Lateral mobility of FcγRIIa is reduced by protein kinase C activation

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Abstract The lateral mobility of membrane proteins can reflect the extent of various protein–protein interactions. Using the fluorescence recovery after photobleaching technique, we have studied the lateral mobility of human FcγRIIa and some FcγRIIa mutants expressed in either P388D1 cells, a mouse macrophage-like cell line, or in Chinese hamster ovary (CHO) cells [1]. After treatment with phorbol myristate acetate (PMA), only the FcγRIIa molecules capable of mediating rapid endocytosis of immune complexes exhibited a reduced lateral diffusion coefficient with respect to untreated controls. Wild type FcγRIIa expressed in CHO cells, and nonfunctional FcγRIIa mutants expressed in P388D1 cells did not show any differences upon PMA treatment. This finding suggests that protein kinase C activation evokes additional protein–protein interactions with the cytoplasmic domain of functional FcγRIIa, which reduced receptor lateral mobility. The identity of these putative interacting proteins and the nature of the interactions remain to be elucidated.

Key words: Fc receptor; Protein kinase C; Fluorescence recovery after photobleaching; Lateral mobility

1 Introduction

Human FcγRIIa (CD32) is widely expressed on hematopoietic cells, including macrophages, neutrophils, and platelets, and, like other multichain immune response receptors, is activated after cross-linking by immune complexes or antigens [2]. FcγRIIa participates in the endocytic clearance of soluble immune complexes, phagocytosis of large particles, and the oxidative burst. The receptor has a modified immune receptor tyrosine activation motif (ITAM), which consists of two YXXL motifs with the tyrosines separated by 15 amino acids, instead of the 10–11 residues found in the consensus sequence for other members of the family. A functional study of a set of mutants in the cytoplasmic domain of FcγRIIa has revealed that the COOH-terminal YXXL motif is required for $[Ca^{2+}]_i$ flux and phagocytosis following stimulation, but is not required for endocytosis of immune complexes. Several mutations in the NH_2-YXXL motif (Tyr^{252} to Phe, and Leu^{255} to Glu) result in a more severely crippled phenotype, which will endocytose only very extensively cross-linked immune complexes. Signal transduction by FcγRIIa in macrophages is associated with the activation of Src kinases, the tyrosine kinase $p72^{syk}$, and tyrosine phosphorylation of numerous cellular substrates. These results suggest that various cytoplasmic molecules interact with the cytoplasmic domain of the receptor following its activation.

There is evidence that protein kinase C plays a role in FcγRIIa-mediated responses, because phorbol esters increase phagocytosis in neutrophils and macrophages [3–8]. Furthermore, Zheleznyak and Brown have shown that the PKC activation is a required early step for FcR mediated phagocytosis by human monocytes [9]. Incubation of monocytes with IgG-opsonized targets increased membrane-associated PKC which precedes the ingestion. Phorbol myristate acetate inhibits the $[Ca^{2+}]_i$ flux due to FcγRIIa activation and also inhibits the tyrosine phosphorylation of Shc, an SH2 adapter protein [10]. These results suggest that one or more PKC isozymes interacts either with FcγRIIa or with other cytosolic factors in the signaling complex.

In this work, we devised an in situ means to test the proposition that FcγRIIa interacts with additional cytoplasmic or cytoskeletal components after phorbol esters induce phosphorylation of cellular components. This test is based upon the fact that the lateral mobility of a given membrane protein will reflect the extent of its interactions with other membrane proteins as well as with proteins in the cytoplasm and pericellular matrix [11]. Using the fluorescence recovery after photobleaching (FRAP) technique to measure changes in receptor lateral mobility associated with receptor activation, we can therefore infer changes in protein–protein interactions upon FcγRIIa activation. Furthermore, by comparing the lateral mobility of wild type FcγRIIa and cytoplasmic domain mutants, we can largely confine our attention to the interactions of cytoplasmic domain FcγRIIa with cytosolic proteins.

However, because FcγRs are activated by cross-linking of receptors induced by multivalent immune complexes or, experimentally, by cross-linking anti-FcγR antibodies, lateral mobility measurements under normal receptor activation conditions are not feasible. To circumvent this difficulty, we employ PMA to intervene in the putative signaling pathway downstream from the cross-linking step.

FcγRIIa and mutants with either cytoplasmic domain truncations or amino acid substitutions were transfected into P388D1 and Chinese hamster ovary (CHO) cells ([1], Lin and Unkeless, unpublished). We then compared the lateral mobility

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Abbreviations: FRAP, fluorescence recovery after photobleaching; PBS, PBS(+), phosphate-buffered saline with Ca^{2+} and Mg^{2+}; PMA, phorbol myristate acetate; FcγR, receptor for the Fc domain of immunoglobulin G; wt, wild type.

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of wild type FcγRIIa in the P388D1 and CHO lines to that of FcγRIIa mutants expressed in P388D1 cells. Mutant FcγRIIa include Δ264 competent to endocytose immune complexes but not to phagocytose EIgG or flux $[Ca^{2+}]_i$ (Δ264), and mutations (Y252F, Δ233, and Δ207) that cripple or inactivate endocytosis as well as phagocytosis and $[Ca^{2+}]_i$ flux. After PMA treatment, only those FcγRIIa molecules capable of mediating rapid endocytosis of immune complexes exhibited reduced lateral mobility suggesting that, indeed, PKC activation did induce additional protein–protein interactions with the cytoplasmic domain of functional receptor.

2. Materials and methods

2.1. Constructs of human FcγRIIa cDNAs and transfected cell lines

Constructs of wild type human FcγRIIa and mutants and transfection protocols for macrophages have been described in detail [1]. The Y252L mutation was made by oligonucleotide-directed mutagenesis [12]. Stable transfectants of the wild type huFcγRIIa and its mutant forms have been expressed in a mouse macrophage-like cell line, P388D1 cells, and in CHO cells.

All the permanently transfected cells were grown in DMEM-H medium supplemented with 5% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 0.2 mg/ml G-418 (Gibco, Grand Island, NY). P388D1 cells were plated on 12 × 12-mm coverslip in 35-mm dishes about 0.5–1 h before the microscopic studies, while the CHO cells were plated for 24 h before use.

2.2. Antibody labels for FRAP studies

FITC-conjugated monoclonal antibody IV.3 IgG was purchased from Medarex (Hanover, NH). The plated cells were labeled with 30 μg/ml antibody in PBS(+) at room temperature for 10 min. When murine C3H 10T1/2 cells were incubated with the IV.3 antibody, the level of staining was negligible.

2.3. Protein kinase C activation and inhibition

50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO) in PBS(+) was used for activating PKC. The PMA was added immediately before and remained throughout the FRAP experiment. 50 ng/ml of the non-active phorbol ester 4α-phorbol 12,13-didecanoate (4αPDD) (Sigma, St. Louis, MO) was used as a control. 100 nM of Calphostin C (Calbiochem, CA) in PBS(+) was used for inhibiting PKC activity. The Calphostin C was added with labeling antibody and remained throughout the FRAP experiment.

2.4. Fluorescence recovery after photobleaching (FRAP)

FRAP measurements were made at room temperature employing a 40× oil objective (n.a., 1.3) to focus the laser beam to a diameter of approximately 2.0 μm. A pulse mode recording and low monitoring power were employed to minimize the fading during the experiment [13]. The 488 nm line of a Spectra-Physics 164–07 argon ion laser (Mountain View, CA) was used. Spots on the cell were bleached for 20 ms using a laser power of about 50 mW. The recovery phase was interrogated with beam powers of about 5 μW. A single curve was recorded per cell, and each coverslip was used for less than one hour after antibody labeling.

2.5. Analysis of PKC-δ

P388D1 cells transfected with wt FcγRIIa were maximally stimulated by ligating the receptor to a glass substrate. Proteins were covalently coupled to glass as described [14]. Glass petri dishes were acid washed with 20% H_2SO_4, neutralized with 0.1 N NaOH, rinsed with water, dried, and derivatized with 3-aminopropyltriethoxysilane (Sigma) for 4 min at room temperature (rt). After rinsing with phosphate-buffered saline (PBS), the dishes or coverslips were incubated with 0.25% glutaraldehyde (Sigma) for 30 min followed by incubation with 5 μg/ml of F(ab')2 anti-mouse IgG (Jackson Immunoresearch) in PBS for 1 h at rt. The dishes were rinsed with PBS, and cells (1×10^6/6 cm dish) suspended in medium containing 2% FCS were allowed to adhere for 30 min at 37°C. Finally, mAb IV.3 Fab was added to a final concentration of 1 μg/ml and incubated for 30 min at 37°C.

Control cells or cells treated with PMA (100 ng/ml, 30 min at 37°C) were activated as described, lysed in 0.5% NP-40, and immunoprecipitated with anti-phosphotyrosine mAb 4G10. Immunoprecipitates were collected with protein A-agarose (Pierce, Rockford, IL). Lysates and immunoprecipitates were subjected to SDS-PAGE, electroeluted onto nitrocellulose, and immunoblotted with either mAb 4G10, or with a specific rabbit anti-PKCδ IgG, which is directed against a peptide epitope (657–673) of rat PKCδ and not crossreactive with α, βI, βII, γ, ε or ε or η isozymes (Santa Cruz Biotechnology, Santa Cruz, CA). Bound rabbit IgG or mouse IgG was detected using alkaline phosphatase-conjugated secondary antibody (Jackson Immunoresearch) and detected with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt (Pierce) as substrate.

3. Results and discussion

3.1. Lateral mobility in resting state

The huFcγRIIa mutants examined for lateral mobility covered a wide range of cytoplasmic domain modifications. The mutants differ in their signaling capabilities. The non-functional truncation mutants Δ207 and Δ233 have cytoplasmic domains of two and twenty-eight amino acids. Δ264, which has a cytoplasmic domain of fifty-nine amino acids, has lost the ability to mediate phagocytosis of erythrocytes coated with IV.3 Fab (E-IV.3 Fab) and to stimulate a $[Ca^{2+}]_i$ flux, but endocytoses immune complexes normally. The point mutant Y252L has a Tyr-to-Leu substitution at position 252, which results in a failure to mediate phagocytosis or $[Ca^{2+}]_i$ flux. This mutant will only mediate endocytosis of extensively cross-linked complexes compared to wild type receptor (Lin and Unkeless, unpublished results).

The wild type huFcγRIIa is functional in P388D1 cells but not in CHO cells [1], and the pattern of tyrosine phosphorylation following receptor cross-linking in P388D1 cells is comparable to that seen in the human monocytic cell line U937. The $P388D_1$ cells thus appear to possess the cytoplasmic factors required for huFcγRIIa mediated responses. By comparing the lateral mobility of FcγRIIa and its various mutants, we hoped to be able to monitor the interactions between these factors and the FcγRIIa cytoplasmic domain.

The lateral mobility data in terms of diffusion coefficient (D) and mobile fraction for wild type huFcγRIIa and its mutants are compared in Table 1. The diffusion coefficients for all the constructs are at the high end of the range for membrane spanning proteins [15]. The implication is that there is little interaction between FcγRIIa cytoplasmic domain and cytosolic factor(s) in the resting state. Progressive truncation of the cytoplasmic domain to twenty-eight and two amino acids (Δ233 and Δ207) resulted in a progressive depression in D, compared to wt FcγRIIa expressed in either CHO or P388D1 cells. One possible interpretation is that the intact cytoplasmic domain inhibits either transmembrane or extracellular domain interactions, and thus the FcγRIIa cytoplasmic domain truncations result in a more highly aggregated receptor and hence lower mobility than the native FcγRIIa.

3.2. Lateral mobility in PMA-treated cells

FcγRs are activated by cross-linking of receptors induced by multivalent immune complexes or, experimentally, by cross-linking anti-FcγR antibodies. Under these circumstances lateral mobility measurements are not feasible. In order to intervene in the signal transduction pathway downstream from the cross-linking step, receptor lateral mobility was measured after

Table 1
Lateral mobility of Fc receptors

Constructs	Endocytic competence[a]	50 ng/ml PMA	DC ($\times 10^{-10}$ cm^2/s)	Mobile fraction (%)	Number of measurements
Wild type in P388D1	+++	–	13.9 ± 2.1	53.6 ± 4.1	56
		+	6.4 ± 0.7*	53.1 ± 3.8	91
		+(+Cal-C)[b]	14.9 ± 2.0**	70.8 ± 4.8	42
Δ264 in P388D1	++	–	14.4 ± 2.2	51.3 ± 5.1	40
		+	7.2 ± 1.0*	48.0 ± 4.9	43
Y252L in P388D1	+	–	7.1 ± 0.7	71.0 ± 4.0	20
		+	7.4 ± 0.8**	67.0 ± 3.0	20
Δ233 in P388D1	–	–	9.8 ± 1.0	65.8 ± 3.5	16
		+	10.4 ± 1.9**	65.6 ± 4.6	17
Δ207 in P388D1	–	–	8.5 ± 1.1	61.8 ± 4.4	28
		+	8.3 ± 1.0**	53.9 ± 4.0	23
Wild type in CHO	–	–	15.6 ± 1.5	78.8 ± 1.7	25
		+	16.7 ± 1.3**	79.9 ± 1.9	25

*Student's t-test, $P < 0.01$, mean ± S.E.M.
**Not significant with reference to cells not treated with PMA.
[a]+++ = phagocytic competent; ++ = endocytose immune complex; + = endocytose large immune complex; –, incompetent.
[b]100 nM of Calphostin added (see section 2).

incubation with PMA. This strategy was adopted because there is evidence to suggest that PKC participates in FcγR signaling. Diacylglycerol has been reported to potentiate macrophage FcγR-mediated phagocytosis [4]. The activity of protein kinase C was elevated after EIgG stimulation [8], and immune complex endocytosis was inhibited by PKC inhibitors [1,5,9]. Furthermore, preceding ingestion of IgG-opsonized targets by monocytes, there is increased membrane-associated PKC [9]. PMA also blunted the $[Ca^{2+}]_i$ flux triggered by FcγRIIa ligation, and inhibited the tyrosine phosphorylation of Shc [10]. It was expected that the additional interactions evoked by PKC activation would be reflected in reductions of huFcγRIIa lateral mobility, because the lateral mobility of membrane proteins can be altered by binding to other protein(s). For example, on the external surface of the plasma membrane, the diffusion coefficient of Thy-1 is reduced by about 70% by binding to anti-Thy-1 IgG [13].

The effect of PMA treatment on mobility of wild type FcγRIIa and its mutants is given in absolute terms in Table 1 and relative terms in Table 2. It is striking that the lateral diffusion of FcγRIIa was reduced by 50% following PMA treatment only in those cell lines capable of endocytosis of immune complexes (wt FcγRIIa and the Δ264 truncation transfected into P388D1 cells). The Δ264 mutation does not support either $[Ca^{2+}]i$ flux or phagocytosis [1]. This diffusion coefficient reduction, mediated by PMA treatment, can be blocked by adding Calphostin C, a PKC inhibitor. The inactive phorbol ester, 4αPDD, had no effect, ruling out a nonspecific effect of lipid binding. The diffusion coefficient of Y252L, a severely crippled FcγRIIa mutant that endocytoses immune complexes only at high cross-linker concentration, was not affected by PMA, nor were the nonfunctional deletion mutants Δ233 and Δ207. Finally, the diffusion coefficient of wt FcγRIIa transfected into CHO cells, in which the receptor does not support phagocytosis, immune complex endocytosis, or $[Ca^{2+}]i$ flux was unaffected by PMA.

These observations provide the first in situ evidence suggesting the PKC activation evokes interaction(s) between the FcγRIIa cytoplasmic domain and cytoplasmic protein(s). The PKC-δ isoform is highly expressed in myeloid cells [16] and is tyrosine-phosphorylated upon activation with PMA [17]. Indeed, following incubation of P388D1, cells with PMA, tyrosine phosphorylation of an 74,000 M_r protein (the size of PKC-δ) was induced (Fig. 1). Furthermore, immunoblotting of an anti-phosphotyrosine immunoprecipitate with an anti-PKC-δ antibody revealed the presence of tyrosine phosphorylated PKC-δ in both P388D1, cells stimulated by FcγRIIa ligation, and treated with PMA (Fig. 1). In vitro, Src is reported to phosphorylate PKC-δ, but acts only on the activated form of the enzyme, following PMA activation [18]. And, PKC-δ is reported to associate with FcεRIβ chain and to phosphorylate the γ-chain on threonine in vivo [19]. It is possible that other PKC isoforms are also activated by FcγRIIa cross-linking. Thus it is likely that activation of FcγRIIa activates one or more PKC isoforms, which would in turn prime receptors for endocytosis of immune complexes, even on those receptors that

Table 2
Effects of PKC activation and inhibition on Fc-R lateral mobility

Receptor	Agent added[a]	Relative mobility[b] ($D_{agent}/D_{control}$)
WT	–	1.0
WT	PMA	0.46 ± 0.09
WT	4αPDD	0.97 ± 0.08
WT	PMA + Calphostin	1.07 ± 0.20
WT (in CHO)	PMA	1.07 ± 0.13
Y252L	PMA	1.04 ± 0.14
Δ264	PMA	0.50 ± 0.10
Δ233	PMA	1.06 ± 0.22
Δ207	PMA	0.98 ± 0.16

[a]See section 2 for details.
[b]Formula for standard error: $c = a/b$, $(\Delta c)^2 = (\Delta a/b)^2 + (a\Delta b/b^2)^2$.

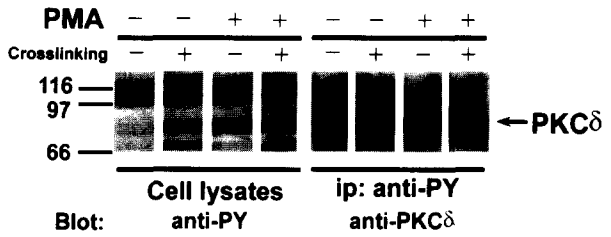

Fig. 1. Tyrosine phosphorylation of PKC-δ. P388D1 cells transfected with FcγRIIa were plated on goat anti-mouse IgG $F(ab')_2$ coated glass dishes in either presence or absence of 100 ng/ml PMA, and the receptors were then activated by adding IV.3 Fab (1 μg/ml) for 30 min at 37°C. Cells were then scraped and lysed in 0.5% NP-40 lysis buffer. The cell lysates were blotted with mAb 4G10, and the 4G10 immunoprecipitates were probed with a polyclonal anti-PKC-δ IgG.

may not have bound immune complexes yet. This 'priming' may involve additional interactions with cytoplasmic factors that serve to reduce the diffusion coefficient. The identity of the putative interacting proteins and the nature of the interactions remain to be elucidated.

The ability to detect membrane protein–cytoplasmic factor interactions by reductions in the lateral mobility of the membrane protein may offer a general method for studying interactions of this type, in situ. A generic cytoplasmic domain–interaction detector could be envisioned with the capability of detecting changes in the lateral mobility of the detector protein. Such a detector molecule could be constructed as a chimera produced by fusing the ectodomain and transmembrane domain from a protein with high diffusion coefficient with the cytoplasmic domain of a protein under investigation. This detector molecule could be expressed in a cell type which does not normally express the wild type ectodomain of the reporter molecule. Detector proteins exhibiting a large D would allow a greater change in D to be detected upon additional interactions with cytosolic factors.

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