the blots were treated with a secondary antibody, rabbit anti-mouse IgG. After another wash with buffer A, the blots were treated with a solution containing ^{125}I-labelled Protein A [1 μCi/ml in 5% (w/v) BSA]. The blots were then washed extensively in buffer A supplemented with 0.05% (v/v) Tween 20, dried and exposed overnight at −70 °C on Kodak X-Omat film with intensifying screens. Control immunoblots probed with secondary antibody alone had no detectable phosphotyrosine signal.

Use of autocamtide-2 to monitor the activity of CAMPKII in neutrophils

CAMPKII is a multifunctional protein kinase involved in the signal transduction of $[Ca^{2+}]_i$ increases [12]. On activation by Ca^{2+}/calmodulin it autophosphorylates and becomes fully active even in the absence of Ca^{2+} [12]. A rise in $[Ca^{2+}]_i$ in stimulated cells can thus be monitored by measuring the activity of CAMPKII in lysates of prestimulated cells [13]. Kinase activity can be assayed by using a synthetic substrate specific for CAMPKII, autocamtide-2 [13].

CAMPKII activity in neutrophils was determined by a procedure previously used for assaying a histone H4 protein kinase in the Triton X-100 lysates of prestimulated cells [17]. Neutrophils (2×10^7 cells/ml) suspended in HBSS were treated with or without mAb IV.3 (10 μg/ml) for 10 min at room temperature. Cells were washed and stimulated with goat anti-mouse IgG $F(ab')_2$ (50 μg/ml) for 1 min at 37 °C. Portions (20 μl) of cells were then withdrawn and added to a reaction mixture (20 μl) containing 10 mM Hepes (pH 7.2), 20 mM $MgCl_2$, 40 μM [γ-^{32}P]ATP (1×10^3–4×10^3 c.p.m./pmol), 0.1% Triton X-100, 5 mM EGTA, 5 mM sodium orthovanadate, 10 μM autocamtide-2 and 1 μM peptide inhibitor of cyclic AMP-dependent protein kinase. The reaction was carried out at room temperature for 10 min and stopped by adding 20 μl of cold 10% trichloroacetic acid. Proteins in the samples were precipitated at 4 °C for 30 min and removed by centrifugation. Samples (20 μl) of supernatant were then spotted on P81 phosphocellulose papers. The papers were washed, dried and counted to determine the amount of ^{32}P incorporated into autocamtide-2 as described [13,17].

RESULTS

Stimulation of respiratory burst by cross-linking of FcγRII by mAb IV.3 in human neutrophils

Cross-linking of FcγRII on neutrophils by treatment with mAb IV.3 followed by goat anti-mouse IgG $F(ab')_2$ fragment induced $O_2^{-\cdot}$ production (Figure 1a). Pretreatment of the neutrophils with either antibody alone caused no detectable increase in $O_2^{-\cdot}$ generation. Treatment of the cells pretreated with mAb MOPC 141, an antibody with the same isoform (IgG2b) as mAb IV.3, had no effect (Figure 1b). The results indicate that the response to mAb IV.3 was specific. Increased concentrations of the secondary cross-linking antibody goat anti-mouse IgG $F(ab')_2$ after mAb IV.3 enhanced $O_2^{-\cdot}$ production, and the effect was saturated at 50 μg/ml (Figure 1b).

Induction of tyrosine phosphorylation of proteins by mAb IV.3 in human neutrophils

Cross-linking of FcγRII induced increases in tyrosine phosphorylation of a number of proteins with apparent molecular masses 150, 120, 110, 95, 75, 55, 49.5 and 43 kDa (Figures 2 and 3). There was no increase in protein tyrosine phosphorylation in the absence of cross-linking antibody, and the secondary antibody alone had no effect (Figure 2). Control cells pretreated with mAb MOPC 141 showed no increase in protein tyrosine phosphorylation, indicating that the response to mAb IV.3 was specific (Figure 2). Increases in tyrosine phosphorylation of pp150, pp120 and pp75 occurred as early as 10 s and reached a maximum 40 s after stimulation. The phosphorylation event in these phosphoproteins was transient: dephosphorylation was taking place by 1 min and was almost complete at 5 min (Figure 3). In contrast, the phosphorylation level of pp55 reached a

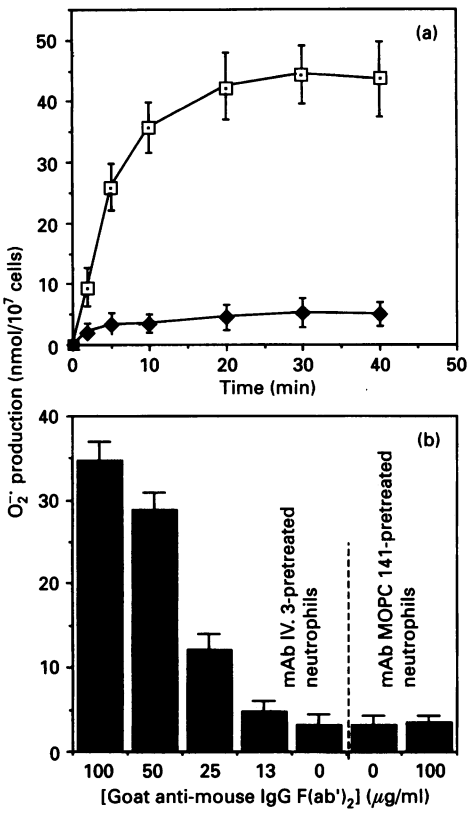

Figure 1 Time course and dose–response curve of $O_2^{-\cdot}$ production in human neutrophils after FcγRII cross-linking

(**a**) Neutrophils were treated with 10 μg/ml mAb IV.3 for 10 min at room temperature. They were then washed twice and stimulated with (□) or without (◆) 50 μg/ml goat anti-mouse IgG (F(ab′)$_2$ fragments for the time indicated. (**b**) mAb IV.3- or mAb MOPC 141-pretreated neutrophils were stimulated with different concentrations of goat anti-mouse IgG F(ab′)$_2$ fragment for 10 min as indicated. $O_2^{-\cdot}$ generation was measured as described in the Materials and methods section. Each point represents the mean ± S.D. for three individual experiments.

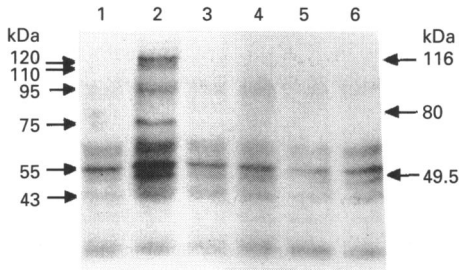

Figure 2 Phosphorylation of neutrophil proteins on tyrosine in response to cross-linking of FcγRII

Neutrophils (2.4×10^7 cells/ml) were exposed to either 10 μg/ml mAb IV.3 (lanes 1 and 2) or 10 μg/ml mAb MOPC 141 (lanes 3 and 4) for 10 min at room temperature. Untreated cells are shown in lanes 5 and 6. Cells were washed twice and then stimulated for 1 min with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) 50 μg/ml goat anti-mouse IgG F(ab′)$_2$. The reaction was stopped by adding SDS stopping buffer, and samples were analysed by SDS/PAGE (7.5% gel): The proteins were transferred to nitrocellulose paper, and probed with anti-phosphotyrosine mAb and ^{125}I-labelled Protein A, and autoradiographed.

maximum at 40 s after stimulation and remained at a high level for 10 min (Figure 3). The extent of tyrosine phosphorylation was proportional to the concentration of goat anti-mouse IgG

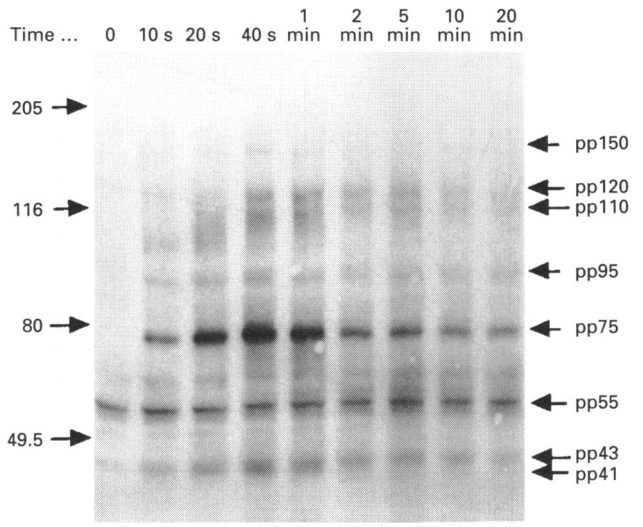

Figure 3 Time course of protein tyrosine phosphorylation in response to FcγRII cross-linking in human neutrophils

Neutrophils (2.4×10^7 cells/ml) were treated with 10 μg/ml mAb IV.3 for 10 min at room temperature. Cells were washed twice and stimulated with 50 μg/ml goat anti-mouse IgG (F(ab′)$_2$ fragment for the times indicated. The reaction was stopped by adding SDS stopping buffer, and electrophoresed in SDS/7.5%-polyacrylamide gel. Phosphotyrosine-containing proteins were detected by Western blotting as indicated in the Materials and methods section.

(F(ab′)$_2$ used to cross-link the FcγRII. Maximum $O_2^{-\cdot}$ generation and tyrosine phosphorylation occurred at approximately the same concentration of cross-linking antibody (50 μg/ml) (Figures 1b and 4).

Effect of genistein on mAb IV.3-induced protein tyrosine phosphorylation and respiratory burst in human neutrophils

Genistein is a tyrosine kinase inhibitor which has been used to study the role of tyrosine phosphorylation in transmembrane signalling [19]. Pretreatment of human neutrophils with genistein leads to a dose-dependent reduction in both tyrosine phosphorylation and $O_2^{-\cdot}$ generation induced by FcγRII cross-linking. With almost 90% inhibition of tyrosine phosphorylation of pp150, pp75 and pp55, $O_2^{-\cdot}$ generation was inhibited by 60%. Flavone, an inactive analogue of genistein, had no effect on tyrosine phosphorylation of pp150 and pp55 and $O_2^{-\cdot}$ production but partially inhibited tyrosine phosphorylation of pp75 and pp120 (Table 1).

The Trypan Blue-exclusion test indicated that genistein caused no significant loss of cell viability. Stimulation of a histone H4 protein kinase activity [17] by cross-linking of FcγRII was not inhibited by genistein (results not shown). We also observed that the $O_2^{-\cdot}$ generation stimulated by phorbol ester was not inhibited by genistein, as has been reported by others [20].

Effect of PT on mAb IV.3-induced tyrosine phosphorylation

PT-sensitive G-proteins have been implicated in signal transduction in neutrophils stimulated by chemotactic factors [21]. Treatment of neutrophils with PT at final concentrations of 0.5–1.0 μg/ml for 1 h inhibits many neutrophil responses stimulated by fMet-Leu-Phe [21]. It is of interest to determine whether

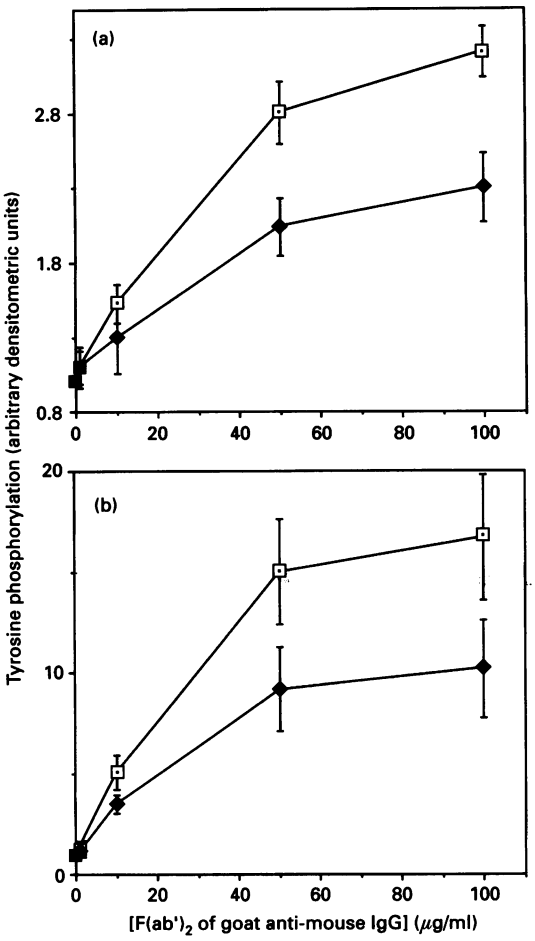

Figure 4 Dose–response curves of tyrosine phosphorylation of proteins in neutrophils after FcγRII cross-linking

mAb IV.3-pretreated neutrophils (2×10^7 cells/ml) as described in Figure 1 were stimulated with the indicated concentrations of goat anti-mouse IgG $F(ab')_2$ for 1 min. The reaction was stopped, and samples were processed as described in the text. Autoradiographs of the resulting blot were quantified by scanning densitometry [18]. The absorbance at zero concentration of goat anti-mouse IgG $F(ab')_2$ was arbitrarily taken as 1.0. Results of densitometric analysis of pp150 (⊡), pp55 (◆) (**a**) and pp120 (◆), pp75 (⊡) (**b**) are shown. Each point represents the mean ± S.D. of three experiments.

G-proteins are involved in transmitting FcγRII signalling. Table 2 shows that pretreatment of neutrophils with 1 μg/ml PT had no effect on tyrosine phosphorylation induced by FcγRII cross-linking. The PT used in this study was functionally active, as it inhibited completely $O_2^{-\cdot}$ production induced by fMet-Leu-Phe, as has been reported by others [22].

Effect of BAPTA/AM on mAB IV.3-induced tyrosine phosphorylation

Next, we examined the role of Ca^{2+} in mAb IV.3-induced tyrosine phosphorylation of various proteins. It has been indicated that Ca^{2+} mobilization is an early event in neutrophils stimulated by chemotactic factors or FcγR activation [22,23]. Pretreatment of human neutrophils with 50 μM BAPTA/AM, an intracellular Ca^{2+} chelator, had no effect on protein tyrosine phosphorylation (Table 3). We have shown previously that BAPTA/AM treatment inhibits the $[Ca^{2+}]_i$ rise induced by fMet-Leu-Phe [24]. In Table 5 (see below), we also demonstrate that BAPTA/AM treatment inhibits the increase in CAMPKII activity caused by cross-linking of FcγRII. These results indicate that the BAPTA/AM treatment was effective.

Effect of OA on mAb IV.3-induced tyrosine phosphorylation

OA, a potent inhibitor of protein phosphatases 1 and 2A, has been widely used to study the roles of serine/threonine phosphatases in cell activation [25,26]. Here we examined its effect on mAb IV.3-induced tyrosine phosphorylation of proteins. mAb IV.3-induced tyrosine phosphorylation of pp55 pp75, pp120 and pp150 was enhanced by pretreatment of cells with 1 μg/ml OA (Table 4), that of pp75 being particularly sensitive.

Effect of BAPTA/AM and genistein on mAb IV.3-stimulated CAMPKII activity

Cross-linking of FcγRII by mAb IV.3 can induce a rise in $[Ca^{2+}]_i$ [10,23]. As an increase in $[Ca^{2+}]_i$ has been shown to stimulate intracellular CAMPKII in GH3 cells [13], we studied the effect of FcγRII cross-linking on neutrophil CAMPKII activity using autocamtide-2, a specific substrate for this enzyme [13,27]. We have observed activation of neutrophil CAMPKII caused by pretreatment with either fMet-Leu-Phe or A23187 (agents known to induce increases in $[Ca^{2+}]_i$), but not with phorbol ester or granulocyte–macrophage colony-stimulating factor (agents that

Table 1 Effect of genistein on mAb IV.3-induced tyrosine phosphorylation of diverse protein substrates and respiratory burst in human neutrophils

mAb IV.3-treated cells were incubated at 37 °C with different concentrations of genistein or flavone for 15 min. Cells were then stimulated with goat anti-mouse IgG $F(ab')_2$ and incubated for 1 min for measurement of tyrosine phosphorylation or for 10 min for measurement of $O_2^{-\cdot}$ production, as described in the Materials and methods section. Densitometry was performed with a laser scanner. The results are scans from three autoradiographs. The extent of inhibition was determined by using the following formula: percentage inhibition = {1 − [experiment (inhibitor-pretreated)]/[positive control (dimethyl sulphoxide pretreated)]} × 100%. Each value represents the mean ± S.D. The 100% value for $O_2^{-\cdot}$ production was 3.58 ± 0.42 nmol/10 min per 10^6 cells.

	Percentage inhibition				
	Tyrosine phosphorylation				
Conditions	pp55	pp75	pp120	pp150	$O_2^{-\cdot}$ generation
Dimethyl sulphoxide control	0	0	0	0	0
+ Genistein (30 μM)	26.3 ± 2.9	54.8 ± 6.8	34.2 ± 4.1	47.3 ± 3.3	38.4 ± 4.5
+ Genistein (150 μM)	91.1 ± 7.6	84.6 ± 9.2	57.2 ± 6.1	84.1 ± 6.1	62.7 ± 7.7
+ Flavone (150 μM)	4.5 ± 0.4	28.6 ± 3.8	20.8 ± 2.4	9.8 ± 0.6	5.8 ± 0.6

Table 2 Effect of PT on mAb IV.3-induced tyrosine phosphorylation of diverse protein substrates in human neutrophils

Cells were incubated with or without 1 μg/ml PT for 50 min at 37 °C before incubation with mAb IV.3 for 10 min at room temperature. The cells were subsequently washed and resuspended in HBSS containing 1 μg/ml PT and stimulated by goat anti-mouse IgG $F(ab')_2$ fragment for 1 min. Tyrosine phosphorylation of proteins was measured as described in the Materials and methods section. Densitometry was performed with a laser scanner. Absorbance of controls was arbitrarily taken as 1.0. The Table presents the results of scans from three autoradiographs. Each value represents the mean ± S.D.

	Autoradiograph densitometry (relative to control)			
Conditions	pp55	pp75	pp120	pp150
Control	1.00	1.00	1.00	1.00
+ PT (1 μg/ml)	0.86 ± 0.09	0.77 ± 0.08	0.97 ± 0.11	0.95 ± 0.07
Cross-linking of FcγRII	1.42 ± 0.16	9.18 ± 1.09	6.02 ± 0.73	2.21 ± 0.27
+ PT + cross-linking of FcγRII	1.47 ± 0.15	8.88 ± 1.04	5.78 ± 0.81	2.82 ± 0.32

Table 3 Effect of BAPTA/AM of mAb IV.3-induced protein tyrosine phosphorylation

mAb IV.3-treated cells were incubated at 37 °C with or without 50 μM BAPTA/AM for 30 min. Cross-linking of FcγRII was then stimulated with goat anti-mouse IgG $F(ab')_2$ fragment for 1 min. Protein tyrosine phosphorylation was measured as described in the Materials and methods section. Densitometry was performed as described in Table 2. Results are means ± S.D.

	Autoradiograph densitometry (relative to control)			
Conditions	pp55	pp75	pp120	pp150
Control	1.00	1.00	1.00	1.00
+ BAPTA/AM	1.09 ± 0.18	1.17 ± 0.12	1.2 ± 0.11	1.12 ± 0.13
Cross-linking of FcγRII	1.12 ± 0.17	4.13 ± 0.68	3.23 ± 0.47	1.98 ± 0.17
+ BAPTA/AM + cross-linking of FcγRII	1.23 ± 0.19	4.42 ± 0.71	3.13 ± 0.45	2.12 ± 0.29

Table 4 Effect of OA on mAb IV.3-induced tyrosine phosphorylation of diverse protein substrates in human neutrophils

mAb IV.3-treated cells were incubated at 37 °C with or without OA for 30 min. Cross-linking of FcγRII was then stimulated with goat anti-mouse IgG $F(ab')_2$ fragment for 1 min. Protein tyrosine phosphorylation was measured as described in the Materials and methods section. Densitometry was performed as described in Table 2. Results are means ± S.D.

	Autoradiograph densitometry (relative to control)			
Conditions	pp55	pp75	pp120	pp150
Control	1.00	1.00	1.00	1.00
+ OA (1 μg/ml)	1.08 ± 0.09	4.33 ± 0.56	2.03 ± 0.36	0.92 ± 0.08
Cross-linking of FcγRII	3.01 ± 0.24	9.92 ± 1.47	7.69 ± 1.02	4.08 ± 0.33
+ OA + cross-linking of FcγRII	3.17 ± 0.21	27.23 ± 3.36	11.18 ± 1.84	5.16 ± 0.39

Table 5 Effect of BAPTA/AM and genistein on mAb IV.3-induced activation of neutrophil CAMPKII

mAb IV.3-treated cells (2×10^7 cells/ml suspended in HBSS) were incubated with or without BAPTA/AM (50 μM, 30 min) or genistein (150 μM, 15 min). Cross-linking of FcγRII was then stimulated with goat anti-mouse IgG $F(ab')_2$ fragment for 1 min. Portions (20 μl) of the cells were withdrawn and the activity of CAMPKII was assayed as described in the Materials and methods section using autocamtide-2 as substrate. The kinase activity derived from untreated cells (control) is designated as 1.00 which is approx. 0.98 ± 0.08 pmol/min per 10^6 cells.

Conditions	CAMPKII activity (relative to control)
Control	1.00
+ BAPTA/AM	0.91 ± 0.09
Cross-linking of FcγRII	4.31 ± 0.54
+ BAPTA/AM + cross-linking of FcγRII	1.34 ± 0.2
+ Genistein	0.95 ± 0.07
+ Genistein + cross-linking of FcγRII	1.23 ± 0.08

do not induce $[Ca^{2+}]_i$ increase) (C.-K. Huang and J. Colasanto, unpublished work).

The effect of cross-linking of FcγRII on neutrophil CAMPKII activity was studied. As shown in Table 5, it induced a large increase in CAMPKII activity. Pretreatment of the cells with BAPTA/AM inhibited the stimulation of CAMPKII activity. Pretreatment of neutrophils with genistein also inhibited the stimulation of CAMPKII induced by FcγRII cross-linking. Neither BAPTA/AM (50 μM) nor genistein (150 μM) [28], when added directly to the assay mixture, inhibited CAMPKII activity (results not shown).

DISCUSSION

It has been reported that cross-linking for FcγRs on human neutrophils by heat-aggregated IgG stimulates phosphorylation of a number of proteins on the tyrosine residue [8,9]. However, it is important to examine whether protein tyrosine

phosphorylation can be mediated via FcγRII alone in human neutrophils. The use of mAb IV.3, a monoclonal antibody to FcγRII, has allowed an independent evaluation of the specific roles of FcγRII-medicated signal transduction. Cross-linking of FcγRII by treatment of neutrophils with the Fab fragment of mAb IV.3 followed by goat anti-mouse IgG $F(ab')_2$ induces the release of $O_2^{-\cdot}$ along with tyrosine phosphorylation of several proteins (Figures 1, 2 and 3). The two events have similar dose–responses but tyrosine phosphorylation occurs earlier. Pretreatment of neutrophils with 150 μM genistein inhibited tyrosine phosphorylation of most proteins by 80–90%, and $O_2^{-\cdot}$ generation was also inhibited by a maximum of 62% (Table 1). The results indicate that mAb IV.3-induced tyrosine phosphorylation plays a major role in $O_2^{-\cdot}$ production. However, as the inhibition of $O_2^{-\cdot}$ production is not as complete as that of tyrosine phosphorylation, other signalling pathways may also be involved. Recently, we observed that the increases in $O_2^{-\cdot}$ production and CAMPKII activity induced by Fc receptor cross-linking were completely inhibited by staurosporine (10 μM), suggesting that protein kinases other than the genistein-sensitive tyrosine kinases may also be involved.

In neutrophils stimulated by chemotactic factors, PT-sensitive G-proteins have been implicated in the transduction of signals for $O_2^{-\cdot}$ generation [21]. With respect to FcγR-mediated $O_2^{-\cdot}$ production, the effect of PT treatment may depend on the nature of the IgG [11]. Soluble immune complexes induce activation of neutrophils via a PT-sensitive pathway, whereas insoluble immune complexes induce $O_2^{-\cdot}$ production through a PT-insensitive pathway. Our results (Table 2) show that PT has no effect on protein tyrosine phosphorylation in FcγRII-cross-linked neutrophils. As the signalling pathways induced by immune complexes may involve the co-cross-linking of multiple isotypes of Fc receptors, further work is required to elucidate the PT-sensitive pathways.

It has been shown that cross-linking of FcγRII in human neutrophils causes Ca^{2+} mobilization [10,11]. Table 3 shows that mAb IV.3-induced protein tyrosine phosphorylation is insensitive to BAPTA/AM treatment. This result suggests that FcγRII-induced protein tyrosine phosphorylation is upstream of Ca^{2+} mobilization.

Pretreatment of neutrophils with OA greatly enhances mAb IV.3-induced protein tyrosine phosphorylation (Table 4), suggesting that serine/threonine phosphatases (phosphatase 1 and 2A) may regulate the activities of tyrosine kinases or tyrosine phosphatases or the structure of kinase substrates. In neutrophils, OA has been shown to affect the cytoskeletal structure and to magnify and prolong the oxidative burst elicited by fMet-Leu-Phe, but inhibits the response to phorbol esters [25,30,31]. We also observed that OA can enhance the $O_2^{-\cdot}$-generating response by 50% and significantly prolong the activation of CAMPKII induced by FcγRII cross-linking. These results suggest a regulatory role of serine/threonine phosphatase in neutrophil tyrosine phosphorylation and $O_2^{-\cdot}$-generating response.

The pattern of FcγRII-cross-linking-induced tyrosine-phosphorylated proteins as described here is similar to that reported in human neutrophils, the rat basophilic leukaemia cell line, RBL-2H3 (both stimulated by heat-aggregated IgG) [8] and the monocytic cell line THP-1 [23] but not platelets [32] (both stimulated by mAb IV.3). In platelets, activation of FcγRII induces tyrosine phosphorylation of FCγRII itself [32]. None of the tyrosine-phosphorylated protein shown in Figure 2 corresponds to FcγRII. In neutrophils, tyrosine phosphorylation of FCγRII (40 kDa) was detectable only after immunoprecipitation, but no reproducible increase in receptor tyrosine phosphorylation occurred in FcγRII-cross-linked cells (results not shown). The nature of the many tyrosine-phosphorylated neutrophil proteins remains to be studied. Some candidates may be: PLC-γ1 (150 kDa), mitogen-activated protein kinase (41 kDa) and tyrosine kinase *syk* (72 kDa). Cross-linking of FcγRI or FcγRII on U937 cells resulted in a rapid and transient phosphorylation of PLC-γ1 on tyrosine residues [33]. Tyrosine phosphorylation of PLC-γ1 may be essential for the increase in $[Ca^{2+}]_i$ induced by FcγRII cross-linking. This hypothesis is supported by the finding that genistein is able to block the intracellular tyrosine phosphorylation (Table 1), Ca^{2+} increase [11] and CAMPKII activation (Table 5) induced by mAb IV.3. Co-cross-linking the phosphotyrosine phosphatase CD45 and FcγR inhibits both tyrosine phosphorylation and Ca^{2+} mobilization [23]. In neutrophils treated with fMet-Leu-Phe or granulocyte–macrophage colony-stimulating factor, a major tyrosine-phosphorylated protein pp41 has been shown to be the activated mitogen-activated protein kinase [18]. In Figure 2, a tyrosine-phosphorylated protein designated pp41 was also observed in FcγRII-cross-linked neutrophils. However, the level of tyrosine phosphorylation of pp41 observed in this study is much lower than that observed in neutrophils treated with either fMet-Leu-Phe or granulocyte–macrophage colony-stimulating factor [18]. FCγRII cross-linking induces the tyrosine phosphorylation of a protein kinase, *syk*, in HL-60 cells [34]. A similar phosphoprotein, pp75, was observed in this study. Figure 3 shows that there is rapid and transient tyrosine phosphorylation of pp75 on cross-linking of FcγRII. The phosphorylation of pp75 is greatly enhanced by OA pretreatment (Table 4). Association of FcγRII with tyrosine kinase *fgr* in human neutrophils was recently reported [35]. Whether the tyrosine-phosphorylated protein, pp55 (Figures 2 and 3), is related to *fgr* remains to be ascertained.

The characteristics of tyrosine phosphorylation induced by FcγRII cross-linking described in this paper have some similarity to those of Fc receptor-mediated phagocytosis [36,37]. Both are independent of PT-sensitive G-proteins and $[Ca^{2+}]_i$ increases but are inhibited almost completely by genistein (150 μM). In contrast, $O_2^{-\cdot}$ generation induced by either FcγR cross-linking (this study) or aggregated IgG [38] is only partially inhibited by genistein.

Very little is known about the downstream events occurring after the increase in $[Ca^{2+}]_i$ in stimulated neutrophils. In this study we report the activation of CAMPKII by FcγRII cross-linking. The effect is inhibited by pretreatment with either BAPTA/AM or genistein, suggesting that CAMPKII may act downstream of tyrosine phosphorylation and play a role in transducing the Ca^{2+} increase signal. A detailed study and characterization of CAMPKII in human neutrophils is currently being undertaken.

In conclusion, tyrosine phosphorylation of neutrophil proteins occurs soon after cross-linking of FcγRII. The increase in protein tyrosine phosphorylation requires neither PT-sensitive G-proteins nor an increase in $[Ca^{2+}]_i$ but can be regulated by protein phosphatases. Furthermore, protein tyrosine phosphorylation may be an early signal functionally linked to FcγRII-mediated signal transduction leading to CAMPKII activation and $O_2^{-\cdot}$ release in human neutrophils.

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REFERENCES

1 Ravetch, J. V. and Kinet, J.-P. (1991) Annu. Rev. Immunol. **9**, 457–492
2 van de Winkel, J. G. L. and Anderson, C. L. (1991) J. Leukocyte Biol. **49**, 511–524
3 Miettinen, H. M., Rose, J. K. and Mellman, I. (1989) Cell **58**, 317–327

4 Huang, C.-K. and Sha'afi, R. I. (1993) Protein Kinases in Blood Cell Function, CRC Press, Boca Raton, FL
5 Perlmutter, R. M., Marth, J. D., Ziegler, S. E., Garvin, A. M., Pawar, S., Cooke, P. M. and Abraham, K. M. (1988) Biochim. Biophys. Acta **948**, 245–257
6 Benhamou, M., Gutkind, J. S., Robbins, K. C. and Siraganian, R. P. (1990) J. Immunol. **87**, 5327–5333
7 Park, D. J., Min, H. K. and Rhee, S. H. (1992) J. Biol. Chem. **266**, 1496–1501
8 Connelly, P. A., Farrell, C. A., Merenda, J. M., Conklyn, M. J. and Showell, H. J. (1991) Biochem. Biophys. Res. Commun. **177**, 192–197
9 Kusunoki, T., Higashi, H., Hosoi, S., Hata, D., Sugie, K., Mayumi, M. and Mikawa, H. (1992) Biochem. Biophys. Res. Commun. **183**, 789–796
10 Odin, J. A., Edberg, J. C., Painter, C. J., Kimberly, R. P. and Unkeless, J. C. (1991) Science **254**, 1785–1788
11 Crockett-Torabi, E. and Fantone, J. C. (1990) J. Immunol **145**, 3026–3032
12 Hanson, P. I. and Schulman, H. (1992) Annu. Rev. Biochem. **61**, 559–601
13 Jefferson, A. B., Travis, S. M. and Schulman, H. (1991) J. Biol. Chem. **266**, 1484–1490
14 Boyum, A. (1968) Scand. J. Lab. Invest. **21**, 77–85
15 Mayo, L. A. and Curnutte, J T. (1990) Methods Enzymol. **186**, 567–585
16 Huang, C.-K., Laramee, G. R. and Casnellie, J. E. (1988) Biochem. Biophys. Res. Commun. **151**, 794–801
17 Huang, C.-K. and Laramee, G. R. (1988) J. Biol. Chem. **263**, 13144–13151
18 Gomez-Cambronero, J., Colasanto, J. M., Huang, C.-K. and Sha'afi, R. I. (1993) Biochem. J. **291**, 211–217
19 Akiyama, T. and Ogawarw, H. (1991) Methods Enzymol. **201**, 362–370
20 Utsumi, T., Klostergaard, J., Akimaru, K., Edashige, K., Sato, E. and Utsumi, K. (1992) Arch. Biochem. Biophys. **294**, 271–278
21 Becker, E. L., Kermode, J. C., Naccache, P. H., Yassin, R., Marsh, M. L., Munoz, J. J. and Sha'afi, R. I. (1985) J. Cell Biol. **100**, 1641–1648
22 Walker, B. A. M., Hagenlocker, B. E., Stubbs, E. B., Sandborg, R. R., Agranoff, B. W. and Ward, P. A. (1991) J. Immunol. **146**, 735–741
23 Rankin, B. M., Yocum, S. A., Mittler, R. S. and Kiener, P. A. (1993) J. Immunol. **150**, 605–616
24 Huang, C.-K., Laramee, G. R., Yamazaki, M. and Shi'afi, R. I. (1990) J. Cell. Biochem. **44**, 221–228
25 Garcia, R. C., Whitaker, M., Heyworth, P. G. and Segal, A. W. (1992) Biochem. J. **286**, 687–692
26 Cohen, P., Holmes, C. F. B. and Taukitani, Y. (1990) Trends Biochem. Sci. **15**, 98–102
27 MacNicol, M., Jefferson, A. B. and Schulman, H. (1990) J. Biol. Chem. **265**, 18055–18058
28 O'Dell, T. J., Kandel, E. R. and Grant, S. G. N. (1991) Nature (London) **353**, 558–560
29 Reference deleted
30 Kreienbuhl, P., Keller, H. and Niggli, V. (1992) Blood **80**, 2911–2919
31 Lu, D., Takai, J. A., Leto, T. L. and Grinstein, S. (1992) Am. J. Physiol. **262** (Cell Physiol. **31**), C39–C49
32 Huang, M. M., Indik, Z., Brass, L. F., Hoxie, J. A., Schreiber, A. D. and Brugge, J. S. (1992) J. Biol. Chem. **267**, 5463–5473
33 Liao, F., Shin, S. H. and Rhee, S. G. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3659–3663
34 Agarwal, A., Salem, P. and Robbins, K. C. (1993) J. Biol. Chem. **268**, 15900–15905
35 Hamada, F., Aoki, M., Akiyama, T. and Toyoshima, T. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 6305–6309
36 Rosales, C. and Brown, E. J. (1992) J. Biol. Chem. **267**, 5265–5271
37 Greenberg, S., Chang, P. and Silverstein, S. C. (1993) J. Exp. Med. **177**, 529–536
38 Bianca, V. D., Grzeskowiak, M., Stefano, D. and Rossi, F. (1993) J. Leukocyte Biol. **53**, 427–438

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