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# Tyrosine Phosphorylation is an Early Signaling Event Common to Fc Receptor Crosslinking in Human Neutrophils and Rat Basophilic Leukemia Cells (RBL-2H3)

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**SUMMARY** Phosphotyrosine-containing proteins were detected by western blotting of whole cell lysates of purified human neutrophils or rat basophilic leukemia cells (RBL-2H3) using a polyclonal anti-phosphotyrosine antibody. When either cell type was stimulated with the appropriate Fc crosslinking agent, heat-aggregated IgG for the neutrophil or DNP-HSA for the IgE-sensitized RBL-2H3, a rapid increase in the phosphotyrosine content of several proteins was observed. The kinetics and specificity of both responses suggest that Fc receptor crosslinking activates a receptor-associated tyrosine kinase, probably a member of the *src* family of tyrosine protein kinases. The subsequent tyrosine phosphorylation events are likely to be important in Fc receptor-mediated stimulus-response coupling in inflammatory cells. 

The IgE receptor (FcεR) expressed on mast cells and the IgG receptors (FcγRI-III) expressed on human neutrophils are members of the Ig gene superfamily (1-3). Antigen-induced crosslinking of IgE-occupied FcεR and immune-complex induced crosslinking of FcγRII and FcγRIII results in the release of secretory granules and their contents which contain potent mediators of inflammation (1-3). The long-term consequences of FcR crosslinking include the synthesis and secretion of a variety of lymphokines (4). Regulatory phosphorylation on serine and threonine residues has long been implicated in the control of stimulus-secretion coupling, primarily as reactions subsequent to the activation of phospholipase C (5). In the present study we demonstrate that FcR crosslinking transmits additional intracellular signals by stimulating the phosphorylation of tyrosine residues in both the human neutrophil and the rat basophilic leukemia cell line, RBL-2H3.

Abbreviations: DNP-HSA, dinitrophenol coupled to human serum albumin; FcεR, Fc receptor for IgE; FcγR, Fc receptor for IgG; α-TNP, antibody to trinitrophenol that recognizes DNP-HSA; PMA, phorbol 12-myristate 13-acetate; I, ionomycin; GM-CSF, granulocyte-macrophage colony stimulating factor; HBSS, Hank's buffered salt solution; HPMNS, human polymorphonuclear leukocytes/neutrophils.

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## Materials and Methods

Materials: α-TNP was isolated as described in (6). Human IgG (Sigma), endotoxin free (data not shown), was aggregated by heating at 63°C for 20 min. Insoluble material was removed by centrifugation prior to separation of aggregated from monomeric IgG by Superose 12 gel filtration chromatography. Phorbol myristate acetate (PMA) and ionomycin were obtained from Sigma. Human recombinant GM-CSF was purchased from Collaborative Research and human recombinant C5a was obtained by the method of Franke et al. (7).

Cell Culture: RBL-2H3 cells were cultured in minimal essential media with Earle's salts supplemented with 20% heat-inactivated fetal bovine serum. 2mM glutamine, 50 units penicillin/ml and 50 μg streptomycin/ml at 37°C in a humidified atmosphere containing 5% $CO_2$. RBL-2H3 cells were harvested by treatment with 0.25% trypsin in buffered saline containing 0.02% EDTA.

Tyrosine Phosphorylation Studies:

SDS-PAGE (8) of whole cell lysates was followed by western blotting with polyclonal anti-phosphotyrosine antibody prepared by the protocol described in Kamps and Sefton (9). Immune complexes were detected by autoradiography using [$^{125}$I]-protein A (ICN). The specificity of the antibody used was tested by incubation of four identical western blots with 2.5 mM phosphoserine, 2.5 mM phosphothreonine, 2.5 mM phosphotyrosine or in the absence of phosphoamino acid (10). Incubation with phosphotyrosine abolished subsequent [$^{125}$I]-protein A binding, while blots incubated with either phosphoserine or phosphothreonine were similar to the untreated control (data not shown).

-Human neutrophils were isolated from whole blood (11). and suspended in Hank's buffered saline solution containing $Mg^{2+}$ and $Ca^{2+}$ (HBSS), were pre-equilibrated at 37°C for 5 min prior to the addition of heat-aggregated IgG or buffer. The final assay concentration was 2.5 x $10^6$ neutrophils in a 0.05 ml volume. Reactions were terminated by the addition of 0.15 ml of 100°C 2X sample buffer (4% SDS, 0.2M dithiothreitol, 10% β-mercaptoethanol, 10mM Na phosphate buffer, pH 7.0, 20% glycerol, 0.03% bromphenol blue), followed by heating for 10 min at 100°C.

-RBL-2H3 cells washed in HBSS were sensitized for 30 min at 37°C with 0.3 μg IgE/ml. After centrifugation the cells were resuspended in HBSS and equilibrated at 37°C for 5 min prior to the addition of antigen (dinitrophenyl-human serum albumin) or buffer. The final assay concentration was 1.5 x $10^6$ cells in 0.05 ml. Reactions were terminated as described for neutrophils.

## Results

Since crosslinking of Fc receptors leads to complex secretory events in the RBL-2H3 rat basophilic leukemia cell line (12) it was of interest to assess the effect of crosslinking on tyrosine phosphorylation. Crosslinking of IgE-occupied Fc receptors with DNP-HSA resulted in the rapid phosphorylation of a number of RBL-2H3 proteins on tyrosine residues (Figure 1a and b). Tyrosine phosphorylation in response to antigen addition was extremely rapid with maximum phosphorylation occurring at 20 seconds post-stimulation. Significant dephosphorylation took place by 5 minutes, although measureable phosphorylation of several substrates persisted at least 15 minutes after antigen addition. As shown in Figure 2, tyrosine phosphorylation was dependent on the antigen concentration. Half-maximal phosphorylation of the 69 kDa substrate occurred at approximately 30 ng DNP-HSA/ml, a value within an order of

magnitude of the concentration of antigen required for half-maximal release of N-acetylglucosaminidase from these cells (~300 ng/ml) (6).

Human neutrophils express several distinct receptors for the Fc portion of the IgG molecule (2,3). Crosslinking of such receptors by soluble immune complexes is thought to play an important role in the initiation of the inflammatory response (2,3). If tyrosine phosphorylation is a signal transduction mechanism common to crosslinking of Fc receptors, one would expect to observe a response in the human neutrophil similar to that shown above for the RBL-2H3 cell. As demonstrated in Figure 3a and b, crosslinking of human neutrophil Fc receptors by heat-aggregated IgG led to a rapid increase in the phosphotyrosine content of a number of proteins, several of which are similar in apparent molecular weight to those observed in the RBL cell line. In particular, phosphotyrosine-containing proteins of approximately 70 kDa and 45 kDa appeared *de novo* following stimulation of the neutrophil and the RBL cell. The time required for maximal increases in phosphorylation of the neutrophil substrates, approximately 3 min, was slightly longer than that seen in the RBL cell. The extent of tyrosine phosphorylation of the neutrophil substrates was proportional to the concentration of heat-aggregated IgG used to crosslink the Fc receptors and monovalent IgG was unable to stimulate tyrosine phosphorylation at identical concentrations (Figure 4).

Although Fc receptor crosslinking in human neutrophils does not appear to stimulate a significant increase in the concentration of intracellular calcium (13), it was of interest to determine if pharmacological agents which bypass receptor-regulated events and artificially increase intracellular calcium and/or activate protein kinase C affect the patterns of tyrosine phosphorylation observed in the RBL-2H3 cell and the human neutrophil. Results shown in Figure 5 indicate that the calcium ionophore, ionomycin, and the phorbol ester, PMA, had no effect on tyrosine phosphorylation in the RBL-2H3 cell. Treatment of neutrophils with phorbol ester alone stimulated the incorporation of phosphotyrosine into proteins of 115 kDa and 42 kDa not seen with other stimuli. Ionomycin alone was without effect, but the combination of PMA and ionomycin stimulated the phosphorylation of the PMA-specific targets as well as additional proteins which appear to be a subset of the proteins affected by Fc receptor

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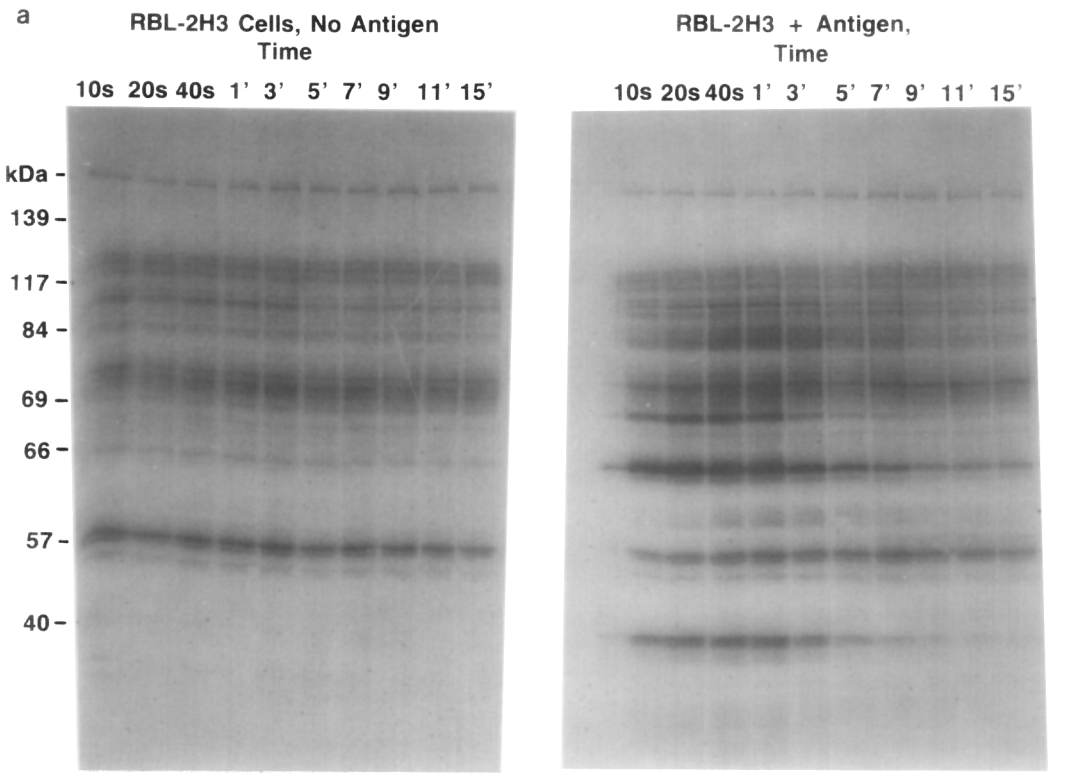
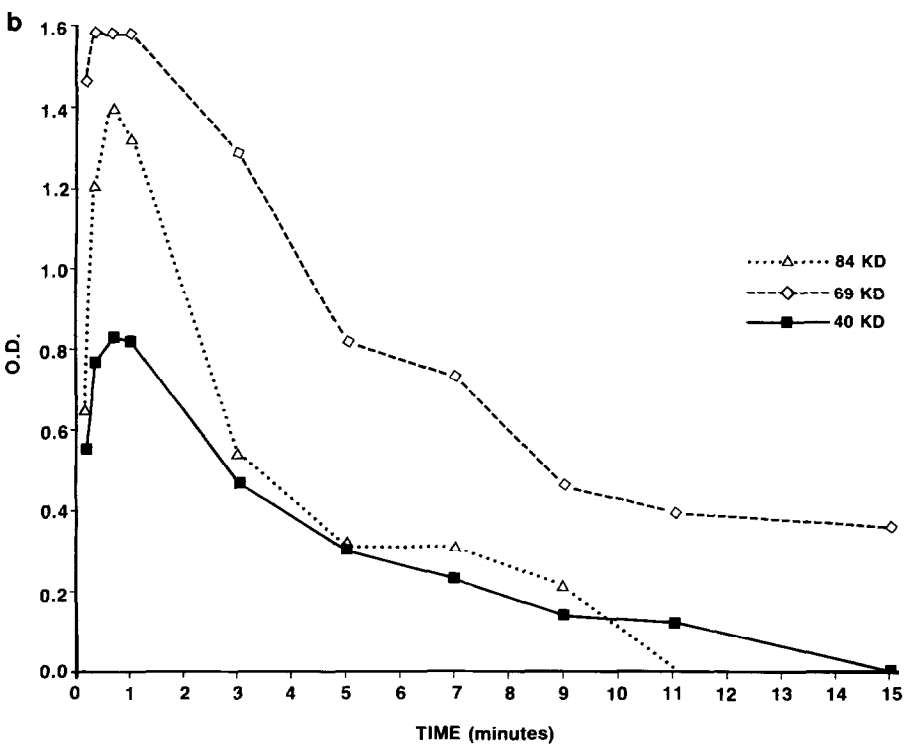
Figure 1. **FcεR crosslinking stimulates an increase in the phosphotyrosine content of multiple substrates in RBL-2H3 cells.** Sensitized RBL-2H3 cells were prepared as described under Methods. DNP-HSA was added at a concentration of 0.1 μg/ml and 50 μl aliquots of the treated cells were withdrawn at 10s, 20s, 40s, and 1, 3, 5, 7, 9, 11, and 15 min. Cell aliquots were immediately diluted in Laemmli buffer and heated as described under Methods. An identical time course was carried out with cells incubated in the absence of DNP-HSA. a. Phosphotyrosine-containing proteins were detected by western blotting using a polyclonal anti-phosphotyrosine antibody (9). The phosphotyrosine content of proteins of Mr 139, 117, 84, 69, 66, 57, and 40 kDa was increased by the addition of DNP-HSA. b. The film shown in Fig. 1a. was scanned using a Molecular Dynamics laser scanner and the optical densities of the 84, 69 and 40 kDa bands were quantitated using Quantity-one from Protein Data Bases, Inc.. All figures are representative of at least 3 individual experiments.


a
RBL-2H3 Cells, No Antigen
Time
10s 20s 40s 1' 3' 5' 7' 9' 11' 15'
RBL-2H3 + Antigen,
Time
10s 20s 40s 1' 3' 5' 7' 9' 11' 15'
kDa
139
117
84
69
66
57
40


b
O.D.
TIME (minutes)
84 KD
69 KD
40 KD

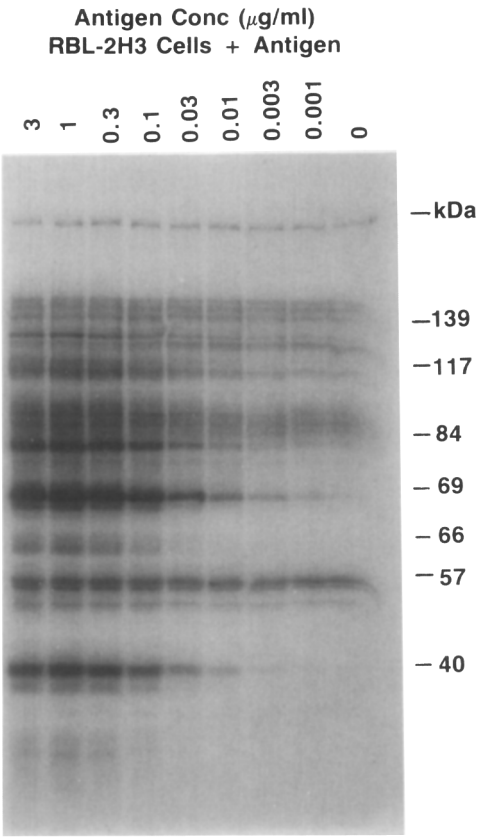



Figure 2. **Tyrosine phosphorylation in the RBL-2H3 cell line is a function of the concentration of added DNP-HSA.** Fifty μl of sensitized RBL-2H3 cells were delivered to tubes containing DNP-HSA at a final concentration of 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 3 μg/ml. Reactions were terminated after a 45 sec incubation and phosphotyrosine-containing proteins detected as described above.

crosslinking. Neutrophil receptors for chemotactic factors, such as the complement fragment C5a, are coupled to the activation of phospholipase C and, as a result, ligand binding activates $Ca^{2+}$/calmodulin-dependent protein kinases as well as protein kinase C (5, 13). As seen in Figure 5, treatment of human neutrophils with an optimal concentration of C5a resulted in the rapid tyrosine phosphorylation of a broad protein band of approximately 120 kDa and several lower molecular weight substrates common to stimulation with aggregated IgG and PMA. In summary, these results suggest that Fc

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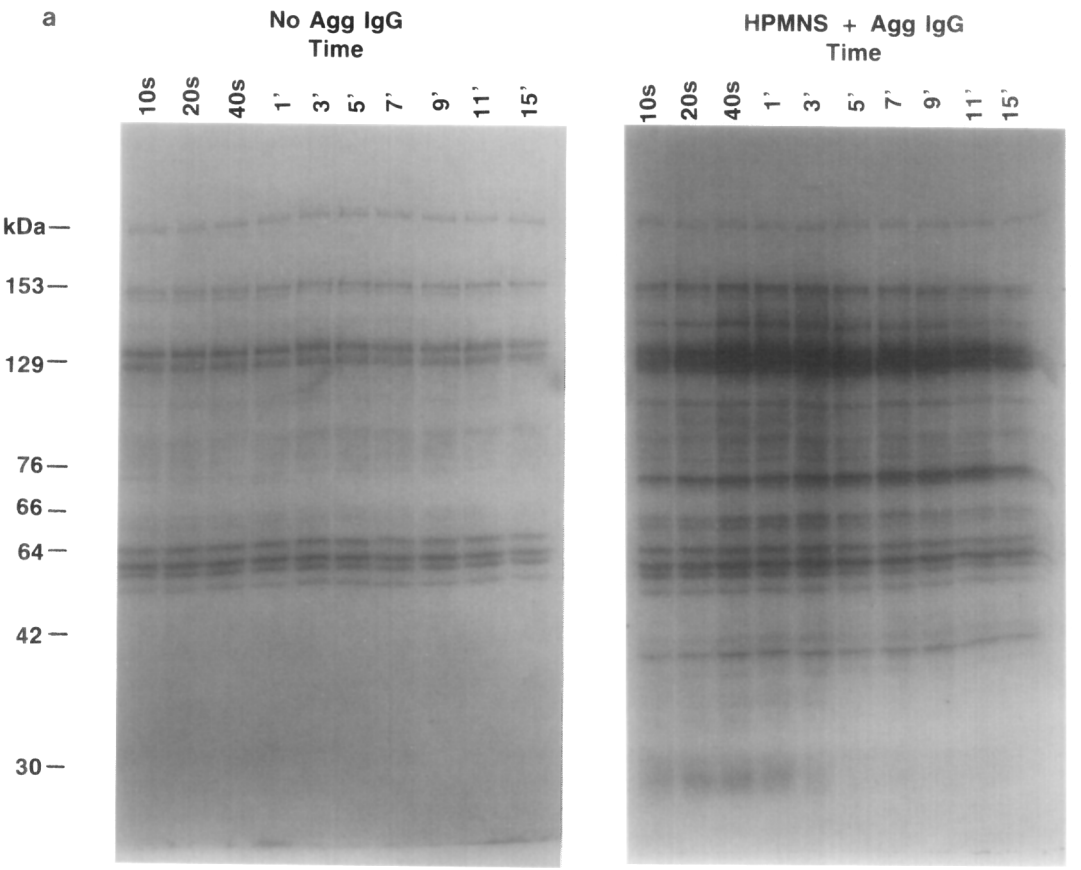
Figure 3. **FcγR crosslinking stimulates an increase in the phosphotyrosine content of multiple substrates in the human neutrophil.** Human neutrophils (HPMNS), prepared as described in the Methods section, were incubated in the absence or presence of 100 μg aggregated IgG/ml for the following times, 10, 20 and 40 sec and 1, 3, 5, 7, 9, 11 and 15 min. a. Phosphotyrosine-containing proteins were detected as described above. The phosphorylation of proteins of Mr 153, 129, 76, 66, 64, 42 and 30 kDa was stimulated following the addition of aggregated IgG. b. The autoradiogram was analyzed as described above and the kinetics of the phosphorylation of the 76, 66 and 42 kDa bands are presented in graphic form.


a
No Agg IgG
Time
10s
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40s
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5'
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7'
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kDa
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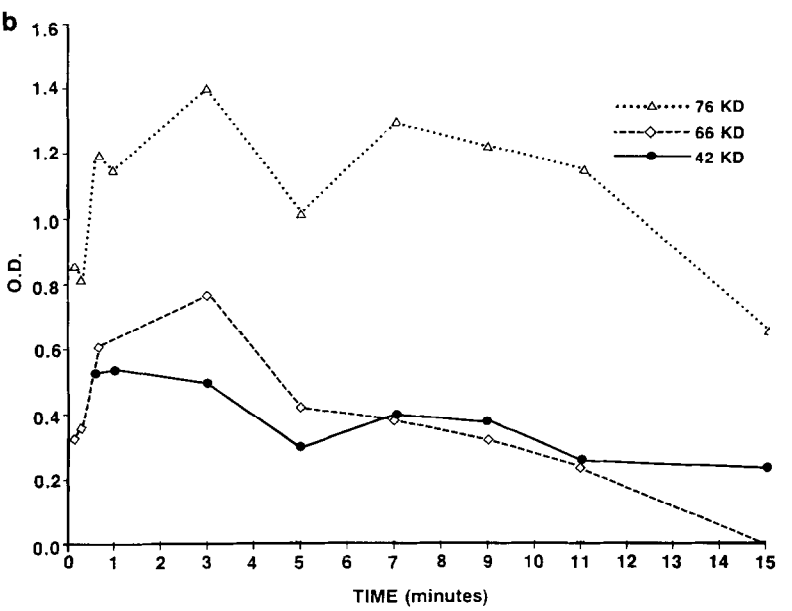
129
76
66
64
42
30


b
O.D.
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TIME (minutes)
76 KD
66 KD
42 KD

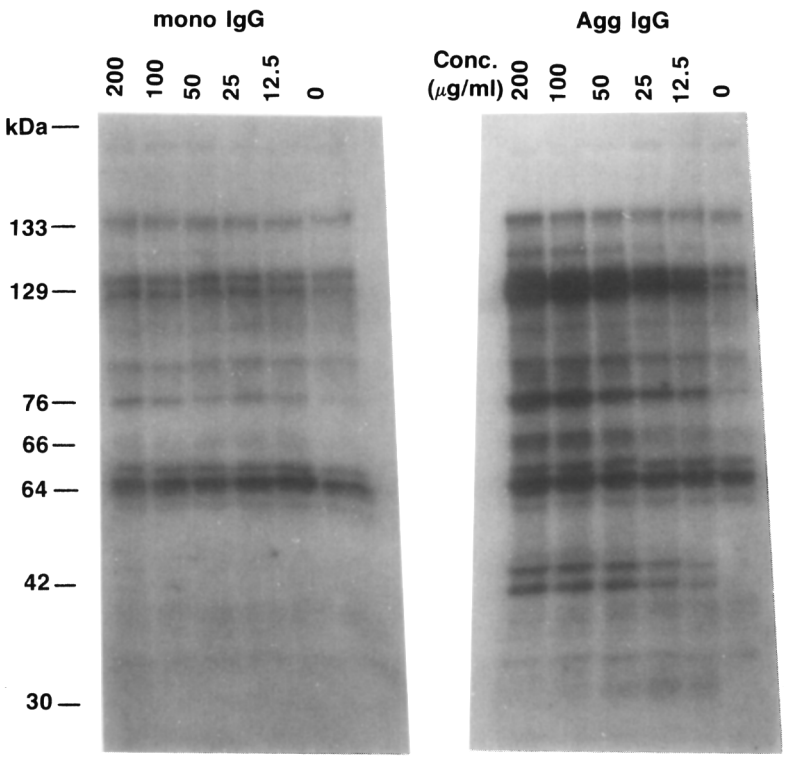



Figure 4. **Tyrosine phosphorylation in the human neutrophil is dependent on the concentration of aggregated IgG.** Human neutrophils (HPMNS), prepared as described above, were incubated for 3 min in the presence of 12.5, 25, 50, 100 and 200 μg/ml heat-aggregated or monomeric IgG. Samples were prepared for phosphotyrosine western blots as described previously. Phosphorylation of the 76 kDa band was half-maximal at approximately 50 μg aggregated IgG/ml.

receptor-crosslinking in the human neutrophil mediates tyrosine phosphorylation by a mechanism that cannot be fully reproduced simply by phospholipase C activation. Finally, tyrosine phosphorylation in response to the cytokine, GM-CSF, was examined (Figure 5) and shown to be distinct from that observed with other stimuli. Treatment with GM-CSF led to an increase in the phosphorylation of a high molecular weight protein, approximately 145 kDa.

## Discussion

In the present study we demonstrate that appropriate crosslinking of Fc receptors on the rat basophilic leukemia cell line, RBL-2H3, and on human neutrophils stimulates the phosphorylation of a number of proteins on tyrosine residues. Similar RBL-2H3 results were published by Benhamou *et al.* (14) while this work was in progress. Responses in both cells were extremely rapid, with a significant response observed as early as 10 sec following the addition of cross-linking agent. These results suggest that tyrosine phosphorylation may regulate the earliest post-receptor events in receptor-mediated exocytosis. Similar studies in T lymphocytes demonstrate that receptor-mediated tyrosine phosphorylation precedes phospholipase C activation (15).

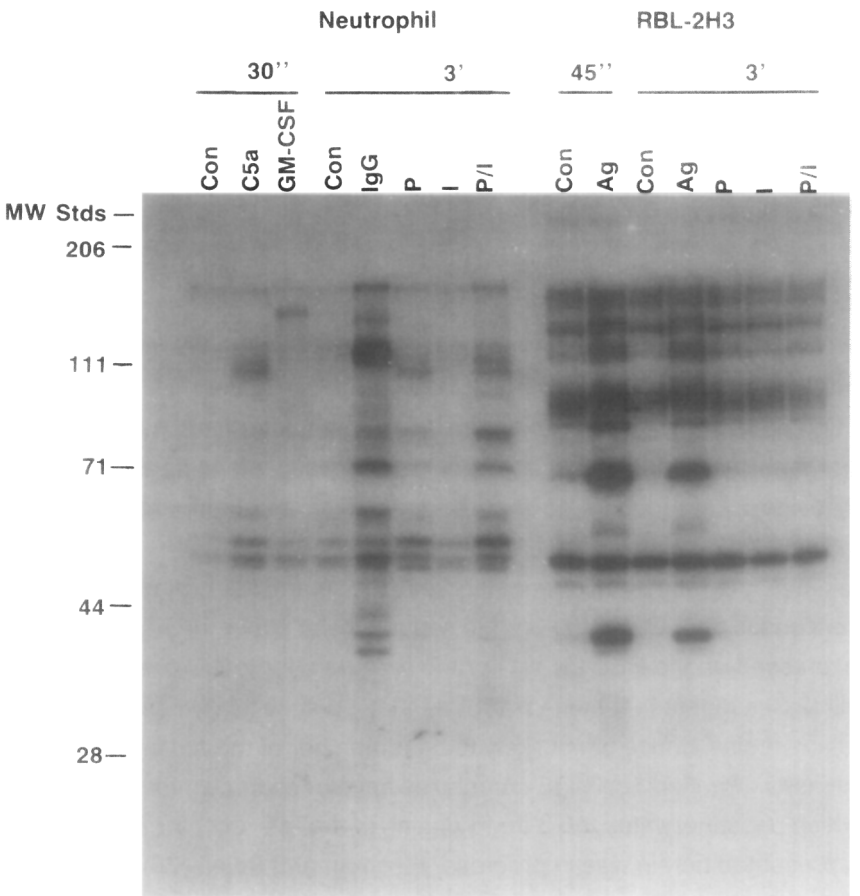



Figure 5. **Stimulation of tyrosine phosphorylation in the neutrophil by GM-CSF and C5a and in the human neutrophil and the RBL-2H3 cell by PMA/Ionomycin.** Human neutrophils (HPMNS), prepared as described above, were treated as follows: 30 sec with either 300 nM C5a or 100 ng GM-CSF/ml or 3 min with 100 µg aggregated IgG/ml, 100 ng PMA/ml, 250 ng ionomycin/ml and both PMA and ionomycin (P/I). RBL-2H3 cells were prepared as described previously and treated for 45 sec and 3 min with 3 µg DNP-HSA/ml or for 3 min with 100 ng PMA/ml, 250 ng ionomycin (I)/ml or both PMA and ionomycin (P/I). Phosphotyrosine blotting was carried out as described previously.

The pattern of phosphorylated proteins (their number and molecular weights) were similar in RBL-2H3 cells of rat basophilic origin and human neutrophils suggesting that Fc receptor signalling and subsequent activation steps may involve secretion-associated proteins conserved across both cell type and species. Several components of the phagocytic NADPH oxidase complex, p47 and p67, contain SH3 (src homology) domains, pinpointing them as potential substrates for an Fc receptor-regulated tyrosine kinase (16, 17).

Some differences were observed when comparing phosphorylation events in the two cell types. Antigen-triggered tyrosine phosphorylation in RBL-2H3 cells was more rapid than that seen in immune complex-treated neutrophils. RBL-2H3 cells and neutrophils responded differently to treatment with PMA, a potent activator of protein kinase C, and

ionomycin, an ionophore used to elevate levels of intracellular $Ca^{2+}$ (5) . These agents had no effect on the tyrosine phosphorylation patterns observed in the RBL-2H3 cell line, suggesting that phospholipase C activation does not mimic antigen-induced phosphorylation. These data agree with the recently published results of Benhamou et al. (14). In contrast, both PMA and ionomycin affected tyrosine phosphorylation in the human neutrophil, although neither alone nor in combination did they fully reproduce the response seen following stimulation with heat-aggregated IgG. Characteristic patterns of tyrosine phosphorylation, distinct from those observed with aggregated IgG, PMA, or ionomycin, were observed in neutrophils following stimulation with the complement fragment (chemotactic factor), C5a, or the cytokine, GM-CSF, suggesting that tyrosine phosphorylation is not an uncommon regulatory mechanism in these cells. Consistent with these findings is the recent observation that translocation of the *fgr* protein-tyrosine kinase occurs as a consequence of neutrophil activation with formyl-Met-Leu-Phe. (18).

The rapidity of the response to receptor crosslinking and the lack of any tyrosine kinase concensus domains in the protein sequences of either the high-affinity receptor for IgE expressed on the RBL-2H3 (1) or the receptors for IgG expressed on the human neutrophil (2,3) suggest that these receptors may be specifically associated with an intracellular tyrosine kinase, most likely to be a member of the *src* family of tyrosine protein kinases. Purified FcεR-IgE complexes have been shown to contain a tyrosine kinase which phosphorylates the β and γ chains of the IgE receptor *in vitro* (19). While the work presented herein was in progress, Elseman and Bolen (20) published preliminary data supporting this hypothesis by showing that cross-linking of FcεR activates *src* family members, *lyn* in the RBL cell and *yes* in the mouse mast cell line PT-18. An analogous situation exists in human lymphocytes, where the tyrosine protein kinase, *fyn*, appears to associate with a component of the T cell receptor complex (21) and *lyn* with the B cell antigen receptor (22).

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