Regulation of Phagocytosis and $[Ca^{2+}]_i$ Flux by Distinct Regions of an Fc Receptor

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The binding of multivalent immunoglobulin G complexes to Fc receptors ($Fc_\gamma Rs$) on macrophages activates multiple immune functions. A murine macrophage cell line, but not a fibroblast cell line, that was transfected with human $Fc_\gamma RIIA$ mediated phagocytosis and an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) flux upon cross-linking of human $Fc_\gamma RIIA$. Transfected macrophages that expressed a truncated receptor lacking 17 carboxy-terminal amino acids phagocytosed small antibody complexes. However, only wild-type transfectants phagocytosed labeled erythrocytes and fluxed $[Ca^{2+}]_i$. Thus, the cytoplasmic domain of human $Fc_\gamma RIIA$ contains distinct functional regions.

FC RECEPTORS ($Fc_\gamma Rs$) FOR IMMUNOglobulin G (IgG) link humoral and cell-mediated immune responses. Activation of $Fc_\gamma Rs$ requires cross-linking of multiple receptors by immune complexes. In neutrophils and macrophages, the binding of immune complexes to $Fc_\gamma Rs$ activates phagocytosis, production of oxidative metabolites, antibody-dependent cell-mediated cytotoxicity (ADCC), and production and release of inflammatory mediators. Human $Fc_\gamma Rs$ belong to a multigene family divided into three subclasses on the basis of differences in structure and avidity for IgG: $huFc_\gamma RI$, $huFc_\gamma RII$, and $huFc_\gamma RIII$. Within a $huFc_\gamma R$ subclass, receptors have nearly identical extracellular domains but differ in their intracellular domains and in their ability to activate cells (*1*), which suggests that the intracellular domains are involved in signal transduction. An individual receptor form can trigger multiple cellular responses. For example, cross-linking of $huFc_\gamma RII$ on macrophages can initiate both an oxidative burst (*2*) and phagocytosis (*3*). At some point a bifurcation of the signal pathway must occur. How multiple signal pathways are activated by $huFc_\gamma RII$ is not clear, because $Fc_\gamma Rs$ have no known enzymatic activity, nor do they structurally resemble G protein–coupled receptors.

To identify intracellular regions required for signaling, we transfected cDNAs encoding $huFc_\gamma RIIA$, the predominant $huFc_\gamma RII$ of neutrophils and macrophages (*4*), and a series of cytoplasmic truncations of $huFc_\gamma RIIA$ into $P388D_1$ cells, a mouse macrophage-like cell line, and into CHO cells, a hamster fibroblast cell line. In the cytoplasmic domains of $huFc_\gamma RIIA$ and $muFc_\gamma RII_2$, which is endogenously expressed on $P388D_1$ cells, two negatively charged regions and intervening tyrosine and leucine residues are conserved (*4, 5*). CHO cells do not express any native $Fc_\gamma R$ and thus provide a control for the cell specificity of $huFc_\gamma RIIA$-mediated functions in $P388D_1$ cells.

Nonsense mutations in the cytoplasmic coding region of a $huFc_\gamma RIIA$ cDNA clone were introduced by cDNA amplification with polymerase chain reaction (PCR) antisense primers that contained the desired nucleotide alteration (*6*). The most extensive truncation, Δ207, encoded a protein that had all but two putative cytoplasmic amino acid residues eliminated. The second and third truncations, Δ233 and Δ264, encoded proteins that retained, respectively, 28 and 59 residues of the 76–amino acid cytoplasmic domain. The Δ233 truncation terminates before the first conserved, charged amino acid coding region, and the Δ264 truncation terminates after the second.

The wild-type and truncated cDNAs were subcloned into the pcEXV-3 mammalian expression vector (*7*) and then transfected into CHO and $P388D_1$ cells by the calcium-phosphate coprecipitation method (*8*). $P388D_1$ cells (P-FcRIIA) or CHO cells (C-FcRIIA) that were transfected and that expressed wild-type $huFc_\gamma RIIA$ were identified by their ability to bind the Fab fragment of a mouse monoclonal antibody (MAb) to $huFc_\gamma RIIA$, MAb IV.3, in comparison with mock-transfected $P388D_1$ cells (P-NEG) or CHO cells (C-NEG) (Fig. 1). There was no significant binding to $huFc_\gamma RIIA$-expressing cells of an isotype control MAb. Mutant $huFc_\gamma RIIA$-expressing transfectants were identified by the same method.

The $P388D_1$ cell line expresses approximately 3×10^5 $muFc_\gamma RII$ and $muFc_\gamma RIII$ per cell (*9*). Scatchard analyses of direct binding assays with ^{125}I-labeled MAb IV.3 were done for one CHO transfectant and for one $P388D_1$ transfectant. Expression of $huFc_\gamma RIIA$ on the other $P388D_1$ and CHO transfectants was determined by comparative fluorescence according to cell type after the binding of MAb IV.3 Fab and treatment with fluorescein isothiocyanate (FITC)–conjugated goat $F(ab')_2$ antibody to mouse IgG. Expression levels varied between 3.1×10^5 and 6.0×10^5 receptors per cell for CHO transfectants and between 6.8×10^5 and 9.0×10^5 for $P388D_1$ transfectants.

We assayed internalization of antibody-$Fc_\gamma R$ (Ab-$Fc_\gamma R$) complexes by coating cell suspensions with MAb IV.3 Fab at 4°C and then cross-linking the Fab with goat $F(ab')_2$ antibody to mouse IgG. After incubation at 37°C to allow the cells to internalize receptor-bound complexes, the cells were returned to 4°C and assayed for complexes remaining on the cell surface by flow cytom-

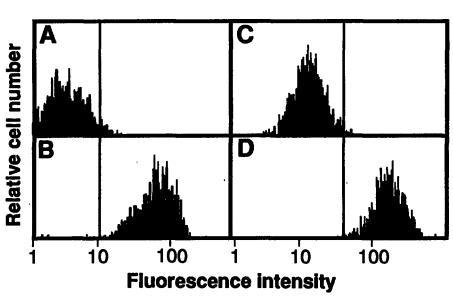

Fig. 1. Screening transfectants. Suspensions of transfected cells (2.5×10^6 per milliliter) were incubated at 4°C with MAb IV.3 (1 μg/ml; Medarex) for 1 hour, washed by centrifugation, and labeled with FITC-conjugated goat $F(ab')_2$ antibody to mouse IgG (20 μg/ml) at 4°C for 1 hour. After washing, 2000 cells per sample were analyzed on a Coulter Epics cell sorter. (**A**) Negative control $P388D_1$ (P-NEG) and (**C**) dihydrofolate reductase ($DHFR^-$) CHO cells (*23*) (C-NEG) were transfected with a vector (1 μg/ml) that contains a neomycin resistance gene, LKK444 (*24*), or a DHFR minigene, pMG1 (*23*), respectively, by the calcium phosphate coprecipitation method (*8*). (**B**) $P388D_1$ (P-FcRIIA) and (**D**) $DHFR^-$ CHO cells (C-FcRIIA) were transfected with $huFc_\gamma RIIA$ cDNA (*25*) subcloned into pcEXV-3 (18 μg/ml) (*26*). Transfection of $P388D_1$ cells required the addition of chloroquine (25 to 100 μM) to the transfection media. Transfected $P388D_1$ cells were grown in DME (Hazleton Biologics) supplemented with 5% FCS (Intergen, Purchase, New Jersey) and 200 mg/l G418 (Sigma Chemical). Transfected CHO cells were grown in hypoxanthine-deficient DME supplemented with 10% dialyzed FCS, 16 μM thymidine, and 300 μM proline. All cell lines were cloned before analysis. Scatchard analyses of direct binding assays utilizing ^{125}I-labeled MAb IV.3 (10^6 cpm/μg) (*22*) were done as described (*9*). Unless stated otherwise, all antibodies were purchased from Organon Teknika-Cappel (West Chester, Pennsylvania). Cells were maintained in a 10-mM Hepes-buffered Hank's balanced salt solution without Ca^{2+} or Mg^{2+} (pH 7.5) that was supplemented with 0.1% dextrose for all procedures unless otherwise noted.

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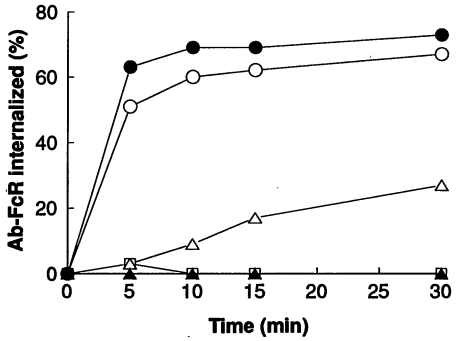

Fig. 2. Time course of internalization of cross-linked huFc$_\gamma$RIIA. After incubation with MAb IV.3 Fab (1 μg/ml) at 4°C for 30 min, suspensions of the P-FcRIIA (●) or C-FcRIIA (△) cells (2.5×10^6/ml) were incubated with goat F(ab')$_2$ antibody to mouse IgG (20 μg/ml) at 4°C for 20 min. To cross-link native muFc$_\gamma$R on P-FcRIIA cells (○), MAb 2.4G2 (*9*) and goat F(ab')$_2$ antibody to rat IgG were substituted. The cells were shifted to 37°C for the indicated time periods and washed at 4°C before labeling the remaining surface complexes with FITC-conjugated rabbit F(ab')$_2$ antibody to goat IgG (30 μg/ml) at 4°C for 30 min. After cross-linking either the native muFc$_\gamma$R or huFc$_\gamma$RIIA and incubating the cells at 37°C, we measured the cell surface levels of noncross-linked muFc$_\gamma$R (□) or huFc$_\gamma$RIIA (▲) by staining at 4°C. The fluorescence of cells maintained at 4°C throughout was used as the control for 0% internalization. Background cell fluorescence levels represented 100% internalization. Background cell fluorescence was determined by omitting MAb IV.3 or MAb 2.4G2. Shifts in logarithmic fluorescence values as compared with background cell fluorescence were converted to linear fold increases in fluorescence. To calculate the percentage of antibody complexes internalized by labeled cells incubated at 37°C, we used the following equation: 1 − [(fold increase in fluorescence of 37°C cells − 1) ÷ (fold increase in fluorescence of 4°C cells − 1)]. All experiments were repeated on at least three separate days. A representative experiment is shown.

etry (Fig. 2). For P-FcRIIA cells, typically >70% of the huFc$_\gamma$RII-specific complexes were rapidly internalized, with a plateau after 10 min. Similar results were obtained when the native muFc$_\gamma$Rs were labeled with MAb 2.4G2 (*9*), a rat antibody that is specific for muFc$_\gamma$RII and muFc$_\gamma$RIII, and were cross-linked with a goat F(ab')$_2$ antibody to rat IgG. Internalization of either human or mouse Fc$_\gamma$Rs did not affect the other. In contrast to the P-FcRIIA cells, C-FcRIIA cells internalized huFc$_\gamma$RIIA-specific complexes only at a slow steady rate; <30% of the complexes were internalized after 30 min. The initial rate of internalization in P-FcRIIA cells was 18 times that of C-FcRIIA cells.

Loss of huFc$_\gamma$RIIA from the surface was due to internalization and not shedding, because total cell-bound counts remained constant before (1968 ± 267) (mean ± SEM) and after (1929 ± 335) a 15-min incubation of P-FcRIIA cells at 37°C with ^{125}I-labeled complexes (*10*). Cell fluorescence was reduced 50% when P-FcRIIA cells were incubated at 37°C with complexes labeled with fluorescein (*10*), which is quenched in acidic cellular compartments (*11, 12*). Internalization of the Ab-huFc$_\gamma$RIIA complexes was inhibited >80% at 23°C, compared with 37°C in P-FcRIIA cells, but internalization in C-FcRIIA cells was not markedly changed (Table 1). Thus, the internalization of Ab-Fc$_\gamma$R complexes by P-FcRIIA cells mirrors macrophage phagocytosis of opsonized particles with respect to temperature sensitivity and delivery to intracellular, low-pH phagosomes (*13*). The internalization in C-FcRIIA cells may be due to activation of a slow, temperature-insensitive endocytic process common to all cells (*13*). In addition, macrophage phagocytosis specifically mediated by huFc$_\gamma$RII probably requires protein kinase C (PKC) (*14, 15*) and tyrosine kinase activation (*16*). Treatment of P-FcRIIA cells for at least 20 min with a PKC inhibitor (calphostin C) or a tyrosine kinase inhibitor (genistein) blocked internalization of Ab-huFc$_\gamma$RIIA complexes. At the inhibitor concentrations used, cell viability (trypan blue exclusion) was >85%, and huFc$_\gamma$RIIA expression in control P-FcRIIA cells was unaffected by either inhibitor.

Table 1. Regulation of internalization by huFc$_\gamma$RIIA. The ability of huFc$_\gamma$RIIA transfectants to internalize Ab-huFc$_\gamma$RIIA complexes during a 15-min incubation at 23°C versus 37°C was determined. The effect of calphostin C (1.2 μM; Kamiya Biomedical), genistein (10 μg/ml; UBI, Incorporated, Lake Placid, New York), or BAPTA-AM (100 μM; Molecular Probes) on a 15-min internalization at 37°C was also determined. Inhibitors were incubated with the cells for 30 min at 37°C and also included throughout the internalization procedure. The percentage of transfected cells that internalized at least one E-IV.3 Fab during a 20-min incubation at 37°C is compared with P-FcRIIA cells treated with BAPTA-Am (100 μM). Data are the mean internalization percentages (±SEM) of at least three independent experiments.

Internalization conditions	Transfected cell line internalization (%)		
	P-FcRIIA	P-Δ264	C-FcRIIA
Ab-huFc$_\gamma$RIIA complexes			
Control 37°C	72 ± 7	66 ± 9	20 ± 15
23°C	11 ± 1	9 ± 3	14 ± 1
Calphostin C	29 ± 18	39 ± 14	
Genistein	32 ± 8	18 ± 14	
BAPTA-AM	68 ± 4		
E-IV.3 Fab			
Control	30 ± 2	1 ± 1	0
BAPTA-AM	5 ± 4		

We examined huFc$_\gamma$RIIA-mediated internalization of opsonized particles by incubating the transfectants with MAb IV.3 Fab–coated erythrocytes (E-IV.3 Fab) for 20 min at 37°C (*17*). Although P-FcRIIA and C-FcRIIA (wild-type) cells were both able to rosette E-IV.3 Fab, only P-FcRIIA cells were able to internalize the opsonized erythrocytes (Table 1). The average percentage of P-FcRIIA cells that internalized at least one E-IV.3 Fab was 30 ± 2% (mean ± SEM). Neither cell type bound or internalized uncoated erythrocytes, nor did mock-transfected P388D$_1$ cells bind or internalize E-IV.3 Fab (Fig. 3). In both phagocytic assays, huFc$_\gamma$RIIA-mediated internalization was dependent on factors expressed in the macrophage cell line, P388D$_1$, but not found in the fibroblast cell line, CHO.

To determine which regions of huFc$_\gamma$RIIA are required for phagocytosis, transfected cells expressing truncated huFc$_\gamma$RIIA proteins were analyzed for the internalization of both Ab-huFc$_\gamma$RIIA complexes and E-IV.3 Fab. To compensate for possible differences due to the varying number of huFc$_\gamma$RIIAs per cell among the transfectants, we normalized the level of E-IV.3 Fab internalization relative to the internalization of positive control erythrocytes coated with rabbit IgG (EA) by each P388D$_1$ transfectant. The Fc domain of rabbit IgG will bind to huFc$_\gamma$RII and muFc$_\gamma$R (*1*). P388D$_1$ cells transfected with the two shorter truncated receptors (P-Δ207 and P-Δ233) internalized <20% of the Ab-huFc$_\gamma$RIIA complexes and no E-IV.3 Fab (Fig. 3). P-Δ264 cells internalized Ab-huFc$_\gamma$RIIA complexes as efficiently as cells expressing the wild-type receptor, P-FcRIIA. As in P-FcRIIA cells, internalization of Ab-huFc$_\gamma$RIIA complexes in P-Δ264 cells was highly sensitive to temperature and was blocked by PKC and tyrosine kinase inhibitors (Table 1). In contrast, E-IV.3 Fabs were not internalized by P-Δ264 cells. Because all P388D$_1$ transfectants internalized muFc$_\gamma$R-specific antibody complexes and EA (Fig. 3), the phagocytic apparatus in each P388D$_1$ transfectant is functional. None of the CHO transfectants could efficiently internalize the Ab-huFc$_\gamma$RIIA complexes or opsonized erythrocytes, though they could avidly bind these particles. Thus the phagocytosis of small antibody complexes, although similar to the phagocytosis of opsonized erythrocytes in macrophage specificity, did not require the 17 COOH-terminal amino acids of huFc$_\gamma$RIIA.

The oxidative burst initiated by huFc$_\gamma$RII cross-linking on macrophages reportedly requires a rapid increase in the intracellular concentration of Ca^{2+} $[Ca^{2+}]_i$, which is released from intracellular stores (*2*). To

detect changes in $[Ca^{2+}]_i$ in huFc$_\gamma$RIIA-transfected cells, we loaded the cells with Indo-1-AM (a calcium fluorophore) (*18*). Sites of huFc$_\gamma$RIIA were saturated with MAb IV.3 Fab and cross-linked with goat F(ab')$_2$ antibody to mouse IgG at 37°C (*19*) (Fig. 4). The P-FcRIIA transfectant had an immediate flux in $[Ca^{2+}]_i$, which increased by 320 ± 82 nM (mean ± SEM) before returning to a baseline level of 102 ± 20 nM. Neither MAb IV.3 Fab nor goat anti-mouse IgG F(ab')$_2$ alone could trigger this increase in $[Ca^{2+}]_i$. Addition of excess EDTA to chelate Ca^{2+} in the buffer only partially reduced this response. In contrast, $[Ca^{2+}]_i$ did not increase in CHO cells transfected with huFc$_\gamma$RIIA, which had a baseline $[Ca^{2+}]_i$ of 157 ± 57 nM. When the amount of MAb IV.3 Fab bound to P-FcRIIA was reduced to the amount bound to C-FcRIIA, $[Ca^{2+}]_i$ still increased after cross-linking. Thus, this huFc$_\gamma$RIIA-mediated increase in $[Ca^{2+}]_i$ comes at least partially from internal stores and requires factors found in macrophages. No cells expressing truncated huFc$_\gamma$RIIA proteins had any $[Ca^{2+}]_i$ increase. Buffering by BAPTA-AM (*20*) of intracellular free Ca^{2+} in P-FcRIIA cells blocked any calcium increase (Fig. 4) and the internalization of labeled erythrocytes but did not affect the rapid internalization of Ab-huFc$_\gamma$RIIA complexes (Table 1). Possibly, both the huFc$_\gamma$RIIA-mediated oxidative burst and the internalization of large opsonized particles depend on an $[Ca^{2+}]_i$ flux, which was abrogated by deleting the 17 COOH-terminal amino acids of huFc$_\gamma$RIIA.

The functional comparisons between P388D$_1$ and CHO cells transfected with wild-type huFc$_\gamma$RIIA indicate that the huFc$_\gamma$RIIA-mediated phagocytic response and the $[Ca^{2+}]_i$ flux require a macrophage signaling machinery not present in fibroblasts. A flux in $[Ca^{2+}]_i$ by P388D$_1$ cells expressing wild-type huFc$_\gamma$RIIA, P-FcRIIA, was required for internalization of E-IV.3 Fab but not of small Ab-huFc$_\gamma$RIIA complexes. Thus P-Δ264 cells expressing a truncated huFc$_\gamma$RIIA, which could not mediate an $[Ca^{2+}]_i$ flux, could still internalize small Ab-huFc$_\gamma$RIIA complexes, though they

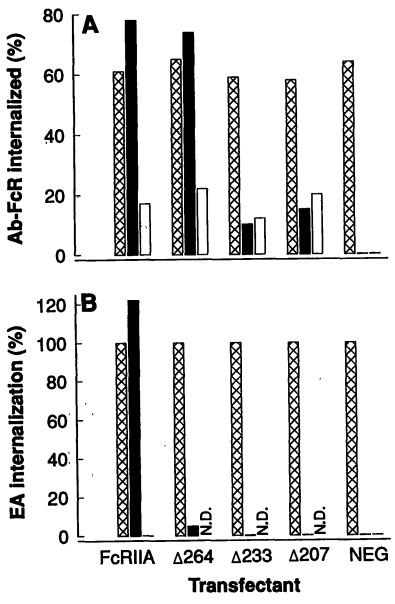

Fig. 3. Phagocytosis mediated by huFc$_\gamma$RIIA. (**A**) The percentage of bound muFc$_\gamma$R-specific antibody complexes internalized by each P388D$_1$ transfectant is shown as a positive control (crosshatched bars). The percent internalization of bound huFc$_\gamma$RII-specific antibody complexes by each P388D$_1$ (closed bars) and CHO (open bars) transfectant is shown. The percent of bound Ab-Fc$_\gamma$R complexes internalized during a 15-min incubation at 37°C was calculated as in Fig. 2. Experiments were repeated on at least three separate days. Representative experiments are shown. (**B**) The internalization of E-IV.3 Fab (*17*) by P388D$_1$ (closed bars) and CHO (open bars) transfectants was normalized relative to the internalization of EA by the corresponding P388D$_1$ transfectant. EA internalization was defined as 100% (crosshatched bars). Erythrocytes (E) (1×10^8 per ml) and huFc$_\gamma$RIIA-transfected cells (5×10^6 per ml) were mixed at 4°C, pelleted, and then incubated for 20 min at 37°C to stimulate internalization. Noninternalized E were lysed by a brief incubation in hypotonic media. The percentage of cells that internalized at least one erythrocyte was determined microscopically. The mean values of three independent experiments are shown. N.D. = not done.

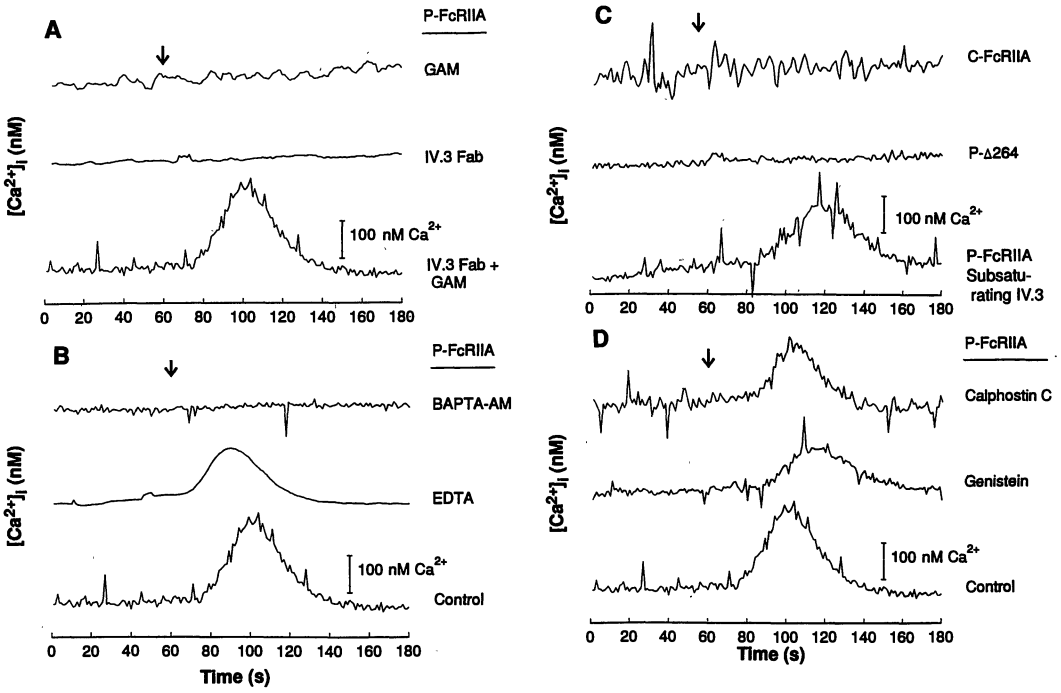

Fig. 4. (**A**) Intracellular calcium increase stimulated by cross-linking huFc$_\gamma$RIIA. After obtaining a baseline $[Ca^{2+}]_i$, we added (↓) a final concentration of 35 μg/ml of goat F(ab')$_2$ antibody to mouse IgG (GAM) (Tago) to initiate huFc$_\gamma$RIIA cross-linking on MAb IV.3 Fab–labeled P-FcRIIA cells (*19*). In control experiments, only GAM or IV.3 Fab were added. (**B**) Effect of extracellular and intracellular free Ca^{2+} chelators. The huFc$_\gamma$RIIA-mediated $[Ca^{2+}]_i$ flux in P-FcRIIA cells treated with EDTA (10 mM) or BAPTA-AM (100 μM) for 15 min is compared with untreated cells. (**C**) Cell type and receptor specificity. The huFc$_\gamma$-RIIA-mediated $[Ca^{2+}]_i$ flux in C-FcRIIA and P-Δ264 cells is compared with that of P-FcRIIA cells labeled with a subsaturating concentration of MAb IV.3 Fab (0.2 μg/ml) before cross-linking with GAM. (**D**) Effect of protein kinase inhibitors. The huFc$_\gamma$RIIA-mediated $[Ca^{2+}]_i$ flux in P-FcRIIA cells, treated with calphostin C (1.2 μM) and genistein (10 μg/ml) as in Table 1, is compared with untreated cells. All experiments were repeated at least three times on different days. Representative tracings are shown. Suspensions of transfected cells (10^7 per ml) were incubated with indo-1-AM (5 μg/ml; Molecular Probes) for 15 min at 37°C. After the cells were washed and resuspended in a Ca^{2+}- and Mg^{2+}-free physiologic saline buffer, they were incubated with MAb IV.3 Fab (1 μg/ml; a saturating concentration) at 37°C for 5 min. The cells were washed and resuspended in a 1.1-mM Ca^{2+} and 1.6-mM Mg^{2+} physiologic saline buffer for 5 min at 37°C. The stirred cell suspension was then transferred to an SLM 8000 fluorimeter. Because indo-1 fluorescence is measured at both 405 and 490 nm and the ratio is calculated from these readings, the indo-1 signal is independent of both intracellular indo-1 concentration and cell number (*18*). We converted the indo-1 fluorescence emission ratio (405 nm/490 nm) with excitation at 355 nm to $[Ca^{2+}]_i$ using the method of Grynkiewicz *et al.* (*18*). We determined the maximal emission ratio by lysing cells in 1% Triton X–100 and the minimal ratio by adding EDTA (40 mM).

could not internalize E-IV.3 Fab. Greater truncation of huFc$_\gamma$RIIA abolished all phagocytic function. This differentiation between huFc$_\gamma$RII-mediated internalization of small complexes and large particles may explain some of the conflicting reports concerning the requirement of an $[Ca^{2+}]_i$ flux for huFc$_\gamma$RII-mediated phagocytosis in macrophages (*3, 21*). Apparently, the cytoplasmic tail of huFc$_\gamma$RIIA contains distinct functional regions for initiating the internalization of small complexes versus an $[Ca^{2+}]_i$ flux and the internalization of large particles. Thus, multiple signaling molecules may interact with the cytoplasmic domain. Bifurcation of the signal pathways utilized by huFc$_\gamma$RIIA to activate multiple effectors begins at the level of the receptor.

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25. The huFc$_\gamma$RIIA cDNA clone was generously donated by J. Kochan (Hoffman–La Roche). The truncated huFc$_\gamma$RIIA cDNAs were constructed by oligonucleotide primer–directed in vitro mutagenesis with the PCR (*6*). All cDNAs were sequenced by the Brookdale Molecular Biology Center, Mount Sinai Medical Center, before subcloning into the Eco RI restriction site of pcEXV-3 and contained only the expected mutation. The vector pcEXV-3 contains an SV-40 early gene promoter and polyadenylation signal.
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Low Affinity Interaction of Peptide-MHC Complexes with T Cell Receptors

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The interaction of antigen-specific T cell receptors (TCRs) with their ligands, peptides bound to molecules of the major histocompatibility complex (MHC), is central to most immune responses, yet little is known about its chemical characteristics. The binding to T cells of a labeled monoclonal antibody to the TCR was inhibited by soluble class II MHC heterodimers complexed to different peptides. Inhibition was both peptide- and TCR-specific and of low affinity, with a $K_D = 4 \times 10^{-5}$ to 6×10^{-5} M, orders of magnitude weaker than comparable antibody-antigen interactions. This finding is consistent with the scanning nature of T cell recognition and suggests that antigen-independent adhesion precedes TCR engagement.

THE T CELL RECEPTOR POLYPEPtides occur as either αβ or γδ heterodimers in close association with the monomorphic CD3 polypeptides on the surface of T cells (*1*). Similar to antibodies, T cell receptor genes consist of multiple V-, J-, and D-like gene segments that join together in various combinations to endow individual T cells with unique specificities (*1*). Analyses of TCR sequences suggest that their protein structure is similar to that of immunoglobulin Fab' fragments (*2*). Despite these similarities, however, the integral membrane character of both TCRs and their ligands (*3*) may necessitate very different characteristics of interaction versus antibody-antigen recognition. In addition, self peptide–MHC complexes are also important for TCR selection in the thymus (*4*). To study these interactions better, we have recently expressed both a TCR heterodimer (*5*) and a class II MHC heterodimer, E^k (*6*) in a glycolipid-anchored (GPI) form, allowing ready solubilization by enzymatic cleavage. Cells bearing the GPI-linked E^k can present peptide antigens to most T cell hybridomas with a fine specificity that is indistinguishable from cells expressing wild-type molecules (*6*). In addition, the cleaved, soluble E^k protein can efficiently bind antigenic peptides and stimulate T cells of the appropriate specificity when bound to a plate (*6*).

We have approached the question of TCR affinity for its ligand by measuring the ability of soluble peptide-E^k complexes to compete with a monoclonal antibody (MAb) to the TCR. The formula of Cheng and Prusoff (*7*)

$$K_D^A = \frac{IC_{50}}{1 + ([B]/K_D^B)}$$

allows one to derive the K_D of a competitor, A, for its receptor using a ligand of known K_D and a determination of what concentration of A results in a 50% inhibition (IC_{50}) of B. In this case, the unknown is peptide-MHC and the reference ligand is the MAb KJ25 (*8*) whose binding is ablated by mutations in the CDR2 region of $V_\beta 3$, mutations that also disrupt T cell recognition (*9*). We used the T cell line 5C.C7 (*10*), which recognizes moth cytochrome c (MCC) and the hybridoma 228.5 (*11*), which is specific for a Lys^{99} to Glu^{99} variant, MCC(99E) (*12*). This hybridoma expresses a VDJ_β sequence identical to that of 5C.C7, and its α chain differs only in the V-J junctional re-

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