Phosphorylation-independent Ubiquitylation and Endocytosis of FcγRIIA*

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Endocytosis of the Fc receptor FcγRIIA depends on a functional ubiquitin conjugation system, and the receptor becomes ubiquitylated upon ligand binding. Phosphorylation of tyrosines in FcγRIIA by Src family kinases is thought to be the initiating event in its signaling. However, although the Src family kinase inhibitor PP1 inhibited both ligand-induced phosphorylation of FcγRIIA and phagocytosis in ts20 cells expressing FcγRIIA, it did not inhibit receptor ubiquitylation or endocytosis of soluble ligands. Conversely, genistein and the proteasomal inhibitor MG132 did not inhibit receptor phosphorylation but strongly inhibited both receptor ubiquitylation and endocytosis. A region of the receptor lying within the immunoreceptor tyrosine-based activation motif was found to be necessary for both ubiquitylation and endocytosis. Ubiquitylation occurs at the plasma membrane before internalization. Endocytosis of FcγRIIA is dependent on clathrin but independent of the adaptor protein AP-2. These findings point to a novel mechanism for ubiquitylation and endocytosis of this immunoreceptor.

Although it has long been appreciated that polyubiquitylation of cytoplasmic proteins can target them for degradation by the proteasome, several additional roles for ubiquitylation have emerged. These include functions in the nucleus and in regulating the trafficking of membrane proteins (1). In particular, ubiquitylation of surface receptors can act as a signal both for their endocytosis and their intracellular sorting for degradation in multivesicular bodies (2, 3).

FcγRIIA, a receptor for IgG-containing immune complexes, is widely expressed in human leukocytes (4). Upon binding of IgG-containing complexes, FcγRIIA triggers cell activation and mediates internalization of the immune complexes. We previously showed that cross-linking of FcγRIIA induces ubiquitylation of the receptor (5). Furthermore, endocytosis of soluble immune complexes by FcγRIIA depends on the presence of a functional ubiquitin conjugation system, whereas actin-driven phagocytosis of large antibody-coated particles does not (5).

An important question for understanding the regulation of receptor trafficking is how ligand binding leads to ubiquitylation of the receptor and/or associated proteins. FcγRIIA signals for cell activation by virtue of the presence in its cytoplasmic domain of an immunoreceptor tyrosine-based activation motif (ITAM)2 consisting of the consensus sequence YXXLX(7–12)YXXL. Such motifs are present in activating Fc receptors, the signaling subunits of the T cell and B cell receptors, and other immunoreceptors (4, 6). The earliest step in signaling from ITAM-containing receptors is thought to be phosphorylation of the tyrosine residues in the ITAM by Src family kinases. The phosphorylated ITAM tyrosines then recruit kinases of the Syk/ZAP-70 family and other effectors to initiate downstream responses such as phagocytosis (4).

Furthermore, as ubiquitylation of surface receptors has come to be recognized as a common mechanism for their regulation, a model has emerged wherein receptor ubiquitylation is triggered by, and secondary to, ligand-induced receptor phosphorylation. This is thought to be the case for several plasma membrane proteins in yeast, including Ste2p and Ste6p (7, 8). Similarly, in the case of mammalian receptor tyrosine kinases, the receptors for epidermal growth factor and hepatocyte growth factor (Met) are ubiquitylated through the action of the E3 ubiquitin ligase Cbl after its binding to phosphorytrosines in the activated, autophosphorylated receptors (9, 10).

In light of this current understanding of ITAM-mediated signaling and receptor ubiquitylation, we investigated the relationship between Src family kinase-mediated phosphorylation of FcγRIIA and its ubiquitylation. We found that ubiquitylation and endocytosis of FcγRIIA are independent of the canonical Src family kinase-mediated signaling pathway, indicating a distinct route of signaling downstream of Fc receptors and more generally a novel mechanism for triggering ubiquitylation upon receptor engagement.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Fetal bovine serum, α-minimal essential medium, and G418 were from Wisent (St. Bruno, Canada), and G418 was from Wisent (St. Bruno, Canada). The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; SFK, Src family kinase; aIgG, aggregated IgG; siRNA, small interfering RNA; E3, ubiquitin-protein isopeptide ligase.
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Rabbit anti-Myc antibody A-14 was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to μ2 subunit of AP-2 (AP50) was from BD Biosciences. Mouse anti-Myc 9E10 and anti-ubiquitin antibody P4G7 were from Covance (Berkeley, CA). Monoclonal antibody IV.3 was purified from hybridoma supernatants. 4G10 anti-phosphotyrosine antibody has been previously described (11). Cy3-, Cy5-, and horseradish peroxidase-conjugated secondary antibodies and streptavidin were from Jackson Immunoresearch Laboratories. Rhodamine-transferrin was from Molecular Probes. Paraformaldehyde was from Calbiochem. Supersignal West Pico chemiluminescent substrate, Restore It stripping buffer, and Ultralink protein G beads were from Pierce. Human IgG, protease inhibitors, antiserum, Restore It stripping buffer, and Ultralink protein G beads were from Sigma-Aldrich.

Cell Culture, DNA Constructs, and Transfection—ts20 cells were grown at 34 °C and 5% CO₂ in α-minimal essential medium +10% fetal bovine serum. cDNA for dynamin I K44A was kindly provided by Dr. S. Schmid. cDNA for dynamin II K44A was prepared as described (12). cDNAs for wild-type and mutant forms of FcγRIIA were cloned into pcDNA3.1/Myc-His (Invitrogen) and were expressed with C-terminal Myc-His₉ tags. Truncation and mutagenesis of FcγRIIA was performed by PCR. Transfections were performed with FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Constructs for expression of Myc-tagged ubiquitin and lysine-mutated ubiquitin were kindly provided by Dr. G. Lukacs (Hospital for Sick Children, Toronto). Stable cell lines expressing FcγRIIA and mutant receptors were selected with G418 (0.5 mg/ml). For treatment of cells with inhibitors, cells were pretreated for 2 h (MG132), 1 h (genistein, Me2SO), or 30 min (PP1) by addition of the inhibitor directly to the culture medium at 100 μg/ml (genistein), 30 μM (PP1), or 20 μM (MG132).

siRNA—siRNA sequences targeting the μ2 subunit of AP-2 (μ2–2) and the heavy chain of clathrin (cch-2) were as previously described (13) and were synthesized as Option C siRNAs by Dharmacon; control nonspecific siRNA was also from Dharmacon. Cells were transfected with the siRNAs by nucleofection (Amaxa) on day 0 and day 2; after the second transfection, cells were plated on plastic coverslips (Sarstedt) or in wells and were analyzed on day 4.

Phagocytosis and Endocytosis Assays—For phagocytosis assays, 3 μm polystyrene beads were opsonized with 50 μg of human IgG/100 μl of 1% bead solution in phosphate-buffered saline. ts20-IIA cells were incubated with beads at 34 °C for 30 min and then washed. Beads remaining outside the cells were labeled with Cy3 anti-human antibody. Cells were fixed with 4% paraformaldehyde and cell nuclei labeled with 4′,6-diamidino-2-phenylindole. Phagocytic index (number of particles taken up per 100 FcγRIIA-expressing cells) was determined by microscopy for at least 300 cells per condition in each experiment. For endocytosis assays, human IgG was aggregated at 62 °C for 20 min at a concentration of 10 mg/ml in phosphate-buffered saline, followed by centrifugation at 16,000 × g for 10 min to remove insoluble aggregates. The supernatant was used at the 1:40–1:100 dilution to induce endocytosis by incubation with ts20-IIA cells for 30 min at 34 °C. Cells were then washed; in some experiments, aggregated IgG (aglgG) remaining outside the cell was detected by incubating for 10 min on ice with Cy3- or Cy5-labeled anti-human secondary antibodies before cell permeabilization. Cells were then washed, fixed with 4% paraformaldehyde, and permeabilized with –20 °C methanol, and total aglgG was detected by incubation with Cy3 or Cy5 anti-human antibodies. For experiments in which endocytosis of rhodamine-transferrin was also measured, it was added for the last 10 min of endocytosis at a concentration of 5 μg/ml. Endocytosis of FcγRIIA was also measured by binding receptors with anti-FcγRIIA monoclonal antibody IV.3, cross-linking the IV.3 with goat anti-mouse antibodies to induce endocytosis, and following disappearance of the cross-linking antibody from the cell surface using Cy5 donkey anti-goat antibodies by flow cytometry as previously described (5). Experiments with fluorescently labeled cross-linking antibody confirmed that the disappearance was due to internalization rather than loss into the medium.

Immunoprecipitation and Western Blotting—ts20 cells growing in 10-cm plates were lysed by addition of 1 ml of cold radiolabeled buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 2 mM Na₃VO₄, 10 μM phenylarsine oxide, 50 mM NaF, 20 mM NEM, 0.1% protease inhibitor mixture in phosphate-buffered saline). Cells were removed by scraping, transferred to Eppendorf tubes, and incubated on ice for 20 min. Insoluble material was removed by centrifugation at 16,000 × g, 4 °C for 10 min. The receptor was immunoprecipitated by addition of 1 μg of anti-Myc antibody (A-14) or IV.3 and 25 μl of protein G beads. The samples were nuted overnight at 4 °C and then washed three times with cold TBS-T (TBS with 0.1% Tween 20). The samples were solubilized in Laemmli’s sample buffer and resolved by SDS-PAGE. For Western blotting, the samples were transferred to nitrocellulose membranes (Bio-Rad), which were then blocked overnight in 3% bovine serum albumin in TBS. The membranes were probed by incubating for 3 h with primary antibody at a 1:1000 dilution in blocking buffer, followed by a 30-min incubation with anti-mouse horseradish peroxidase or streptavidin-horseradish peroxidase when using a biotinylated 4G10 primary antibody. Membranes were washed in TBS-T, developed using Supersignal West Pico chemiluminescent substrate, and visualized using CL-Xposure film (Pierce) or a Genius2 Bioimager (Syngene). Quantitation was performed using Genetools software (Syngene) or by scanning films using a GS-800 densitometer with Quantity One software (Bio-Rad).

Microscopy and Immunofluorescence—Fixed and mounted cells were viewed using a Zeiss Axiovert 200M microscope with a ×40 oil immersion objective and an ORCA ER camera (Hamamatsu). Green fluorescent protein, rhodamine/Cy3, Cy5, and 4′,6-diamidino-2-phenylindole were visualized using standard filter sets.

RESULTS

Endocytosis of FcγRIIA Is Independent of Src Family Kinases—We tested the effects on FcγRIIA-mediated internalization processes of inhibition of Src family kinases (SFK) using the
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FIGURE 1. Endocytosis of FcyRIIA does not depend on Src family kinase activity. A, ts20 cells expressing FcyRIIA (ts20-IIA) were incubated for 30 min with aggregated human IgG (aglG) in the presence of MeSO carrier (control) (A), 30 μM PP1 (B), or 100 μg/ml genistein (C). aglG was detected with secondary antibodies. Scale bar, 20 μm. Images are representative of five experiments. D, ts20-IIA cells were incubated with or without aglG for 10 min in the absence or presence of 30 μM PP1 or 100 μg/ml genistein as indicated. Receptors were immunoprecipitated from cell lysates with rabbit anti-Myc antibodies and blotted with anti-phosphotyrosine antibody 4G10 or mouse anti-Myc. The extent of basal (unstimulated) phosphorylation varied between experiments; the effects of inhibitors are representative of five experiments. E, phagocytosis of IgG-coated 3 μm beads (white bars) and endocytosis of cross-linking antibodies (black bars; measured by flow cytometry as described under “Experimental Procedures”) were assessed in ts20-IIA cells treated under the same conditions as in panels A–C as indicated. Phagocytosis was quantified as phagocytic index and is expressed as percent of control; endocytosis is expressed as amount of antibody internalization in 10 min as percent of control (n = 3 ± S.D. for each).

specific SFK inhibitor PP1. For these experiments, we used Chinese hamster ts20 cells stably transfected with FcyRIIA (henceforth termed ts20-IIA cells). We have previously shown that expression of FcyRIIA in these cells supports both phagocytosis of antibody-coated particles and endocytosis of soluble immune complexes (5). Endocytosis leads to the delivery of both receptor and ligand to lysosomal compartments (5). This heterologous transfection model has the advantage of allowing the study of cells expressing a single defined FcyR species or mutant versions thereof. As expected, treatment of the ts20-IIA cells with PP1 strongly inhibited both cross-linking-induced phosphorylation of FcyRIIA (Fig. 1D) and phagocytosis of large IgG-opsonized particles (Fig. 1E). These findings are consistent with the standard model of signaling for FcR-mediated phagocytosis, in which SFK-mediated receptor phosphorylation is a crucial initiating step (14). We then assessed endocytosis of soluble complexes of aglG mediated by FcyRIIA under the same conditions. Notably, uptake of aglG into endosomes proceeded unimpaired in the presence of PP1 (Fig. 1, A and B), as did endocytosis of cross-linking antibody in a flow cytometry-based uptake assay (5) (Fig. 1E). These observations imply that SFK activity, although essential for phosphorylation of large particles, is dispensable for endocytosis of small soluble ligands.

It was previously reported that FcyRIIA endocytosis is dependent on tyrosine kinase activity, based on its inhibition by the tyrosine kinase inhibitor genistein (15, 16). In light of these earlier observations, we assessed the effect of genistein in our system. Consistent with the previous findings, genistein strongly inhibited endocytosis of aglG (Fig. 1, C and E). Treatment with genistein, however, did not inhibit phosphorylation of the receptor (Fig. 1D) and, consistent with this, had little if any effect on phagocytosis (Fig. 1E). Thus, PP1 and genistein have opposite effects on endocytosis and phagocytosis, underscoring the mechanistic differences between these two modes of FcR-mediated ligand uptake (5).

Ubiquitylation of FcyRIIA Is Independent of Src Family Kinases—Given the previously demonstrated dependence of endocytosis on ubiquitylation, these results imply that ubiquitylation can occur in an SFK-independent manner. To address this, the receptor was immunoprecipitated and its phosphorylation and ubiquitylation examined by immunoblotting. Cross-linking of the receptor upon addition of aggregated IgG led to both tyrosine phosphorylation and ubiquitylation of the receptor (Fig. 2, A and B). It is noteworthy that the major species of ubiquitylated and phosphorylated receptor are distinct entities, based on their difference in molecular weight (Fig. 2, A and B). Although treatment with PP1 inhibited receptor tyrosine phosphorylation, the receptor was still robustly ubiquitylated, indicating that receptor phosphorylation is not required for its ubiquitylation (Fig. 2, A–D). Genistein had opposite effects, corresponding to what was seen for FcyRIIA internalization. Although treatment with genistein did not inhibit phosphorylation of the receptor, it largely abolished ubiquitylation of the receptor (Fig. 2, A–D), correlating with its effect on endocytosis.

Ubiquitylation of FcyRIIA Is Blocked upon Inhibition of the Proteasome—The function of ubiquitylation in trafficking of membrane proteins is generally thought to be distinct from its role in targeting cytoplasmic proteins to the proteasome. Nonetheless, we previously showed that endocytosis of FcyRIIA is inhibited by treatment with clasto-lactacystin β-lactone, a specific inhibitor of the proteasome (5). We have observed similar inhibition of endocytosis upon treatment with MG132, another proteasomal inhibitor; notably, maximal inhibition requires more than an hour of pretreatment (data not shown). We had suggested that inhibition by proteasomal inhibitors could be due to an indirect effect on receptor ubiquitylation, e.g. through depletion of free ubiquitin in the cell (5). Consistent with this model, treatment with MG132 largely abolished receptor ubiquitylation, whereas receptor tyrosine phosphorylation was not inhibited (Fig. 2, A–D). This suggests that the ubiquitylated receptor is not itself a substrate for proteasomal degradation, in which case the amount of ubiquitylated receptor would be expected to increase rather than decrease. Degradation of the
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FIGURE 2. Characterization of FcγRIIA ubiquitylation. A–C, ts20-IIA cells were incubated with or without agIgG for 10 min in the absence or presence of 30 μM PP1, 100 μg/ml genistein, or 20 μM MG132 as indicated. Receptors were immunoprecipitated from cell lysates with rabbit anti-Myc antibodies, and immunoprecipitates were divided and analyzed by SDS-PAGE and Western blotting with anti-ubiquitin antibody P4G7 (A) or anti-phosphotyrosine antibody 4G10 (B). C, blot from panel A was stripped and reprobed with mouse anti-Myc antibodies. Arrows in panels A–C indicate position of ubiquitylated, phosphorylated, and unmodified receptors, respectively. Some spreading of the bands in panels B and C occurs because of comigration with IgG heavy chain. D, quantitation of ubiquitylation (black bars) and phosphorylation (white bars) as in panels A–C from four experiments. Error bars indicate S.D. Levels are expressed relative to that seen with agIgG in the absence of inhibitors. Asterisks indicate significant differences from this condition (p < 0.05). E, ts20-IIA cells were incubated with (+) or without (−) agIgG, and receptors were immunoprecipitated followed by Western blotting with antibodies FK-1 or P4G7. lys, total cell lysate from MG132-treated cells. F, ts20 cells stably transfected with untagged FcγRIIA were transiently transfected with constructs expressing wild type (wt), K48R, or K63R Myc-tagged ubiquitin and treated with agIgG as indicated. Receptors were immunoprecipitated with anti-FcγRIIA antibody IV.3 and blotted with anti-Myc to detect receptor ubiquitylation.

receptor after agIgG addition is inhibited by MG132 treatment (data not shown), but this is presumably an indirect consequence of the block of internalization preventing delivery of the receptor to lysosomal compartments (5).

The molecular weight shift of ubiquitylated FcγRIIA could reflect polyubiquitylation (addition of chains of ubiquitin) and/or multiple monoubiquitylation. To address this, immunoprecipitated FcγRIIA was blotted with antibody FK-1, which only recognizes polyubiquitylated proteins (17), or with P4G7, which recognizes both monoubiquitylated and polyubiquitylated proteins. Ubiquitylated FcγRIIA was detected upon probing with FK-1 (Fig. 2E), indicating that FcγRIIA ubiquitylation includes polyubiquitylation. Polyubiquitin chains can form through linkages involving different lysines in ubiquitin. Expression of mutant forms of ubiquitin in which either lysine 48 or lysine 63 was mutated to arginine did not affect the pattern of ubiquitylation observed (Fig. 2F), suggesting that if polyubiquitin chains are a significant component of FcγRIIA ubiquitylation they do not occur obligatorily through either

\[ \text{Lys-48 or Lys-63 but rather may occur through both or through other lysines.} \]

A Region of FcγRIIA within the ITAM Is Required for Endocytosis—The experiments using PP1 indicated that receptor tyrosine phosphorylation is not required for endocytosis. We also sought to test directly whether the tyrosines present in FcγRIIA play any role in this process. There are three tyrosines in the cytoplasmic domain of FcγRIIA; Tyr-282 and Tyr-298 lie within the ITAM and an additional tyrosine lies at residue 275 (Fig. 3A). Mutant versions of FcγRIIA in which these tyrosines were replaced individually or pairwise by phenylalanyl were expressed in ts20 cells, and their ability to support phagocytosis and endocytosis was tested. Phagocytosis was observed with both a Y275,298F double mutant (96 ± 31% of the level seen for wild type; n = 3) and a Tyr-282 single mutant (16 ± 0.7% of wild type; n = 3). For endocytosis, the Y275,298F double mutant gave uptake very similar to the wild-type receptor as judged by microscopic analysis of uptake of agIgG (Fig. 3, B and C) or by flow cytometry (Fig. 3E). Moreover, truncation of the receptor in the middle of the ITAM after residue 286 also did not impair endocytosis (Fig. 4C) or affect its dependence on ubiquitylation (data not shown). Tyr-282, however, does appear to play an important role in endocytosis. The Y282F mutant receptor is strongly impaired in its endocytosis upon addition of aglG (Fig. 3D) or after receptor cross-linking with antibodies (Fig. 3E).

It would seem that SFK-mediated tyrosine phosphorylation of FcγRIIA is not necessary for its endocytosis, and yet the presence of Tyr-282 is nonetheless required. We noted that, in addition to being one of the ITAM tyrosines, Tyr-282 lies within a motif of the form YXXΦ, where Φ is a bulky hydrophobic residue. Such motifs often act as targeting signals for endocytosis by virtue of their binding to the clathrin-associated adaptor protein AP-2 (18). To further explore this possibility, additional mutagenesis of residues in the vicinity of Tyr-282 was performed, either in the context of the full-length receptor or of the truncated receptor that still had fully functional endocytosis. Mutation of Leu-285 to alanine also impaired endocytosis (Fig. 4, B and F), whereas mutation of Thr-284 to alanine had no effect (Fig. 4, A and F), consistent with Y282MTL functioning as an AP-2 binding motif. However, Met-283 also appeared to be important for endocy-
tosis, in that mutation of this residue to either Ala or Ser also inhibited endocytosis (Fig. 4, D–F).

Endocytosis of FcγRIIA Is Dependent on Clathrin but Independent of AP-2—Given that the identity of the residue in the Tyr+1 position would be unlikely to strongly affect AP-2 binding (18), we directly addressed whether there is a requirement for AP-2 in FcγRIIA endocytosis by using siRNA to down-regulate AP-2 expression. Effective knock down of AP-2 was confirmed by Western blotting (Fig. 5G). Importantly, as a functional assay we confirmed that the knockdown of AP-2 was sufficient to strongly inhibit endocytosis of transferrin (Fig. 5C). Notably, however, endocytosis of aglG by FcγRIIA was not inhibited under these conditions (Fig. 5D), indicating that this adaptor is not essential for FcγRIIA uptake.

Although AP-2 has been considered the major adaptor for clathrin-mediated endocytosis, clathrin-dependent uptake of several receptors has been reported to occur in the absence of AP-2, and it was speculated that such uptake might occur through alternative interactions such as those mediated by ubiquitination (13). Endocytosis of aglG by FcγRIIA in ts20 cells was inhibited by potassium depletion and hypertonic shock, commonly used means of disrupting clathrin-mediated endocytosis; conversely, filipin, which can disrupt clathrin-independent endocytosis (19), only inhibited FcγRIIA endocytosis when used at levels that also blocked clathrin-mediated transferrin uptake (data not shown). To more specifically address the clathrin-dependence of FcγRIIA endocytosis, we also knocked down clathrin expression using siRNA. The efficiency of knockdown was incomplete, with only some fields and groups of cells showing strong inhibition of transferrin uptake. However, in contrast to the lack of effect of AP-2 knockdown, the cells with inhibited transferrin uptake also showed a block in uptake of aglG into endosomes (Fig. 5, E and F). This is also consistent with previous studies showing a strong inhibition of FcγRIIA endocytosis by antisense-mediated knockdown of clathrin (20). Thus, endocytosis of FcγRIIA appears to be dependent on ubiquitylation and clathrin but independent of AP-2.

Ubiquitylation Occurs Early during Endocytosis—Although the apparent dependence of endocytosis on ubiquitylation suggests an early role for the process, it is possible that ubiquityla-

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**FIGURE 3. Role of tyrosines in endocytosis of FcγRIIA.** A, sequence of the C terminus of FcγRIIA. B–D, endocytosis of aglG was assayed in ts20 cells expressing wild-type receptor (B), Y275,298F mutant receptor (C), or Y282F mutant receptor (D). Images are representative of five experiments. E, endocytosis of the same FcγRIIA mutants expressed in ts20 cells was assessed by flow cytometry. Internalization of cross-linking antibody is expressed as percent of initial bound (n = 3 ± S.D.). Y282F and wild-type receptor showed similar expression levels (mean fluorescence intensities of 608 and 739 when analyzed by flow cytometry).

**FIGURE 4. Identification of a region of FcγRIIA important for endocytosis.** A–E, ts20 cells expressing FcγRIIA mutants containing the indicated sequences at residues 282–285 were incubated with aglG for 30 min. The receptors in panels C–E were in addition truncated after residue 286 (trunc). Images are representative of two experiments. F, endocytosis of the same receptors as in panels A–E assessed by flow cytometry and expressed as percent uptake of initial bound cross-linking antibody in 10 min (n = 3, ± S.D.). Asterisks indicate significant inhibition relative to YMTLtrunc (p < 0.05). Expression levels of all receptors were comparable (mean fluorescence intensity of 500 ± 60).
tion might occur after internalization and that its role might instead be to prevent receptor recycling to the plasma membrane from an internalized pool. To address this question, we inhibited progression of endocytosis by expression of dominant negative dynamin. We verified that expression of the dominant negative K44A mutant of dynamin II inhibited transferrin uptake (Fig. 6, A and B), as expected for a clathrin-dependent process. Endocytosis of FcγRIIA is also inhibited by dominant negative dynamin (Fig. 6, A and C). The extent of receptor ubiquitylation upon ag IgG addition in dynK44A-transfected cells was examined. Even with less than 50% transfection efficiency, receptor ubiquitylation was found to increase by 33 ± 13% (n = 3) relative to that in untransfected cells (Fig. 6G, lane 3 versus lane 2). The fact that ubiquitylation increased rather than decreased implies that ubiquitylation occurs at an early step, before internalization from the plasma membrane.

The Region of FcγRIIA Required for Endocytosis Is Also Necessary for Ubiquitylation—Because the role in endocytosis of the region within the FcγRIIA ITAM does not appear to consist of recruiting AP-2, we assessed whether this region of the receptor instead might be involved in triggering receptor ubiquitina...
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ubiquitylation. Indeed, the mutations that impaired endocytosis also all caused a loss of ubiquitylation (Fig. 6G). This suggests that a loss of ubiquitylation accounts for the impaired endocytosis of these mutants.

DISCUSSION

In this work, evidence is presented that ubiquitylation of FcγRIIA upon receptor clustering occurs in a manner independent of SFK-mediated receptor phosphorylation. This is surprising in several respects. On one hand, SFK-mediated FcR phosphorylation is generally regarded as the earliest event triggered after receptor aggregation, and virtually all responses to FcR cross-linking would be expected to be blocked by inhibition of SFKs. Indeed, we confirmed that the uptake of large particles through phagocytosis by FcγRIIA requires SFK activity. Moreover, in multiple systems in which ubiquitylation has emerged as a signal determining receptor trafficking it is thought that the initiating event that drives receptor ubiquitylation is receptor phosphorylation. This is the case for the mammalian receptor tyrosine kinases such as the receptors for epidermal growth factor and scatter factor (9, 10). Similarly, endocytosis of multiple proteins in yeast proceeds through a pathway that involves an ordered process of phosphorylation followed by ubiquitylation (7, 8). In contrast, our findings indicate that tyrosine phosphorylation of FcγRIIA by SFK, although necessary for signaling for phagocytosis, is not required for ubiquitylation or for endocytosis of soluble immune complexes.

Proteasome inhibitors inhibit endocytosis of FcγRIIA (5). We have found that this is likely due to an indirect effect on receptor ubiquitylation. Inhibition of endocytosis by proteasome inhibitors has also been seen for the growth hormone receptor, the leptin receptor OB-Ra, and the gap junction protein connexin-43 (21–23). In these cases also the effect is likely indirect, because in each case receptor ubiquitylation was also impaired (21–23). These results emphasize that it is important to consider possible effects on trafficking events such as endocytosis when interpreting the outcomes of proteasome inhibition.

It is not clear what target of genistein accounts for its inhibitory effects on ubiquitylation and endocytosis. Clearly, it is not the SFK that phosphorylate the receptor, as genistein treatment did not inhibit receptor phosphorylation or phagocytosis. A lack of effect of high concentration genistein on SFK-mediated phosphorylation has also been reported in the context of T cell receptor signaling (24). Although it is possible that there could be a distinct tyrosine kinase involved in ubiquitylation and endocytosis, the effects of genistein were only observed at relatively high concentrations (>30 μg/ml; data not shown), suggesting that a target other than a tyrosine kinase is likely involved.

For the epidermal growth factor receptor, it has been reported that the receptor becomes multiply monoubiquitylated upon activation (17, 25), but recent studies using mass spectrometry reveal a large amount of polyubiquitylation (26). For FcγRIIA, both mono- and polyubiquitylation may contribute to endocytosis. We do not find evidence that polyubiquitin chains are formed obligatorily through either Lys-48 or Lys-63 linkages. Recent findings suggest that multiple, complex chain topologies can occur during polyubiquitylation (27) and furthermore that different topologies can support recruitment of ubiquitin-binding proteins and endocytosis (27, 28). Thus, although the presence of multiple ubiquitins seems to be important for endocytosis (28), their exact orientation may be less so.

A region lying within the ITAM appears to be required for both ubiquitylation and endocytosis. The simplest interpretation is that this region is involved in recruiting a ubiquitin ligase necessary for progression of endocytosis. The exact relationship between ubiquitylation and endocytosis of plasma membrane proteins has been unclear. Clathrin-independent uptake of an epidermal growth factor receptor-ubiquitin chimeric protein has been reported (19). However, in the case of FcγRIIA, uptake appears to be dependent on both the ubiquitylation machinery and clathrin, consistent with recent findings of clathrin- and ubiquitin-dependent endocytosis of other proteins (22, 28, 29). Notably, the AP-2 adaptor protein appears not to be required for FcγRIIA endocytosis. Because the knockdown of AP-2 is not complete, we cannot exclude that FcγRIIA endocytosis might require interaction with AP-2 but occur with a higher affinity than for the transferrin receptor. However, AP-2-independent uptake is consistent with ubiquitylation itself being sufficient to recruit the endocytic machinery, as has been seen with AP-2-independent endocytosis of model proteins containing ubiquitin as their sole internalization signal (28).

Ubiquitylation can lead to recruitment of several ubiquitin-interacting motif-containing proteins implicated in endocytosis, including epsin and eps15. Because of the low affinity of the ubiquitin-interacting motifs in these proteins, the presence of multiple ubiquitins, either on an individual protein or provided in trans on oligomerized receptors, appears to be necessary to allow for significant binding (28, 29). This suggests one possible means by which receptor clustering could directly lead to an increase in ubiquitylation in a manner independent of SFK-mediated phosphorylation. It is possible that there is some degree of constitutive ubiquitylation of FcγRIIA that is opposed by continuous removal of ubiquitin by deubiquitinating enzymes, so that there is little steady-state ubiquitylation of non-cross-linked receptors. Oligomerization of partially ubiquitylated receptors might allow sufficient binding of ubiquitin-interacting motif-containing proteins to stabilize receptor ubiquitylation by protecting it from deubiquitinating enzymes. This would be analogous to a model proposed for FcεRI signaling in which receptor phosphorylation upon multimerization occurs through exclusion of phosphatases (30). In this way, ubiquitylation and the initiation of endocytosis would be highly cooperative processes.

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REFERENCES
1. Schnell, J. D., and Hicke, L. (2003) J. Biol. Chem. 278, 35857–35860
2. Hicke, L., and Dunn, R. (2003) Annu. Rev. Cell Dev. Biol. 19, 141–172
3. Raiborg, C., Rusten, T. E., and Stenmark, H. (2003) Curr. Opin. Cell Biol.
4. Daeron, M. (1997) Annu. Rev. Immunol. 15, 203–234
5. Booth, J. W., Kim, M. K., Jankowski, A., Schreiber, A. D., and Grinstein, S. (2002) EMBO J. 21, 251–258
6. Cannons, J. L., and Schwartzberg, P. L. (2004) Curr. Opin. Immunol. 16, 296–303
7. Hicke, L., Zanolari, B., and Riezman, H. (1998) J. Cell Biol. 141, 349–358
8. Kelm, K. B., Huyer, G., Huang, J. C., and Michaelis, S. (2004) Traffic 5, 165–180
9. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
10. Peschar, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. (2001) Mol. Cell 8, 995–1004
11. Kanakura, Y., Druker, B., DiCarlo, J., Cannistra, S. A., and Griffin, J. D. (1991) J. Biol. Chem. 266, 490–495
12. Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A., and Aderem, A. (1999) J. Exp. Med. 190, 1849–1856
13. Motley, A., Bright, N. A., Seaman, M. N., and Robinson, M. S. (2003) J. Cell Biol. 162, 909–918
14. Greenberg, S., and Grinstein, S. (2002) Curr. Opin. Immunol. 14, 136–145
15. Davis, W., Harrison, P. T., Hutchinson, M. J., and Allen, J. M. (1995) EMBO J. 14, 432–441
16. Ghazizadeh, S., and Fleit, H. B. (1994) J. Immunol. 152, 30–41
17. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) Nat. Cell Biol. 5, 461–466
18. Bonifacino, J. S., and Traub, L. M. (2003) Annu. Rev. Biochem. 72, 395–447
19. Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P., and Polo, S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2760–2765
20. Tse, S. M., Furuya, W., Gold, E., Schreiber, A. D., Sandvig, K., Inman, R. D., and Grinstein, S. (2003) J. Biol. Chem. 278, 3331–3338
21. van Kerkhof, P., Govers, R., Alves dos Santos, C. M., and Strous, G. J. (2000) J. Biol. Chem. 275, 1575–1580
22. Belouard, S., and Rouille, Y. (2006) EMBO J. 25, 932–942
23. Leithe, E., and Rivedal, E. (2004) J. Biol. Chem. 279, 50089–50096
24. Graber, M., June, C. H., Samelson, L. E., and Weiss, A. (1992) Int. Immunol. 4, 1201–1210
25. Mosesson, Y., Shtieglman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J., and Yarden, Y. (2003) J. Biol. Chem. 278, 21323–21326
26. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) Mol. Cell 21, 737–748
27. Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R. W., and Gygi, S. P. (2006) Nat. Cell Biol. 8, 700–710
28. Barriere, H., Nemes, C., Lechardeur, D., Khan-Mohammad, M., Fruh, K., and Lukacs, G. I. (2006) Traffic 7, 282–297
29. Hawryluk, M. J., Keyel, P. A., Mishra, S. K., Watkins, S. C., Heuser, J. E., and Traub, L. M. (2006) Traffic 7, 262–281
30. Young, R. M., Holowka, D., and Baird, B. (2003) J. Biol. Chem. 278, 20746–20752