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The Unique Cytoplasmic Domain of Human FcyRIIIA Regulates Receptor-Mediated Function

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Ligand specificity characterizes receptors for Abs and many other immune receptors, but the common use of the FcR γ-chain as their signaling subunit challenges the concept that these receptors are functionally distinct. We hypothesized that elements for specificity might be determined by the unique cytoplasmic domain (CY) sequences of the ligand-binding α-chains of γ-chain-associated receptors. Among Fcγ receptors, a protein kinase C (PKC) phosphorylation consensus motif [RSSTR], identified within the FcγRIIIα (CD16A) CY by in silico analysis, is specifically phosphorylated by PKCs, unlike other FcRs. Phosphorylated CD16A mediates a more robust calcium flux, tyrosine phosphorylation of Syk, and proinflammatory cytokine production, whereas non-phosphorylatable CD16A is more effective at activation of the Gab2/PI3K pathway, leading to enhanced degranulation. S100A4, a specific protein-binding partner for CD16A-CY newly identified by yeast two-hybrid analysis, inhibits phosphorylation of CD16A-CY by PKC in vitro. Reduction of S100A4 levels in vivo enhances receptor phosphorylation upon cross-linking. Taken together, PKC-mediated phosphorylation of S100A4 modulates distinct signaling pathways engaged by the receptor. Calcium-activated binding of S100A4 to CD16A, promoted by the initial calcium flux, attenuates the phosphorylation of CY, and, acting as a molecular switch, may both serve as a negative feedback on cytokine production pathways during sustained receptor engagement and favor a shift to degranulation, consistent with the importance of granule release following conjugate formation between CD16A effector cells and target cells. This switch mechanism points to new therapeutic targets and provides a framework for understanding novel receptor polymorphisms. The Journal of Immunology, 2012, 189: 4284–4294.
gether, we propose a molecular switch that regulates the dual pathways of effector functions for CD16A. Although initially activating cytokine production, CD16A undergoes a shift to more efficient granule release, favored by S100A4-mediated inhibition of receptor phosphorylation, as might occur during conjugate formation between CD16A effector cells and target cells. Distinct from other FcRs, this switch mechanism highlights new therapeutic targets and provides a framework for understanding novel receptor polymorphisms.

Materials and Methods

Abs and reagents

The following Abs were used: 3G8 anti-CD16 (Abott Biotech); FITC-3G8 (Invitrogen); F(ab')2, 3G8 (prepared by Rockland Immunocchemicals); 32.2 anti-CD64 (Medarex); rabbit anti-CD16 (from Dr. Howard Fleit); rabbit anti-CD16A cytoplasmic tail (CY) and anti-CD64 cytoplasmic tail (CY) (made by Research Genetics); rabbit anti-S100A4 (from Dr. Arthur Polans, University of Wisconsin, Madison, WI); rabbit anti-S100A4 (made by Cocalico Biologicals); 3C7 anti-S100A4 and 7D3 anti-FcR γ-chain mAb (generated in our laboratory); F(ab')2 mouse IgG and F(ab')2 goat anti-mouse IgG (Jackson ImmunoResearch Laboratories); IgE anti-TNP (BD Pharmingen); rabbit anti-IgG, anti-Syk, and anti-Gab2 (Santa Cruz Biotechnology); and mouse IgG (Jackson ImmunoResearch Laboratories). IgE anti-TNP (BD Pharmingen); 5C7 anti-S100A4 and 7D3 anti-FcR (United States Biological); 5B6, a monoclonal antibody specific for murine CD16A (26), was generated by Dr. Daniel Krangel (Yale University School of Medicine, New Haven, CT) and a gift from Dr. Bruce Whittingham (University of Connecticut, Storrs, CT). 3G8 goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and an ECL kit (Amersham Biosciences) were used to detect IgG blots.

Measurement of intracellular calcium

Intracellular Ca2+ concentration ([Ca2+]i) was determined as described previously (26). Briefly, suspensions of cells at 106/ml in Ca2+- and Mg2+-free Hank's PBS (pH 7.4) were incubated with 5 μM Indo-1/AM at 37°C for 40 min. Following incubation, cell suspensions were incubated another 25 min at room temperature, washed, and resuspended to 105 cells/ml in Hank's PBS with 1 mM CaCl2 and MgCl2, 1 mg/ml BSA, and 10 mM HEPES (Hanks+-). Cells were opsonized with 7.5 μg/ml mAb 3G8 F(ab')2 for 40 min at 4°C, washed once, resuspended in Hanks+-, and 2 × 105 cells were immediately transferred to the SLM 8000. With excitation at 488 nm, the fluorescence emission at 510 nm was measured, integrated, and recorded each second. After establishing a baseline for 60 s, goat anti-mouse F(ab')2 was added (20 μM final concentration) and data acquisition was continued for an additional 3.5 min. Each sample was individually calibrated by lysing cells in 1% Triton X-100 to determine the maximal emission ratio and by adding EDTA (20 μM final concentration) to determine the minimal ratio. Alternatively, 2.5 × 106 RBL-2H3 cells expressing [RSSTR] or [RAAAR] CD16A were loaded with 2 μM Fluo-4 and opsonized with 10 μg/ml murine IgE for 20 min on ice. After establishing a baseline, cells were stimulated with goat anti-mouse Ig (20 μg/ml) and further analyzed by FACS analysis for intracellular calcium release.

In vitro kinase assays

Active PKC isoforms (Upstate Biotechnology) in 50 μl kinase buffer (for PKC α, β, and γ, 4 mM MOPS [pH 7.2], 5 mM β-glycerophosphate, 0.2 mM Na2VO3, 0.2 mM DTT, 0.2 mM CaCl2; for PKC δ, ε, η, and ζ, 20 mM HEPES [pH 7.4], 0.03% Triton X-100; for PKC γ, 8 mM MOPS [pH 7.0], 0.2 mM EDTA) with dicyglycerol (5 μM), sonicated phosphatidyl serine (50 μg/ml), 50 μM ATP, 10 mM MgCl2, and 10–20 μCi [32P]ATP (Amersham Biosciences) were incubated with 5 μg GST-fusion proteins for 10 min at 32°C. The proteins were precipitated with 20% TCA, separated by SDS-PAGE, and visualized by autoradiography. In all PKC assays, a second set of samples without [32P]ATP was run in parallel and stained with Coomassie blue for protein loading controls.

Induced CD16A phosphorylation

Cells stably expressing [RSSTR] or [RAAAR] CD16A were metabolically labeled with 32Porthophosphate (27). Labeled cells were incubated with 3G8 F(ab')2 at 10 μg/ml in phosphate-free DMEM at 4°C for 10 min. After washing and resuspending in ice-cold phosphate-free DMEM, cells were incubated with 20 μg/ml F(ab')2 goat anti-mouse at room temperature for 5 min. The control samples were treated the same but incubated on ice all the times, or without addition of F(ab')2 rabbit anti-mouse. Cells were lysed in Triton X-100 lysis buffer (24) on ice for 10 min. Cell lysates or immunoprecipitates were analyzed by immunoblots.

Cytokine analysis

Cytokine production was measured as described previously (13) with modifications. Cells were stimulated in 24-well tissue culture plates (Costar) with 1 μg/ml LPS, surface-absorbed mAb 3G8 F(ab')2, mouse IgE, or F(ab')2 mouse IgG. Wells were coated with absorbed F(ab')2 Ab; Ab, mouse IgG (40 μg/ml), or mouse IgE (40 μg/ml) overnight at room temperature. After washing, cells (5 × 105 cells/ml) were added to the wells and cultured for varying periods of time. Levels of murine or rat cytokines in culture supernatants were quantitated by ELISAs following manufacturers' protocols.

Deaggregation assay

Deaggregation of RBL-2H3 cells was determined by measuring the release of the granule enzyme β-hexosaminidase (25). Briefly, cells were plated on 24-well plates at 1 × 105 cells/well overnight. Monolayer cells were washed with Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA [pH 7.4]). The cells were incubated with 100 μl 5 μg/ml Ab (F(ab')2, 3G8, F(ab')2 mouse IgG, or mouse IgE on ice for 45 min. After washing with Tyrode's buffer, cells were incubated with 100 μl F(ab')2 goat anti-mouse at 20 μg/ml at 37°C for various time points. Cell supernatants (10 μl) were incubated with 30 μl substrate (3 mM p-nitrophenyl-N-acetyl-β-D-glucoaminide in 0.2 M glycine buffer [pH 4.5] at 37°C for 1 h). The reaction was stopped with 1% 100 mM NaCO3-NaHCO3 (pH 10.0) and absorbance at 405 nm was measured. Results are expressed as percentages of the total β-hexosaminidase contents of the cells after subtracting the spontaneous release in the absence of receptor cross-linking.

Measurement of intracellular calcium
for identity. The identity of target plasmid was determined by amplification using vector-specific upper and lower primers provided in the DupLEX-A system. An S100A4-specific upper primer was designed to be compatible with the vector-specific lower primer to screen all of the positive yeast clones. S100A4 primer was 5'-GGG CAA AGA GGG TGA CAA-3'. The interaction of CD16A-CY with S100A4 was confirmed by cotransforming S100A4 and CD16A-CY expression plasmids into a less sensitive yeast strain, EGY 188.

In vitro binding assays

GST-CD16A-CY (12 µg) was incubated with 12 µg GST-S100A4, GST-CD64-CY, or GST in 100 µl KTT buffer (28) with 1 mM CaCl2 or 10 mM EDTA at 4°C overnight. CD16A was immunoprecipitated with rabbit anti-CD16A-CY overnight at 4°C. The precipitates were washed five times with KTT buffer with 1 mM CaCl2 or 10mM EDTA and analyzed by SDS-PAGE and Western blotting. To pull down S100A4 in cell lysate, U937 cells were lysed in Nonidet P-40 lysis buffer with or without Ca2+ but containing 10 mM EDTA. The supernatant was incubated with various GST fusion proteins on glutathione-Sepharose 4B beads overnight at 4°C. The beads were washed with the lysis buffer with or without Ca2+ for three times and analyzed by SDS-PAGE and Western blotting with anti-S100A4 mAb SC7. Blots were then stripped and reprobed with an anti-GST mAb to control protein loading.

Coimmunoprecipitation assays

NK3.3 cells, 293 cells transiently expressing S100A4 and CD16A or CD64, or primary mononuclear cells were lysed in Nonidet P-400 lysis buffer with or without Ca2+ but containing 10 mM EDTA as above. CD16A or CD64 was immunoprecipitated from the supernatants with 3G8 or 197 overnight.

**FIGURE 1.** CD16A is specifically phosphorylated by PKC. (A and B) In vitro kinase assay using PKCs (A) and PKCαβγ (B) with various GST-Fc receptor CY domains (upper panel). Protein gel was stained with Coomassie blue (lower panel). Data are representative of six experiments. (C) Cytoplasmic domain sequences of wild-type ([RSSTR]) and the mutant ([RAAAR]) CD16A. In vitro kinase substrates include separate clones of CD16A-CY with S/T to A mutations within the [RSSTR] motif (RSAAR-1 and -2 represent duplicate assays). Protein gel was stained with Coomassie blue (lower panel). Data are representative of three separate experiments using PKCδ. (D) CD16A-stably transfected P388D1 cells were metabolically labeled with [32P]orthophosphate and treated with 10 µg/ml BIM-I or control reagent BIM-V at 37°C for 60 min prior to receptor cross-linking for 5 min at 25°C. Immunoprecipitated CD16A was detected by autoradiogram and parallel gels were blotted with rabbit anti-CD16A-CY. Data are representative of three experiments. (E) [RSSTR] or [RAAAR] CD16A-stably transfected RBL-2H3 cells were metabolically labeled with [32P]orthophosphate and the receptor was cross-linked for 5 min at 25°C. Immunoprecipitated CD16A was analyzed by autoradiography, Western blotting, and densitometry (upper panel). The density of [32P]CD16A was plotted for each treatment condition (lower panel). Data represent means of three experiments.
at 4˚C. Immunoprecipitates were analyzed by Western blotting with anti-S100A4. Blots were then stripped and reprobed with polyclonal anti–CD16A-CY Abs and/or a polyclonal anti–CD64-CY Ab.

Results
PKC phosphorylates CD16A-CY but not other FcR CYs
The α-chain CYs of FcγRIIIa (CD16A), FcγRIa (CD64), and FcεRI (CD89) each have unique sequences without homology in the human genome. ProfileScan (PROSITE database) suggested several putative PKC phosphorylation motifs in their α-chain CYs although in vitro kinase assays showed essentially no phosphorylation of the CYs of CD89 or CD64. However, CD16A-CY, which contains a potential PKC phosphorylation motif [RSSTR] (Fig. 1C), was strongly phosphorylated by PKCs (Fig. 1A, 1B, Supplemental Fig. 1). Mutation of the CD16A [RSSTR] motif to [RAAAR] abolished the phosphorylation. Mutation of Ser219 and Thr220 to Ala219 and Ala220 also abolished PKC phosphorylation, localizing the phosphorylation site to these two residues (Fig. 1C).

In NK3.3 and P388D1 cells stably expressing human CD16A, CD16A phosphorylation was enhanced by PMA treatment and inhibited by selective PKC inhibitors (Supplemental Fig. 2B, 2C). CD16A phosphorylation was induced by CD16A cross-linking in P388D1 cells stably expressing human CD16A. Selective PKC inhibitors BIM-I and Go6976 inhibited the phosphorylation (Fig. 1D, Supplemental Fig. 2A), indicating that CD16A is phosphorylated by PKC. phosphorylation of CD16A in [RSSTR] but not [RAAAR] CD16A RBL-2H3 cells indicates that the [RSSTR] motif is responsible for the phosphorylation (Fig. 1E).

The PKC motif in CD16A-CY differentially regulates induction of proinflammatory cytokines and degranulation
Activation of monocytes/macrophages by CD16A results in the synthesis and secretion of TNFα, IL-6, and IL-1β (30, 31). The levels of secretion of IL-6, IL-1β, and TNF-α in [RSSTR] CD16A-expressing P388D1 cells following receptor cross-linking were markedly higher than those in [RAAAR] cells expressing...
equivalent levels of CD16A (Fig. 2A, 2B). Comparable levels of γ-chain were associated with CD16A, consistent with the requirement of γ-chain for CD16A surface expression (Fig. 2C). CY-tailless CD16A-expressing cells gave similar results as did [RAAAR] cells (Supplemental Fig. 3). LPS-induced cytokine production was at similar levels in these cell lines (Supplemental Fig. 3 and data not shown). The levels of secretion of IL-4 in [RAAAR] CD16A-expressing RBL-2H3 cells following receptor cross-linking were significantly reduced in comparison with those in [RSSTR] cells expressing equivalent levels of CD16A (Fig. 2D). CD16A cell surface expression was comparable (Fig. 2E) and γ-chain association with CD16A was equivalent (Fig. 2F) in the transfectants. In contrast, the [RAAAR]-transfected cells showed enhanced receptor-mediated degranulation compared with [RSSTR] CD16A-transfected RBL-2H3 cells (Fig. 3A). Both [RSSTR] and [RAAAR] CD16A-expressing cells underwent comparable levels of degranulation when activated through endogenous FcεR, indicating equivalent capacities for degranulation (Fig. 3B). As a complementary approach, RBL-2H3 cells stably expressing [RSSTR] or [RAAAR] CD16A were transiently transfected with wild-type PKCθ or constitutively active PKCθ A148E constructs. Both the wild-type PKCθ and the constitutively active PKCθ resulted in a significant decrease (40.2 and 39.5% reduction at 30 min; \( p = 0.0004 \) and 0.0036, respectively) in receptor-induced degranulation in cells expressing [RSSTR] CD16A when compared with the vector control (Fig. 3C). However, the wild-type and the constitutively active PKC constructs reduced receptor-induced degranulation much less in cells expressing the [RAAAR] receptor (9.4 and 15.2% at 30 min, respectively) (Fig. 3D). Taken together, these results provide strong evidence for the role of PKC-mediated phosphorylation of CD16A α-chain in determining the balance of cell programs initiated by receptor engagement.

The PKC motif in CD16A-CY regulates γ-chain–mediated signaling

The CD16A-CY regulates receptor-mediated early signaling, including calcium mobilization and tyrosine phosphorylation of Syk (Ref. 15 and data not shown). We investigated the role of the

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Receptor-mediated degranulation is augmented in RBL-2H3 cells expressing nonphosphorylatable CD16A. (A) Degranulation in RBL-2H3 stable transfectants expressing CD16A [RSSTR] or [RAAAR] was induced by receptor cross-linking and measured as hexosaminidase activity. Data are means ± SD from three experiments. (B) Degranulation induced by cross-linking FcεR with mouse IgE and goat anti-mouse cross-linking. Results are representative of three experiments with \( p > 0.05 \) for receptor-specific degranulation in CD16A [RSSTR] versus [RAAAR] RBL-2H3 stable transfectants. (C and D) PKCθ wild-type, PKCθ A148E (constitutively active form), or control vector DNA was introduced into CD16A [RSSTR] (C) and [RAAAR] (D) RBL-2H3 stable transfectants. After CD16A cross-linking, degranulation was measured as described. Data are presented as means ± SD of three experiments. *\( p < 0.05 \) by Student paired \( t \) test.
CD16A-CY [RSSTR] motif in receptor-mediated early signaling in cell lines expressing functionally competent endogenous γ-chain (13, 14, 29). Receptor cross-linking induced a rise in [Ca²⁺]i, of 241 nM in RBL-2H3 cells expressing [RSSTR] CD16A, but only 127 nM calcium, on average, in RBL-2H3 cells expressing [RAAAR] CD16A at equivalent levels (Fig. 4A). Endogenous FcγR engagement induced comparable calcium fluxes in both cell lines. Receptor-induced tyrosine phosphorylation of total cellular γ-chain and coprecipitated Syk was reduced markedly in P388D1 cells expressing [RAAAR] CD16A in comparison with [RSSTR] CD16A (Fig. 4B). Furthermore, although association of γ-chain with the CD16A was equivalent in [RSSTR] and [RAAAR] cells, receptor-induced tyrosine phosphorylation of receptor-associated γ-chain and γ-chain-associated Syk was significantly reduced in P388D1 cells expressing [RAAAR] CD16A in comparison with [RSSTR] CD16A (data not shown). Thus, key components of γ-chain-mediated signaling are regulated by the PKC motif in CD16A.

The PKC motif in CD16A-CY regulates degranulation via a Gab2/P13K-dependent pathway

FcγR-induced degranulation is likely mediated by both calcium-dependent and -independent pathways (32). The adaptor protein Gab2 is critical for activation of the P13K/Akt pathway (33) and the Fyn/Gab2/RhoA-dependent, calcium-independent pathway in mast cell degranulation (32). Engagement of CD16A on NK cells initiates a cascade of signaling events including PI3K activation (34), which is required for CD16A-mediated granule exocytosis and ADCC in NK cells (35). The enhanced degranulation in the [RAAAR] CD16A-expressing RBL-2H3 cells is unlikely caused by the calcium-dependent pathway because there is impaired calcium signaling in these cells. We therefore investigated the effect of the PKC motif on the calcium-independent Gab2/P13K/Akt pathway. After CD16A cross-linking, tyrosine phosphorylation of Gab2 was induced in the [RAAAR] but not the [RSSTR] CD16A-expressing RBL-2H3 and P388D1 cells (Fig. 4C and data not shown). Similarly, levels of phosphorylation of Ser⁴⁷³ and Thr⁴⁰⁸ of AKT, an indication of maximum activation of the enzyme, markedly increased in the [RAAAR] CD16A cells following receptor cross-linking compared with that in [RSSTR] CD16A cells (Fig. 4D, 4E). Treatment with wortmannin, a highly selective inhibitor for PI3K with an in vitro IC₅₀ of 2–4 nM for PI3K (36), at levels of 0.3–3 nM markedly reduced receptor-mediated degranulation in the [RAAAR] CD16A-expressing RBL-2H3 cells (10.4 and 29.9% at 0.3 and 3 nM, respectively), but only reduced degranulation modestly in the [RSSTR] CD16A cells (2.1 and 7.3% at 0.3 and 3 nM, respectively) (Fig. 4F). The percentage of β-hexosaminidase release without inhibitor was 17.9% in [RSSTR] CD16A cells and 51.3% in [RAAAR] CD16A RBL-2H3 cells. These results indicate that FcγR-induced degranulation is regulated by the PKC motif in CD16A-CY.

**FIGURE 4.** Early signaling following CD16A cross-linking was altered in cells expressing nonphosphorylatable CD16A. (A) CD16A receptor cross-linking triggered calcium mobilization in RBL cells expressing human CD16A [RSSTR] or [RAAAR] (left panel). Data are representative of three experiments. IgE receptor cross-linking triggered calcium mobilization in RBL cells expressing human CD16A [RSSTR] or [RAAAR] (right panel). Data represent one of five experiments giving similar results. (B) Tyrosine phosphorylation of γ-chain– and γ-chain–associated Syk in P388D1 stable transfectants expressing CD16A [RSSTR] or [RAAAR]. After receptor cross-linking for 5 min at 25°C, cell lysates were immunoprecipitated with polyclonal anti–γ-chain and separated by SDS-PAGE under nonreducing conditions. Tyrosine phosphorylated γ-chain and Syk were analyzed by sequentially blotting with anti-phosphotyrosine (4G10), anti-Syk, and anti-γ-chain. Data are representative of three experiments. (C) After CD16A cross-linking in RBL-2H3 stable transfectants for 5 min, Gab2 in cell lysates was immunoprecipitated and probed sequentially with anti–p-Tyr (4G10) and polyclonal anti-Gab2. (D and E) Cells were stimulated as above and AKT was immunoprecipitated and probed with anti–phospho-AKT Thr⁵⁰⁶ (D), anti–phospho-AKT Ser⁴⁷³ (E), and anti-AKT. (F) RBL-2H3 stable transfectants expressing [RSSTR] or [RAAAR] CD16A were preincubated for 15 min at 37°C with the indicated concentrations of wortmannin or DMSO vehicle (35). The cells were washed and CD16A was cross-linked for 30 min. Degranulation was measured as hexosaminidase activity. Data are summarized from three experiments.
that the enhanced degranulation in [RAAAR] CD16A RBL-2H3 cells is likely mediated by a wortmannin-sensitive, PI3K-dependent pathway.

**S100A4 interacts with CD16A-CY**

To identify protein partners that might interact with the CD16A cytoplasmic domain and contribute to the modulation of CY function, we performed yeast two-hybrid studies using a pEG202-CD16A-CY bait vector and the human PBL cDNA library and identified S100A4 as a specific CD16A-CY–interacting protein (Fig. 5A). To map the region of CD16A-CY that is essential for the interaction with S100A4, a series of CD16A mutant variants were constructed (Fig. 5B and data not shown). These variants were tested for interaction of CD16A-CY with S100A4 using the yeast two-hybrid system. Deletion of the six most C-terminal residues of CD16A abrogated the optimal interaction with S100A4, implicating these to be part of the S100A4 binding site of CD16A (Fig. 5C).

To verify interaction between CD16A-CY and S100A4, we used GST fusion proteins and in vitro pull-down assays to demonstrate that GST-S100A4, but not GST or GST-CD64-CY, was pulled down by GST-CD16A-CY (Fig. 6A). Similarly, GST-CD16A-CY, but not GST-CD64-CY, was pulled down by GST-S100A4 or purified S100A4 (data not shown). Furthermore, GST-CD16A-CY, GST-CD64-CY, GST-CD89-CY, or GST was incubated with U937 cell lysates containing endogenous S100A4, and S100A4 was only pulled down with GST-CD16A-CY in the presence of Ca²⁺ (Fig. 6B). These data demonstrated that the interaction between CD16A-CY and S100A4 is direct, Ca²⁺-dependent, and specific. To confirm that these two proteins interact in cells, CD16A was immunoprecipitated from NK3.3 cell lysates and probed for CD16A and S100A4, which coprecipitated with CD16A in these cells in a Ca²⁺-dependent manner (Fig. 6C). CD16A or CD64, immunoprecipitated from 293 cells transiently expressing CD16A or CD64 and S100A4, showed coimmunoprecipitation of S100A4 with CD16A but not with CD64 in a Ca²⁺-dependent manner (Fig. 6D). Furthermore, S100A4 was communoprecipitated with CD16A but not CD64 in lysates from primary mononuclear cells in the presence of calcium (Fig. 6E), indicating specific interaction of the two proteins at physiological expression levels. To study the subcellular localization of both proteins in freshly prepared primary mononuclear cells, cells were stained for S100A4 (green) and CD16 (red) and examined by scanning laser microscopy. About 20–30% of the cells stained positive for CD16A, presumably being NK cells, monocytes, and macrophages. S100A4 was also present in these cells, with a cytoplasmic distribution and a few patches of colocalization with CD16 (Fig. 6F). When CD16 was cross-linked, enhanced colocalization of CD16A and S100A4 was evident with some comigration to the interior of cells. Thus, modest colocalization of S100A4 with CD16A is enhanced following receptor cross-linking. In cells pretreated with BAPTA-AM, however, S100A4 no longer colocalized with CD16A following receptor cross-linking, indicating that the colocalization is Ca²⁺-dependent. CD64 and S100A4 were not colocalized in receptor cross-linked cells.

**S100A4 inhibits phosphorylation of CD16A by PKC and affects receptor-mediated degranulation**

S100A4 inhibits phosphorylation of its target proteins by PKC and/or CK2 (20, 22, 23, 37). In vitro kinase assays with GST-CD16A-CY and PKC in the presence or absence of S100A4 demonstrated...
FIGURE 6. The interaction of CD16A-CY with S100A4 is direct, specific, and calcium-dependent. (A) GST-CD16A-CY in binding buffer containing 1 mM CaCl$_2$ or 10 mM EDTA and was incubated with GST-S100A4, GST-CD64-CY, or GST. GST-CD16A-CY was precipitated with polyclonal anti–CD16A-CY and sequentially blotted with polyclonal anti-S100A4 and anti-GST ($n=3$). (B) U937 cell lysates were incubated with GST-CD16A-CY, GST-CD64-CY, GST-CD89-CY, or GST on glutathione-Sepharose 4B beads in the presence of 1 mM Ca$^{2+}$ or 10 mM EDTA overnight at 4˚C. The beads were washed and the proteins were resolved and immunoblotted sequentially with anti-S100A4 and anti-GST ($n=3$). (C) Interaction between CD16A and S100A4 in NK3.3 cell line in the presence of 1 mM Ca$^{2+}$ or 10 mM EDTA. Cell lysates were immunoprecipitated with 3G8 and analyzed by sequential immunoblotting with anti-S100A4 and anti–CD16-CY ($n=3$). (D) The 293 cells transiently expressing S100A4 and CD16A or CD64 were lysed with Nonidet P-40 lysis buffer with or without Ca$^{2+}$ 48 h after transfection. CD16A and CD64 immunoprecipitates were blotted with anti-S100A4 and anti–CD16A-CY sequentially. S100A4 expression in the cells was examined by immunoblotting with anti-S100A4 in the cell lysate ($n=3$). (E) Freshly prepared mononuclear cells were incubated with Alexa Fluor 594-conjugated 3G8 F(ab')$_2$ (10 µg/ml) for 40 min at 4˚C to label surface CD16 (upper three panels). Cells were then washed with PBS and cross-linked at 37˚C for 15 min. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 2 min at room temperature. Cells were incubated with Alexa Fluor 488-conjugated anti-CD64 for 40 min at 4˚C to label surface CD64, cross-linked, and analyzed as above. Original magnification ×40 in all panels. Data are representative of six experiments.
that GST-S100A4, but not GST, inhibited phosphorylation of GST-CD16A-CY by PKC (Fig. 7A, Supplemental Fig. 4A). Because GST-S100A4 inhibited phosphorylation of GST-CD16A-CY and p53 but did not affect the phosphorylation of MBP by PKC under the same conditions, we exclude the possibility that GST-S100A4 acts as a competitive substrate or results in substrate inhibition of the kinase (Supplemental Fig. 4B, 4C). Notably, the level of inhibition of CD16A phosphorylation by PKC in the presence of S100A4 was similar to that reported for other target proteins of S100A4m including p53 and liprin-β1 (20–23). To assess the effect of S100A4 binding on CD16A phosphorylation in vivo, phosphorylation of CD16A was measured in S100A4 small interfering RNA (siRNA) or control RNA-treated RBL-2H3 cells. S100A4 was reduced to an undetectable level in S100A4 siRNA-treated cells (data not shown). The ratio of [\(^{32}\)P]CD16A to CD16A protein in anti-CD16 immunoprecipitates increased 40.5% in cells treated with control RNA, whereas the ratio increased 78.6% in S100A4 siRNA-treated cells after receptor cross-linking (Fig. 7B), indicating interference of S100A4 with induced phosphorylation of CD16A. Thus, S100A4 may regulate CD16A-specific functions by modulating its phosphorylation by PKC. Correspondingly, degranulation measured at 10 min after CD16A cross-linking was reduced in cells treated with S100A4 siRNA (Figs. 7C, 8).

Discussion
The [RSSTR] sequence in the cytoplasmic domain of CD16A is phosphorylated by PKC in in vitro kinase assays, in human cell lines, and in primary human cells. The phosphorylation is specific to this motif, and the phosphorylated and nonphosphorylatable states differentially regulate receptor-initiated signaling and downstream cell programs. Phosphorylated CD16A favors more robust tyrosine phosphorylation of the FcRγ-chain with greater flux in [Ca\(^{2+}\)], and production of IL-1β, IL-6, TNF-α, and IL-4, a provocative observation given the hyperactivation of PKC0 in a subset of lupus patients (38). In contrast, the [RAAAR] CD16A leads to greater activation of Gab2 and AKT and greater dependence of degranulation on the PI3K pathway. Furthermore, the calcium-sensitive S100A4, known to inhibit the phosphorylation of its target proteins by PKC and CK2, interacts specifically with CD16A (among FcRs), inhibits phosphorylation of CD16A-CY by PKC in vitro, and modulates in vivo CD16A phosphorylation. Taken together, these data suggest that S100A4 may serve as a modulator of CD16A [RSSTR] phosphorylation. Its calcium-dependent binding of CD16A may diminish further phosphorylation, providing both a negative feedback loop for cytokine production and a mechanism favoring degranulation by receptors newly recruited to structures such as the immunological synapse in conjugate formation.

Among the FcRγ-chain–associated FcRs, total truncation mutants suggest a contribution of the α-chain cytoplasmic domain to receptor signaling and cell programs (29, 39). We hypothesized that Ser/Thr phosphorylation events might provide a window on the mechanism of α-chain contributions, and in silico analysis identified the PKC phosphorylation consensus motif [RSSTR] within the CD16A cytoplasmic domain, whereas FcγRI (CD64) and FcεR (CD89) have CK2 and CK1 sites, respectively. The predicted specificity for kinases has been supported experimentally, suggesting important mechanisms for differential regulation. Further evidence for receptor-specific mechanisms for regulation is provided by the specific interaction of S100A4 with the cytoplasmic domain of CD16A, but not with those of CD64 or CD89. Not only do CD16A and S100A4 coimmunoprecipitate in a Ca\(^{2+}\)-dependent manner in human cell lines and primary mononuclear cells, but they also colocalize in primary mononuclear cells.

S100A4 inhibits phosphorylation of its binding partner proteins by PKC and/or CK2 (20, 22, 23, 37). Consistent with these obser-

![FIGURE 7. S100A4 inhibits phosphorylation of CD16A by PKC and attenuates receptor-mediated degranulation. (A) GST-CD16A (1 µM) was phosphorylated in the absence and presence of various concentrations of GST-S100A4 by active PKC for 30 min at 30˚C (23) (upper panel). A parallel set of samples without [\(^{32}\)P]ATP was stained with Coomassie blue to control protein loading (lower panel). (B) RBL-2H3 [RSSTR] or [RAAAR] CD16A stable transfectants were transfected with S100A4 siRNA or nonsilencing control RNA (45). After 24 h, phosphorylation of CD16A in [\(^{32}\)P] metabolically labeled RBL-2H3 cells was measured as described above. Data are representative of three experiments. (C) RBL-2H3 stable transfectants expressing CD16A were transfected with S100A4 siRNA or control RNA. After 24 h, degranulation was measured at 10 min after CD16A cross-linking at the indicated concentrations. Data represent the means ± SD for six experiments. **p < 0.01, ***p < 0.001 by Student paired t test.]
mediated degranulation. Indeed, overexpression of a constitutively active PKC construct decreased receptor-mediated degranulation. Furthermore, calcium-dependent binding of S100A4 to CD16A diminished further phosphorylation, providing both a negative feedback loop, potentially restricting cytokine production, and a mechanism favoring degranulation.

Nonphosphorylatable CD16A enhanced degranulation, implying that phosphorylation of the PKC motif downregulates receptor-mediated degranulation. Indeed, overexpression of a constitutively active PKC construct decreased receptor-mediated degranulation in cells expressing wild-type, but only minimally in cells with the nonphosphorylatable CY receptor, clearly supporting the notion that phosphorylation of the [RSSTR] motif is responsible for this effect. Although PKCs are important mediators in initiation of many effector functions of Fcγ receptors, including NK cell killing and phagocytosis (40, 41), their effects are no doubt multiple. For example, PKCδ-deficient mast cells exhibited a significantly higher level of degranulation, suggesting that PKCδ is a negative regulator of Ag-induced mast cell degranulation (42). In addition to the well-known calcium-dependent pathway, degranulation in mast cells requires a calcium-independent, Fyn/Gab2/RhoA-dependent pathway, which plays a critical role in the microtubule-dependent translocation of granules to the plasma membrane (32). Gab2 is critical for PI3K recruitment and activation of the PI3K/AKT pathway (33) and Rho GTPases, involved in microtubule organization and granule translocation, and it is a downstream effector of PI3K (reviewed in Ref. 43). Engagement of CD16A on NK cells initiates PI3K activation (34), which is required for CD16-mediated granule exocytosis and ADCC by NK cells (35). Hyperresponsive CD16A-initiated degranulation in RBL-2H3 cells with nonphosphorylatable CD16A is unlikely due to the effects on the calcium-dependent pathway, which is downregulated in cells with a nonphosphorylatable receptor. In contrast, the Gab2/PI3K/AKT pathway is upregulated in cells with nonphosphorylatable CD16A, and degranulation is more sensitive to wortmannin inhibition in these cells. Thus, phosphorylation of CD16A regulates two distinct functions mediated by the receptor via at least two distinct pathways: a calcium-dependent pathway is concurrently upregulated, leading to augmented production of proinflammatory cytokines; and a calcium-independent, PI3K-dependent pathway is downregulated, leading to attenuated degranulation. That activation of PI3K after CD16 cross-linking in human primary monocytes limits the expression of TNFα, IL-1β, and IL-6 (44) supports the notion that PI3K activation plays distinct roles in CD16A-mediated degranulation and production of proinflammatory cytokines.

Our proposed model of PKC-mediated, phosphorylation-dependent balancing of proinflammatory cytokine production and degranulation, with a feedback loop involving the calcium-activated binding of S100A4 (Fig. 8), focuses attention on a critical role of the ligand-binding α-chain of CD16A and other Fc common γ-chain–associated receptors. The calcium-activated binding of S100A4 to CD16A may serve as such an inhibitor modulator on the Syk/cytokine pathways during sustained signaling and favor a shift to degranulation upon conjugate formation between CD16A+ effector cells and target cells. Such a molecular switch points to new therapeutic targets. Furthermore, the subtle modulation of the contributions of the ligand-binding α-chain through genetic variation in the cytoplasmic region, a theme clearly established within the extracellular domain, may provide additional insights into receptor function and into risk for immune-mediated diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


FIGURE S1. FcR α-chain CY are differentially phosphorylated by PKC. Substrates include GST, GST-CD64-CY, CD89-CY, CD32B-CY, CD16A-CY and/or mouse γ-chain. (A) Phosphorylating enzyme was PKCδ. (B) Phosphorylating enzyme was PKCε. (C) Phosphorylating enzyme was PKCη.
**Figure S2.** (A) CD16A-stably transfected P388D1 cells were metabolically labeled with $^{32}$P orthophosphate and incubated with 500nM PKC inhibitor Go6976 or DMSO at 37°C for 60 minutes prior to cross-linking for 5 min at 23°C. Immunoprecipitated CD16A was analyzed by autoradiography, western blotting, and densitometry. The density of $^{32}$P CD16A was plotted for each treatment condition. (B) NK3.3 cells were metabolically labeled with $^{32}$P orthophosphate and incubated with 300ng/ml PMA or DMSO at 37°C for 10 minutes. Immunoprecipitated CD16A was analyzed by autoradiography. (C) CD16A-stably transfected P388D1 cells were metabolically labeled with $^{32}$P orthophosphate and treated with 10 ug/mL BIM-I or control reagent BIM-V at 37°C for 60 minutes prior to treatment of DMSO or PMA at 100 ng/ml for 20 min at 37°C. Immunoprecipitated CD16A was detected by autoradiogram and parallel gels blotted with rabbit anti-CD16A-CY.
Figure S3

**FIGURE S3.** LPS or receptor-mediated production of TNFα (A) and IL-6 (B) in P388D1 stable transfectants expressing CD16A [RSSTR], [RAAAR] or Tail-deletion (TL). Cells were stimulated in a receptor specific manner as described previously (13) or with 1 μg/ml LPS for 24 hours at 37°C. Cytokine proteins in supernatants were measured by ELISA. Data are the mean ±SD from three experiments.
Figure S4

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FIGURE S4. S100A4 inhibits phosphorylation of CD16A in vitro. (A) The phosphorylation of GST-CD16A (1µM) by active PKC (30 min at 30°C; upper panel) was reduced by increasing concentrations of GST-S100A4. A parallel set of samples without ³²P-ATP was analyzed by PAGE and Coomassie staining to show protein loading (lower panel). (B) Similarly, the phosphorylation of p53 (1µM) by active PKC (30 min at 30°C) was reduced by increasing concentrations of GST-S100A4; comparator lanes show similar reductions of phosphorylation of GST-CD16A (1µM) as demonstrated in (A) and (C). (C) Unlike GST-CD16A (1µM), the phosphorylation of MBP (1µM) by active PKC (30 min at 30°C) in the presence of increasing concentrations of GST-S100A4 was unaffected (upper panel). A parallel set of samples without ³²P-ATP was analyzed by PAGE and Coomassie staining to show protein loading (lower panel).