Modulation of FcγRI (CD64) Ligand Binding by Blocking Peptides of Periplakin*

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FcγRI requires both the intracellular domain of the α-chain and associated leukocyte Fc receptor (FcR) γ-chains for its biological function. We recently found the C terminus of periplakin to selectively interact with the cytoplasmic domain of the FcγRI α-chain. It thereby enhances the capacity of FcγRI to bind, internalize, and present antigens on MHC class II. Here, we characterized the domains involved in FcγRI-periplakin interaction using truncated and alanine-substituted FcγRI mutants and randomly mutagenized periplakin. This allowed us to design TAT peptides that selectively interfere with endogenous FcγRI-periplakin interactions. The addition of these peptides to FcγRI-expressing cells modulated FcγRI ligand binding, as assessed by erythrocyte-antibody-rosetting. These data support a dominant-negative role of C-terminal periplakin for FcγRI biological activity and implicate periplakin as a novel regulator of FcγRI in immune cells.

Leukocyte Fc receptors (FcRs)1 are membrane-expressed glycoproteins that bind the constant fragments (Fc) of immunoglobulins (1, 2). FcR cross-linking can trigger a variety of cellular responses including phagocytosis, antigen presentation, and cytokine production. Most FcR exist as multisubunit complexes containing a unique ligand binding α-chain and promiscuous FcR γ-chains that are indispensable for tyrosine-based signals (3–6).

FcγRI (CD64, FcγRIa1) is unique among multisubunit FcR due to a high affinity binding to human IgG, its limited myeloid cell distribution, and a relatively large intracellular domain (7, 8). Products of related genes include FcγRII and FcγRIc isoforms, but these specify low affinity IgG receptors if functionally expressed at all (9–12). Besides a role in antigen clearance, FcγRI (a1) can potently enhance MHC class I and II antigen presentation in vitro and in vivo (13–16). These properties make FcγRI a candidate target for immunotherapy, and concepts are being developed to modulate immune responses by FcγRI-directed agents (16–19). The potential of such therapeutic approaches supports further work to enlarge our knowledge of FcγRI biology.

The Fcγ γ-chain has been studied in great detail and is critically important for FcγRI function; it stabilizes FcγRI α-chain surface expression in vivo (20) and mediates several key functions that require ITAM signaling motifs (21–24). In addition, recent data show that the cytosolic domain of the FcγRI α-chain (FcγRI-CY) could transduce signals leading to cellular effector functions (25, 26). MHC class II antigen presentation assays using IIA1.6 cells co-expressing truncated FcγRI-CY mutants and “signaling-dead” FcγRI-γ chains indicated a motif for antigen presentation in the membrane proximal ~34 aa of FcγRI-CY (25). Deletion of FcγRI-CY in the presence of functional FcγRI-γ chain lowered the kinetics of endocytosis and phagocytosis and abolished interleukin-6 production (26). FcγRI-CY signaling likely involves (de)phosphorylation of its serine residues and may include other mechanisms of post-translational modification (27). Thus far no protein effectors have been described that control FcγRI function by FcγRI-CY interaction. Filamin A (ABP-280) has been shown to bind FcγRI-CY, but no functional consequences are known for this interaction (28).

We recently found periplakin to selectively bind FcγRI-CY and to modulate ligand binding, receptor modulation, and antigen presentation via FcγRI.2 Periplakin represents a 195-kDa protein implicated in cornified envelope assembly and structural stability of epithelia (29–32). Like other members of the plakin family (for review, see Refs. 33 and 34) periplakin associates with the actin and intermediate filament cytoskeleton (32, 35, 36). Periplakin has recently been suggested to be involved in signaling of protein kinase B (37) and G-proteins located downstream of the µ-opioid receptor in neurons (38).

In the present study we characterized the molecular interaction between FcγRI and periplakin. We determined the periplakin binding domain of FcγRI and vice versa by progressive truncations and alanine-scanning mutagenesis of FcγRI-CY and random mutagenesis of periplakin. Peptides of these binding domains and the membrane-translocating TAT sequence (39) were designed to interfere with FcγRI-periplakin interactions in IIA1.6 cells. FcγRI ligand binding was studied in EA-rosette assays.

EXPERIMENTAL PROCEDURES

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1 The abbreviations used are: FcR, Fc receptor; Fc, constant fragments; aa, amino acids; CY, cytosolic tail; ITAM, immunoreceptor tyrosine-based activation motif; mAb, monoclonal antibody; PPL, periplakin; WT, wild type; Bicine, N,N-bis(2-hydroxyethyl)glycine; MHC, major histocompatibility complex; FITC, fluorescein isothiocyanate; EA, erythrocyte-antibody.

2 Beekman, J. M., Bakema, J. E., van de Winkel, J. G. J., and Leusen, J. H. W. (2004) Proc. Natl. Acad. Sci. U.S.A., in press.
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...cloned into pcDNA3.1 HISABC (Invitrogen). The murine FcR...

Z-buffer (60 mM Na2HPO4, 60 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4; WT and truncated FcR...sentative samples are shown, and yellow colors indicate co-localization. Experiments were performed at least three times, yielding essentially similar results.

variants (see Fig. 1) were generated by PCR and EcoRI/SalI-cloned into pGBT9 (Clontech, Palo Alto, CA). Alanine replacement of FcγRI residues 311–325 was achieved by PCR-based cloning techniques (primer mutations and overlap extension PCR) and EcoRI/SalI insertion into pGBT9. pGAD-GH (Clontech) contained C-terminal periplakin (GenBank™ accession number AF001691) clone 2.2 (bp 4620–5361). Glycine replacement of aspartic acid 1694 (D1694G) of FcγRI was stained directly by CD64 monoclonal antibody (mAb) 10.1-FITC (SeroTec, Oxford, UK) in blocking buffer. Periplakin was stained by incubation with polyclonal rabbit serum 5117 (a kind gift of Dr. B. Burgering, Laboratory of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center Utrecht (37)), rinsing in phosphate-buffered saline, and subsequent incubation with goat-α-rabbit-CY3 (Jackson Laboratories, West Grove, PA). Double staining of mIgG1-FITC (Dako, Glostrup, Denmark) and pre-immune serum 5117 in combination with goat-α-rabbit-CY3 served as negative controls as well as staining on mock-transfected cells. The slides were rinsed extensively, mounted in Mowiol containing 2.5% DABCO (1,4-diazabicyclo(2.2.2)octane; Sigma), and examined with a 63× planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser microscope (Leica).

Random Mutagenesis of C-terminal Periplakin—pGAD-GH clone 2.2 served as a PCR template in the presence of dITP and limiting amounts of dATP or dTTP to generate a pool of randomly mutated periplakin PCR products. This method was adapted from a protocol described by Spee et al. (43). Specific PCR characteristics were: 10 ng of pGAD-GH clone 2.2, 10 pmol per primer (pGAD-GH2, 5'-agatctagactag-3', and pGAD-GH3, 5'-gaattgtaattgacagac-3'), 2 units of AmpliTaq Gold, 1× Gold buffer, 8 mM MgCl2, 30 μM dATP, 30 or 200 μM dTTP, 200 μM DCTP, dGTP, and dTTP in a final volume of 50 μL. The PCR program consisted of an initial 5-min incubation at 95 °C and 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 4 min at 72 °C. PCR products were purified, and 100 ng of PCR product with 500 ng of Ecoil/Apal-restricted, gel-purified pGAD-GH and 2 μg of pGBT9-FcγRI-CY was transformed by 1 μl sorbitol, 10 mM Bicine, 3% ethylene glycol in yeast cells. Yeast cells were plated on complete supplement mixture medium without leucine and tryptophan to select for functional plasmids. After 3 days,
colonies were lifted and tested for loss of interaction by the absence of β-galactosidase activity. Plasmids were prepared from β-galactosidase-negative colonies and sequenced. Sequences were aligned using BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html).

EA-rosetting—Human erythrocytes were prepared by Ficoll/Hypaque density centrifugation, stored in sterile Alsever at 4 °C, and used within 2 weeks. Erythrocytes were fluorescently labeled using the PKit26 fluorescent cell linker kit (Sigma) according to the manufacturer's protocol and opsonized by hybridoma supernatant containing mlgG2a anti-human glycosphorin-A1 at 1 h at 2 × 10⁹ erythrocytes/ml at 4 °C (44). Erythrocytes were washed twice with Hepes-buffered RPMI 1640 medium and analyzed by flow cytometry. Cells and free erythrocytes were distinguished by their scatter patterns and autofluorescence in the FL1 channel. The percentage of cells that were FL1-2-positive was expressed as percentage of EA-rossettes. FcγRI surface expression was measured on a FACScalibur flow cytometry system (BD Biosciences) using the F(ab')² fragment of CD64 mAb H22 (Ref. 45; a kind gift of Dr. T. Keler, Medarex, Annandale, NJ) and goat F(ab')² anti-human k-light chain-FITC (Southern Biotech, Birmingham, AL).

TAT Peptides—Fusion peptides of the protein transduction domain of TAT (39, 46) and the binding domains of FcγRI for periplakin (TAT-FcγRI) or vice versa (TAT-PPL) were from Eurogentec (Herstal, Belgium). The sequence of TAT-FcγRI is YGRKKRRQRRRRGVTIR-KELLKKKWDEIE (29-mer), and that of TAT-PPL is YGRKKRRQRRGKLRSQECDWEEISVK (27-mer). Peptides were >95% pure and had standard N and C termini. Control TAT-peptide (YGRKKRRQRRR) consisted of the TAT sequence above and was a kind gift of Dr. P. Coffur (Dept. of Pulmonary Diseases, University Medical Center Utrecht). For transduction, cells were washed twice in Hepes-buffered RPMI 1640 medium containing 5 mM EDTA/EDTA buffer) to remove free extracellular calcium. Cells were incubated for 30 min in 10 μM TAT peptide at 37 °C in EDTA buffer at 5 × 10⁶ cells/ml. Cells were diluted in ice-cold EDTA buffer, washed at 4 °C in buffer without EDTA, and used in EA-rossette assays. Aliquots were analyzed by flow cytometry for FcγRI surface expression and cell viability by annexin V and propidium iodide staining (Roche Applied Science). Periplakin expression was assessed by Western blot using periplakin-recognizing rabbit serum 5117 after transient expression of full-length periplakin (construct kindly provided by Dr. F. Watt, Keratinocyte Laboratory, London Research Institute). For control experiments, TAT peptides were FITC-labeled (Molecular Probes, Leiden, The Netherlands), pulsed as described above, and analyzed by confocal microscopy. Surface IgG was stained by an anti-mouse IgG mAb CY-3 conjugated (Jackson).

RESULTS

Identification of the Periplakin Interaction Domain within FcγRI—To pinpoint the binding domain of FcγRI for periplakin, we tested progressive truncations of FcγRI-CY for interaction with C-terminal periplakin in yeast two-hybrid binding assays (Fig. 1). The minimal binding domain consisted of the membrane-proximal 17 amino acids of FcγRI-CY, as demonstrated by growth of yeast colonies on histidine-depleted media after transformation with FcγRIΔ327 and periplakin (residues 1372–1756). However, in β-galactosidase assays a slightly larger domain of FcγRI (22 residues, FcγRIΔ332) was required for periplakin interaction in yeast cells. Removal of the N-terminal valine (residue 311) or more (312–313) completely abrogated periplakin interaction. However, residues of the GAL4 DNA binding domain directly upstream of the N terminus of FcγRI-CY did not contribute to the interaction, as a stretch of six glycines in between the GAL4 DNA binding domain and FcγRI-CY left binding to periplakin intact (data not shown).

We next mapped the binding domain of FcγRI for periplakin in IIA1.6 transfectants. Stable transfectants of WT-FcγRI, FcγRIΔ342, FcγRIΔ332, and FcγRIΔ315 were co-transfected with C-terminal periplakin clone 3.4 and assessed after 48 h without further stimulation. WT-FcγRI and FcγRIΔ342 co-localized with C-terminal periplakin, showing both proteins to
be present at similar sites in cells. Although periplakin localized to the (sub)plasma membrane area, co-localization of FcγRI-332 and tail-less FcγRI (FcγRIΔ315) with C-terminal periplakin was abrogated, suggesting a loss of interaction.

To assess the relative contribution of residues within FcγRI-CY for periplakin interaction, alanine-scanning mutagenesis was applied to FcγRI-CY residues 311–329, and proteins were assessed for interaction with periplakin in yeast cells. Notably, substitutions were found that either abrogated or improved the interaction between FcγRI and periplakin (Fig. 2A). Alanine replacement of plasma membrane-proximal residues abrogated the interaction, except for Thr-312, whereas single substitutions of the stretch of positively charged residues (KRKKK) and Asp-324 apparently led to a better interaction, except for the last lysine. The increase in β-galactosidase activity may reflect a better-stabilized interaction with periplakin. By alanine substitution of the proximal part of FcγRI-CY, we targeted a sequence that is largely conserved from mouse to man (Fig. 2B). However, we did not detect any interaction between murine FcγRI-CY and human periplakin. The absence of Glu-316 and Trp-323 in the mouse sequence might contribute to the differences between these species.

Together, these results pointed to a periplakin binding domain of FcγRI-CY in juxtaposition to the plasma membrane. Periplakin binding to FcγRI-CY in yeast required a minimal motif of 17 residues within FcγRI-CY, but significant co-localization was only observed when 32 residues of FcγRI-CY were present. This discrepancy was observed consistently and indicated that both systems differ in FcγRI-CY and periplakin binding requirements. For further studies with blocking peptides, we utilized the membrane-proximal 17 residues of FcγRI as a blocking domain for FcγRI-periplakin interaction.

Identification of the Interaction Domain of Periplakin for FcγRI—Random mutagenesis of periplakin (schematically shown in Fig. 3A) was chosen as a tool to define the periplakin domain that interacts with FcγRI. We prepared a library of mutated clone 2.2 cDNAs by PCR amplification in the presence of dITP and screened for loss of interaction with FcγRI in yeast cells (Table I). A 2-fold increase of colonies that acquired a loss-of-interaction phenotype was achieved by the addition of dITP in the PCR. FcγRI-periplakin interaction was lost in 43% (68/158), and 57.3% (55/96) of yeast colonies by lowering dATP and dATP/dCTP concentrations, respectively. By (partially) sequencing plasmids from 41 colonies, a total of 65 missense mutations and 8 premature stop codons were identified. The most C-terminal-located stop codon was introduced after residue 1688, indicating that residues 1510–1688 of periplakin do not contain a full binding site for FcγRI. Only one of the missense mutants had a single amino acid substitution (aspatic acid at position 1694 into glycine (D1694G)) that resulted in a disturbed interaction (Fig. 3B). We confirmed this observation by site-directed mutagenesis of D1694G in the original periplakin 2.2 construct. Interactions were mitigated both in yeast cells (data not shown, n = 2) and transfected IIA1.6 cells (Fig. 3C). The D1694G-mutated clone 2.2 staining was more dominant in the cytoplasm and sometimes followed a filamentous pattern.

Six substitutions were located within the 15 residues adjacent to Asp-1694 of being located at the binding interface, as the tertiary structure of these domains is unknown. These data supported periplakin 1687-1701 to be part of the FcγRI binding domain of periplakin, and we hypothesized that a peptide with this sequence may block such interaction.

Flow Cytometric Analysis of EA-rosetting—Our previous work documented the ligand binding capacity of FcγRI to be increased by C-terminal periplakin transfection. Here, we developed a quick assay to measure FcγRI-ligand binding via the use of EA-rosetting by flow cytometry (i.e. the percentage of cells that bound mIgG2a-sensitized erythrocytes, upper right panel in Fig. 4B). Non-bound cells and FL2-labeled erythrocytes were found in the lower right and upper left quadrants, respectively. We observed 5% background binding to untransfected IIA1.6 cells or unsensitized erythrocytes (data not shown). A 2.5-fold increase in binding of mIgG2a-sensitized erythrocytes to FcγRI was observed upon co-expression of C-terminal periplakin in IIA1.6 cells (Fig. 4C). Binding appeared independent of small differences in surface levels of FcγRI (Fig. 4A) and FcγRI γ-chain signals. Similar differences were observed when EA-rosetting was scored by light microscopy (data not shown, n = 4).

Modulation of EA-rosetting by TAT-Periplakin—Peptides containing a TAT motif and the postulated binding domains of FcγRI and periplakin were designed to block intracellular FcγRI-periplakin interaction. FcγRI surface expression and periplakin levels were unaffected by the addition of TAT pep-
Random PCR mutagenesis of periplakin

Two screens were performed to define the FcγRI binding site within periplakin. Random PCR mutagenesis was achieved by including an artificial fifth nucleotide dITP in PCR in combination with lowered amounts (indicated by the down arrow) of dATP (screen 1) or dATP and dTTP (screen 2). "Control PCR" represents co-transfections of pGBT9 WT FcγRI-CY and linearized pGAD-GH with periplakin inserts generated by PCR without dITP. "Mutagenesis PCR" represents co-transfections of pGBT9 WT FcγRI-CY and linearized pGAD-GH with periplakin inserts generated by PCR with dITP. Percentages and absolute numbers (between parentheses) of colonies that lost interaction between FcγRI and periplakin are indicated. The locations of introduced stop codons (amino acid position) by random mutagenesis PCR are indicated in the last column.

| Screen no. | Control PCR | Mutagenesis PCR | Stop codons |
|------------|-------------|-----------------|-------------|
| I [dATP]  | 27.5% (53/193) | 43.0% (68/158) | 1644, 1649, 1663, 1666, 1676 |
| II [dATP] + [dTTP] | 29.6% (28/95) | 57.3% (55/96) | 1653, 1681, 1688 |

Fig. 4. Flow cytometric analysis of FcγRI-mediated EA-rosette formation. A. stable subcloned IIA1.6 transfectants expressing FcγRI/WT Fcγ γ-chain, FcγRI/mutated Fcγ γ-chain (two independent subclones), and FcγRI/mutated Fcγ γ-chain/periplakin clone 3.4 (two independent subclones) were assessed for FcγRI expression using F(ab')2 fragments of the CD64 mAb H22 and goat F(ab')2 anti-human ƙ light chain FITC by flow cytometry. Black lines represent FcγRI staining, and dotted lines represent isotype control. B. erythrocytes (RBC) were FL2-labeled and sensitized with mlgG2a α-glycophorin A mAb. Subsequently, FcγRI transfectants and sensitized erythrocytes were incubated, and EA-rosetting was assessed by flow cytometry as the percentage of cells bound to mlgG2a-erythrocytes. Lower right quadrants contain non-bound transfectants (that differ in size and FL-1 autofluorescence from erythrocytes), upper left quadrants contain non-bound erythrocytes, upper right quadrants contain transfectants bound to erythrocytes. C. analysis of EA-rosetting of transfectants using erythrocytes sensitized with two concentrations of mlgG2a α-glycophorin A mAb. One representative experiment out-of-four is shown; error bars indicate S.D.

DISCUSSION

In this report we studied the molecular interaction between FcγRI and periplakin. Minimal binding domains were defined and generated as TAT peptides to disrupt intracellular FcγRI-periplakin interactions. TAT-PPL transduction enhanced the capacity of FcγRI to form EA-rosettes in transfected IIA1.6 cells without affecting receptor expression levels (Fig. 5). Because this effect mimicked stable transfection of C-terminal periplakin, it is likely that C-terminal periplakin and TAT-PPL regulate FcγRI by preventing FcγRI-CY binding to endogenous periplakin (Fig. 6). This suggests that endogenous periplakin somehow decreases FcγRI-ligand binding.

We showed that the proximal part of FcγRI-CY binds periplakin. Receptor truncation experiments in yeast cells indicated FcγRI C-terminal residues 333–374 to be fully dispen-
Molecular Characterization of FcγRI-Periplakin Interaction

Fig. 5. Modulation of EA-rosetting by TAT-peptides. A, FcγRI surface expression of stable transfectants expressing FcγRI and the ITAM-mutated FcR γ-chain with or without periplakin clone 3.4 after TAT peptide transduction. FcγRI expression was measured with F(ab′)2 fragments of the CD64 mAb H22 and goat F(ab′)2 anti-human κ light chain FITC by flow cytometry. Filled histograms are non-transduced cells, black lines represent TAT-periplakin-transduced cells, gray lines represent TAT-FcγRI-transduced lines. B, periplakin levels after TAT peptide transduction. Full-length periplakin was transiently overexpressed, and periplakin levels were assessed by Western blot. Tubulin staining was used as a loading control. C, transduction of IIA1.6 cells with FITC-conjugated peptides. Non-TAT control peptide and TAT peptides were FITC-conjugated and pulsed into cells as described under “Experimental Procedures.” The images contain identical numbers of cells. Surface IgG on IIA1.6 cells was CY-3-labeled in the insert by a monoclonal antibody recognizing mIgG2a, which was CY-3 conjugated mAb recognizing mIgG. Cells on IIA1.6 cells were CY-3-labeled in the insert by a monoclonal antibody recognizing mIgG2a, which was CY-3 conjugated mAb recognizing mIgG. Filled histograms show staining with the FITC-conjugated peptide. Error bars indicate S.D.

Fig. 6. Schematic showing the proposed regulatory mechanism of C-terminal periplakin and TAT-periplakin on FcγRI function. A, endogenous periplakin (light gray) binds the periplakin binding site (white box) of FcγRI (black). B, the addition of TAT-periplakin (small light gray boxes) functionally “out-competes” endogenous periplakin binding to FcγRI. C, transfected C-terminal periplakin (hatched light gray boxes) blocks endogenous periplakin-FcγRI interaction similar to TAT-periplakin. D, both C-terminal periplakin and endogenous periplakin are blocked by excess TAT-periplakin.

Ala16 substitution of individual FcγRI-CY residues 311-325 that affected binding to periplakin were found largely conserved from mouse to man (Fig. 2). Residues located directly adjacent to the plasma membrane and more downstream (Lys322, Trp323, and Leu325) abrogated FcγRI binding to periplakin. Remarkably, most single substitutions of the large positive KRRKKK stretch and Asp324 seemed to facilitate binding between FcγRI and periplakin. Overall sequence similarity of FcγRI with mouse FcγRI or other activable human FcR receptors is low (maximally 20%), and no obvious overlapping domains are present. These could not interact with periplakin in yeast two-hybrid studies, although the membrane proximal region of mouse FcγRI shares significant similarity with human FcγRI.

In mice, seven allelic variants of FcγRI have been described,
from which three have altered amino acids in the proximal part of the intracellular domain (49). Although no functional polymorphisms have been assigned to the cysteolic tail of human FcRI, amino acid substitutions that influence periplakin interaction might have an effect on FcRI function in vitro. Notably, the human FcRI b and c isoforms have identical cysteolic tails to FcRIa but contain asparagine at position 324 instead of aspartic acid (11). This cysteolic tail variant exhibited increased interaction with periplakin in yeast two-hybrid binding studies (data not shown). However, the functional relevance of these isoforms is not known at present.

The random mutagenesis PCR suggested periplakin residues 1687–1701 to be part of the FcRI binding domain within periplakin (Fig. 3). Furthermore, the effect of TAT-PPL on FcRI function implies this region to be essential (Fig. 5).

Within this sequence aspartic acid at position 1694 diminished FcRIa but contained asparagine at position 324 instead of aspartic acid (11). This cytosolic tail variant exhibited increased interaction with periplakin in yeast two-hybrid binding studies (data not shown). However, the functional relevance of these isoforms is not known at present.

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