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Cell surface control of the multiubiquitination and deubiquitination of high-affinity immunoglobulin E receptors

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Multiubiquitination of proteins is a critical step leading to selective degradation for many polypeptides. Therefore, activation-induced multiubiquitination of cell surface receptors, such as the platelet-derived growth factor (PDGF) receptor and the T cell antigen (TCR) receptor, may correspond to a degradation pathway for ligand-receptor complexes. Here we show that the antigen-induced engagement of high-affinity immunoglobulin E receptors (FcεRI) results in the immediate multiubiquitination of FcεRI β and γ chains. This ubiquitination is independent of receptor phosphorylation and is restricted to activated receptors. Surprisingly, receptor multiubiquitination is immediately reversible when receptors are disengaged. Therefore, multiubiquitination and deubiquitination of FcεRI receptors is controlled at the cell surface by receptor engagement and disengagement. The rapidity, specificity and, most importantly, the reversibility of the activation-induced receptor multiubiquitination suggest that this process may turn on/off a cell surface receptor signaling function thus far unsuspected.

Key words: deubiquitination/IgE receptor/multiubiquitination/signaling/transmembrane protein

Introduction

Cell surface receptors are increasingly recognized as complexes of multiple subunits. Some of these subunits are used for ligand binding occurring on the cell surface, whereas the others are used for intracellular signaling. In this regard, the immune system has developed a rich variety of antigen-binding receptors. Among these are the T cell antigen (TCR) and the B cell antigen receptors, and Fc receptors such as the high-affinity receptor for immunoglobulin E (FcεRI) and one of the low-affinity receptors for IgG (FcγRIII, CD16). The ligand-binding structures of this class of receptors are distinctive, although they all contain immunoglobulin-like domains (Williams and Barclay,1988; Ravetch and Kinet, 1991). Their signaling subunits share homologous cytoplasmic amino acid motifs which are critical to the process of cell activation (Reth, 1989; Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Romeo and Seed, 1991; Romeo *et al.*, 1992; Wegener *et al.*, 1992). Unlike the growth factor receptors, these receptors lack intrinsic kinase activity. However, within seconds of their engagement with antigen, these receptors activate non-receptor tyrosine and serine/threonine kinases which lead to the phosphorylation of various substrates, including the receptor subunits (Hsi *et al.*, 1989; Benhamou *et al.*, 1990, 1992; June *et al.*, 1990a; Paolini *et al.*, 1991).

The high-affinity receptor for immunoglobulin E (FcεRI) represents a versatile model for studying the function of this class of kinase-coupled receptors. An advantage of this receptor system is that it allows one to study the effect of both receptor engagement and disengagement. This is possible because the ligand IgE binds to its receptor without triggering any detectable intracellular signals. Cell activation requires the binding of multivalent antigens to the receptor-bound IgE and continuous aggregation of receptors. After binding of hapten-specific IgE to the receptor, it is therefore possible to engage receptors with a specific multivalent hapten and to disengage receptors with an excess of monovalent hapten.

FcεRI is a tetrameric complex composed of a ligand-binding α chain, a β chain and a homodimer of γ chains (Kinet, 1990; Ravetch and Kinet, 1991). The γ chains contain a motif of 12 amino acid residues, including two tyrosines, which are essential in cell signaling (Letourneur and Klausner, 1991; Amigorena *et al.*, 1992; Bonnerot *et al.*, 1992; Kinet, 1992; Wirthmueller *et al.*, 1992). The FcεRI γ chains are part of other receptors such as FcγRIII (Hibbs *et al.*, 1989; Ra *et al.*, 1989; Anderson *et al.*, 1990; Letourneur *et al.*, 1991) and TCR (Lanier *et al.*, 1989; Orloff *et al.*, 1990), and are homologous to the TCR ζ chains which are also critical in signal transduction (Weissman *et al.*, 1988). The role of the FcεRI β chain is at present unclear, but this subunit, which crosses the membrane four times and contains two long cytoplasmic domains, may also play some role in transmembrane signaling (Alber *et al.*, 1991). Engagement of FcεRI leads to the immediate phosphorylation of β (serine and tyrosine) and γ (threonine and tyrosine). The phosphorylation signal is specific for activated FcεRI receptor complexes and is immediately reversible upon receptor disengagement (Paolini *et al.*, 1991). Furthermore, FcεRI activates at least two distinct phosphorylation pathways: one leading to phosphorylation and presumably activation of phospholipase C-γ1, and the other to phosphorylation of the receptor and of various undefined substrates (Benhamou *et al.*, 1990, 1992; Park *et al.*, 1991; Adamczewski *et al.*, 1992; Li *et al.*, 1992; Schneider *et al.*, 1992). The rapid phosphorylation/dephosphorylation of FcεRI seems to be a mechanism to couple/uncouple the receptor with specific, yet undefined, substrates, which include a 125 kDa protein (Paolini *et al.*, 1992).

The covalent addition of the 8.5 kDa ubiquitin to proteins is a common means used by eukaryotic cells to target these proteins to degradation (Hershko, 1988; Finley and Chau, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992). The addition of a single ubiquitin moiety is sometimes sufficient

for degradation, but multiubiquitination is required in most cases (Chau *et al.*, 1989; Gregori *et al.*, 1990). Recently, ligand-induced multiubiquitination of the platelet-derived growth factor (PDGF) receptor (Mori *et al.*, 1992) and of the TCR (Cenciarelli *et al.*, 1992) has been reported. A stimulation of several minutes seems to be required to detect receptor multiubiquitination, a period of time which would correspond well with what might be expected for a process of degradation. Now we have found that multiubiquitination of the FcεRI signaling subunits, the β and γ chains, occurs within seconds of receptor engagement and, moreover, is reversible upon receptor disengagement. These characteristics indicate that this process is controlled at the cell surface and suggest a novel role for ubiquitination in receptor-mediated signaling.

Results

Apparent association of multiple phosphoproteins with activated FcεRI

Rat basophilic leukemia cells (RBL-2H3) were incubated with anti-dinitrophenyl (DNP) IgE, labeled *in vivo* with [^{32}P]orthophosphate and stimulated (or not) for 30 s with a multivalent antigen dinitrophenyl[30]−human serum albumin (DNP[30]−HSA). After cell lysis, the cell surface FcεRI receptors were immunoprecipitated with a polyclonal anti-IgE antibody and resolved on two-dimensional gels, the first dimension being a non-equilibrium pH gradient electrophoresis (NEPHGE) and the second an SDS−PAGE (Figure 1). As shown before, the phosphorylated 30 kDa doublet of the β chain is detected in resting cells, while γ chains are only poorly labeled (Figure 1, left panel). In fact, in the exposure shown here the γ chains are not visualized. An additional faint doublet around 55 kDa is also detected under these conditions. After antigen triggering, the phosphorylation of the β chain increases in intensity and a number of new phosphorylated species appear above the β chain around 40, 48 and 66 kDa (Figure 1, right panel). The 55 kDa doublet observed under resting conditions is seen with approximately the same intensity and a new 66 kDa species, more acidic than the one described above, is clearly detected after activation. In the lower part of the gel, the phosphorylated γ chains can now be clearly seen around 14 kDa, and additional species also appear above the γ chains. Thus, a number of phosphoproteins co-precipitate with surface FcεRI following its activation with antigen.

Antigen-mediated multiubiquitination of surface-expressed β and γ chains

To identify with certainty which of the phosphorylated bands described above correspond to the β and γ chains, the immunoprecipitates of FcεRI were also immunoblotted with anti-β (Figure 2, panels A and C) and anti-γ antibodies (Figure 2, panels B and D). Surprisingly, this analysis reveals that many β- and γ-immunoreactive proteins become apparent after antigen stimulation. We compared by two-dimensional peptide mapping the bona fide β (labeled A) with the 40 kDa β-immunoreactive species (labeled B), and the bona fide γ (labeled C) with one of the γ-immunoreactive species (labeled D). It is clear that β and the 40 kDa protein on the one hand, and γ and the γ-immunoreactive species on the other hand, share common segments (Figure 3). This analysis confirms that the larger β- and γ-immunoreactive proteins contain at least β and γ.

The regularity in the increase of the molecular mass of these β- and γ-containing proteins prompted us to investigate whether β and γ could be covalently modified by ubiquitin, a 8.5 kDa protein. Immunoprecipitates of cell surface-expressed FcεRI were analyzed by two-dimensional NEPHGE−PAGE and immunoblotted with anti-ubiquitin antibody. No relevant bands are seen in resting conditions (Figure 2, panels E and F). After activation,

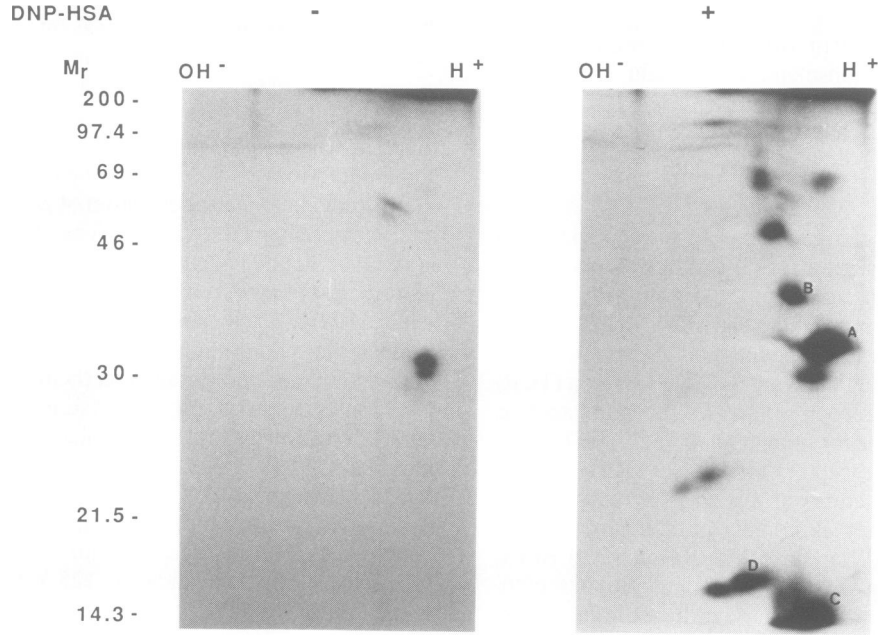

Fig. 1. Aggregation of FcεRI in RBL-2H3 cells induces apparent phosphorylation of various polypeptides associated with β and γ chains. RBL-2H3 cells were saturated with mouse anti-DNP IgE, labeled with [^{32}P]orthophosphate and then incubated at 37°C for 30 s with medium alone (**left panel**) or with DNP[30]−HSA (**right panel**). Immunoprecipitates with an anti-IgE rabbit polyclonal antibody were resolved by NEPHGE−PAGE (13.5% slab gel) under reducing conditions and autoradiography. M_r indicates the relative molecular mass.

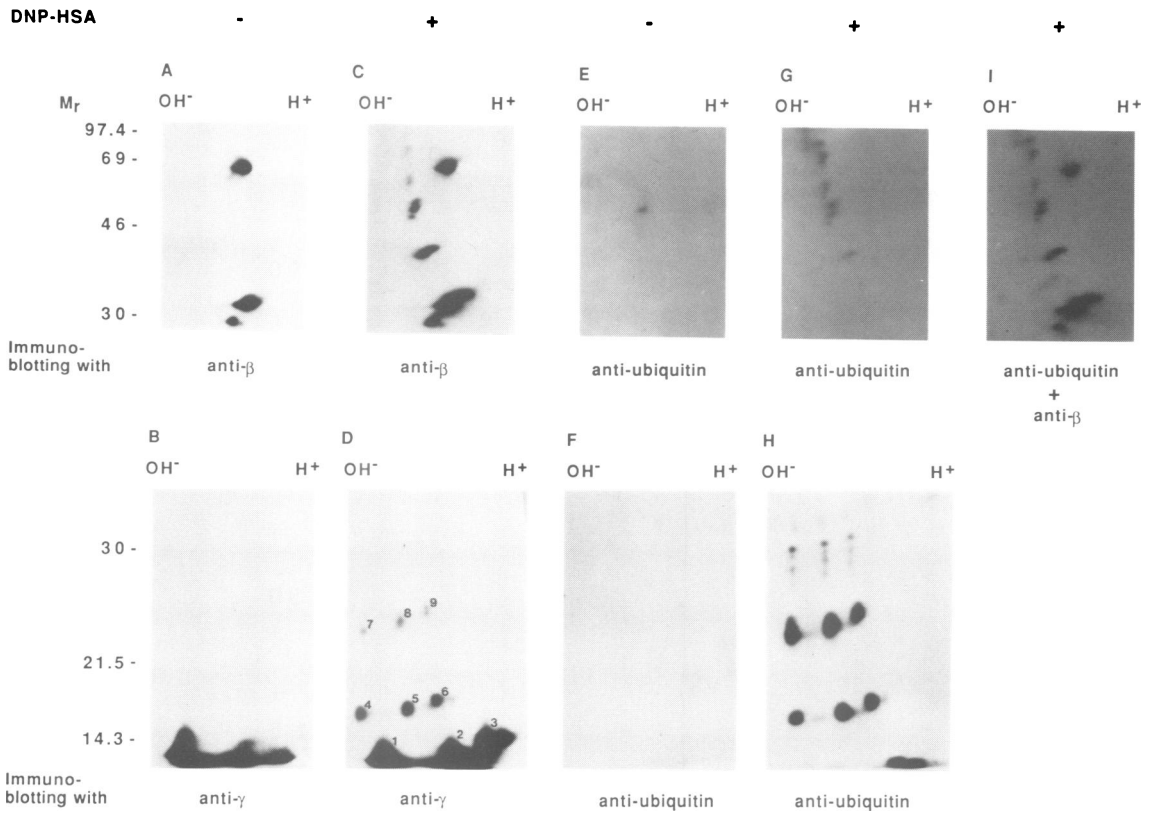

Fig. 2. Aggregation of FcεRI induces multiubiquination of β and γ chains. RBL cells were incubated at 37°C for 30 s in the absence (**A**, **B**, **E** or **F**) or presence (**C**, **D**, **G**, **H** and **I**) of 1 μg/ml DNP−HSA. Lysates were immunoprecipitated with polyclonal anti-IgE antibody and resolved by NEPHGE−PAGE (13.5% slab gels). Proteins were transferred to Immobilon, immunoblotted and detected with enhanced chemiluminescence. Panels A and C, anti-β blotting; panels B and D, anti-γ blotting; panels E−H, anti-ubiquitin blotting; panel I, anti-ubiquitin blotting followed by anti-β blotting. Mol. wt standards are indicated to the left.

the anti-ubiquitin antibody reacts strongly with all of the γ-immunoreactive species, including the non-phosphorylated forms (panel H). Additional bands of higher mol. wt are also detected. The position and distribution of these bands strongly suggest that they correspond to additional forms of ubiquitinated γ which are not seen with anti-γ because of their low abundance. The anti-ubiquitin antibody reacts less strongly with the β-immunoreactive species (panel G). For this reason, the anti-ubiquitin immunoblot was further reacted with anti-β in order to show an exact correspondence between the β-immunoreactive and the ubiquitinated proteins (panel I). This analysis shows that β, like γ, becomes ubiquitinated after activation. Furthermore, the incremental increase in the molecular mass of both β and γ indicates that both chains are in fact multiubiquitinated.

Receptor phosphorylation and ubiquitination occur independently

Comparison of Figure 1 (right panel) and Figure 2 (panels D and H) indicates that there are more ubiquitinated than phosphorylated forms of γ. Our interpretation of the data is as follows: the γ species #1 is recognized by the anti-γ, but not by the anti-ubiquitin antibody, does not appear to be phosphorylated and therefore must correspond to the non-ubiquitinated, non-phosphorylated form; the γ species #2 and #3 (corresponding to the C species of Figure 1) are also non-ubiquitinated and may correspond to the forms of γ with one or two phosphotyrosines, respectively; the

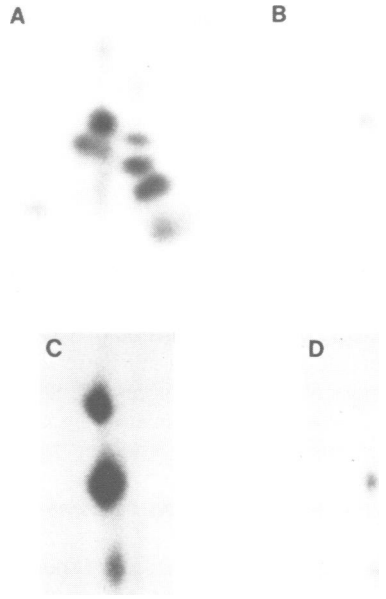

Fig. 3. Two-dimensional tryptic phosphopeptide analysis. Phosphoproteins corresponding to the β chain (**A**), the γ chain (**C**) and the respective ubiquitinated forms (**B** and **D**) were extracted from the gel shown in Figure 1. The phosphopeptides obtained after trypsin digestion were resolved by two-dimensional phosphopeptide analysis. Samples were electrophoresed from right (cathode) to left (anode); chromatography was performed from bottom to top.

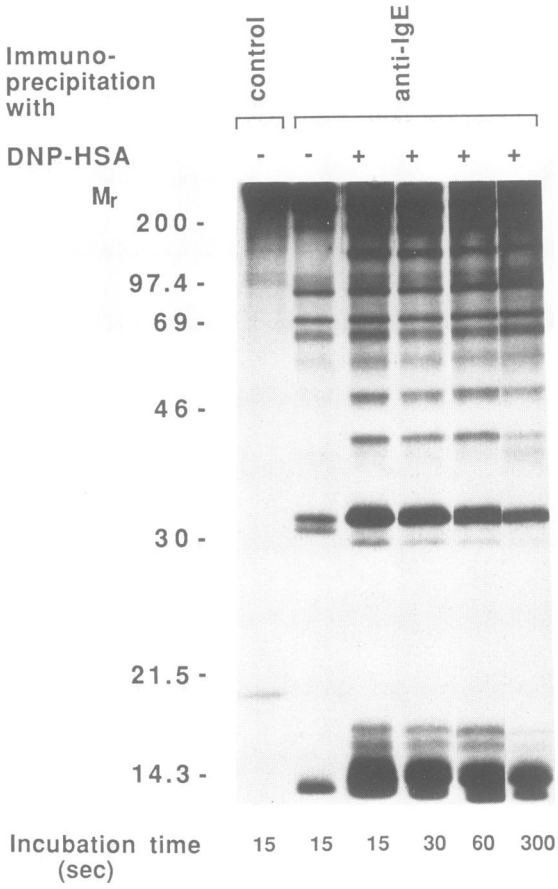

Fig. 4. Time course of β and γ chain ubiquitination. Immunoprecipitates from lysates of ^{32}P-labeled cells using a control antibody (**lane 1**) or anti-IgE antibody (**lanes 2–6**) were analyzed by SDS–PAGE and autoradiography. RBL cells (3×10^7 cells/condition) were saturated with anti-DNP mouse IgE and incubated with medium alone (lanes 1 and 2) or with 1 μg/ml DNP–HSA for the time indicated for individual lanes. Mol. wt standards are on the left.

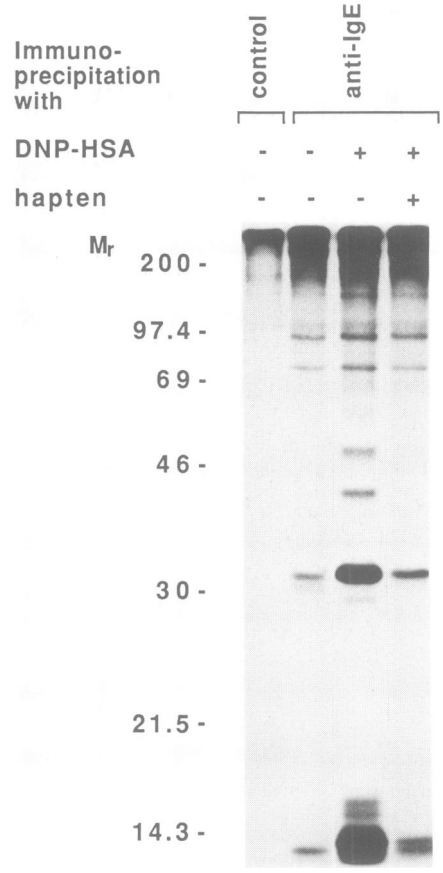

Fig. 5. Apparent disappearance of ubiquinated β and γ after receptor disengagement. Immunoprecipitates from lysates of ^{32}P-labeled cells using a control antibody (**lane 1**) or polyclonal anti-IgE antibody (**lanes 2–4**) were analyzed by SDS–PAGE and autoradiography as for Figure 4. Cells were saturated with anti-DNP mouse IgE and incubated for 30 s with medium alone (lanes 1 and 2) or 500 ng/ml DNP–HSA (lanes 3 and 4). Cells were then chilled on ice, in the presence of cold PBS containing phosphatase inhibitors, or incubated for a further 30 s with 50 μM DNP–lysine (lane 4).

γ species #4 and #7 are likely to correspond to non-phosphorylated forms of γ with one and two molecules of ubiquitin, respectively; the γ species #5, #6, #8 and #9 are both ubiquitinated (#5 and #6 with one, and #8 and #9 with two ubiquitin molecules) and phosphorylated (#5 and #8 with one, and #6 and #9 with two phosphotyrosines). Note that the γ species #6 correspond to D species from Figure 1. Multiubiquitination of γ with various stages of phosphorylation is apparent in Figure 2 (panel H). Therefore, each possible form of γ is represented and it is clear that γ phosphorylation and ubiquination are independent of each other.

Time course of the antigen-induced ubiquination of β and γ

We next investigated in a time-course experiment whether receptor ubiquitination occurs as an early event of the signaling cascade. Cell surface ^{32}P-labeled and IgE-bound FcεRIs were engaged with antigen for 0, 15, 30, 60 or 300 s, immunoprecipitated with anti-IgE, and analyzed by SDS–PAGE and autoradiography. Immunoprecipitates from cells stimulated for 60 s were also analyzed by anti-β, anti-γ and anti-ubiquitin immunoblots (data not shown), which confirmed that the 40 kDa, 48 kDa and the >14 kDa doublet of bands correspond to ubiquitinated forms of β and γ, respectively. Figure 4 shows that these ubiquitinated and phosphorylated forms of β and γ appear as early as 15 s after antigen stimulation.

The multiubiquitination of β and γ is reversible upon receptor disengagement

We then analyzed the fate of the multiubiquitinated receptors after receptor disengagement. RBL-2H3 cells were incubated with anti-DNP IgE and labeled *in vivo* with [^{32}P]orthophosphate and the receptors were engaged (or not) for 30 s with the antigen DNP[30]–HSA. The cells were then further incubated with an excess of the monovalent hapten DNP–lysine to disengage the receptors. Following cell lysis, the cell surface FcεRI receptors were immunoprecipitated with anti-IgE antibody and resolved by SDS–PAGE (Figure 5). As expected, the phosphorylated and ubiquitinated β (40 and 48 kDa) and γ (doublet >14.3 kDa marker) are observed after receptor engagement. After receptor disengagement, however, these phosphorylated species disappear. This could be due to a deubiquitination and/or to a dephosphorylation of the ubiquitinated β and γ or, alternatively, to a selective degradation of ubiquitinated β and γ.

The same immunoprecipitates were also analyzed by

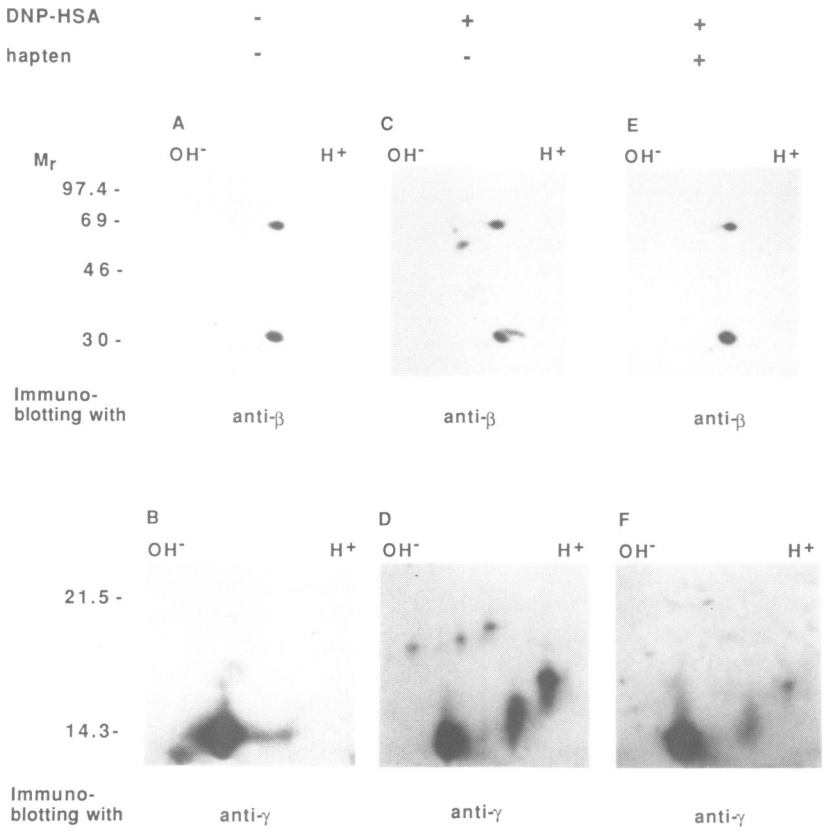

Fig. 6. Deubiquination of β and γ chains following receptor disengagement. RBL cells (10^8 cells/condition) were incubated at 37°C for 30 s in the absence (**panels A** and **B**) or presence (**panels C–F**) of 500 ng/ml DNP–HSA and then chilled on ice or incubated for an additional 30 s with 50 μM DNP–lysine (panels E and F). Lysates were immunoprecipitated with polyclonal anti-IgE antibody and resolved by NEPHGE–PAGE (15% slab gels). Proteins were transferred to Immobilon, immunoblotted as indicated in each panel and detected by enhanced chemiluminescence. Densitometric analysis of the β immunoreactive species in panel C was performed by scanning the blot with an LKB 2222-010 Ultrascan XL Laser Densitometer (LKB, Bromma, Sweden). Data analysis was performed by using LKB 2400 GelScan XL Software (LKB). The total volume (the product of absorbance × area) of the boxed area(s) corresponding to the β immunoreactive forms was 10.744 AU × mm^2, while the total volume of the spots corresponding to the β ubiquitinated forms was 2.825 AU × mm^2.

two-dimensional NEPHGE–PAGE for immunoblotting with anti-β and anti-γ. Figure 6 shows that receptor engagement induces ubiquination of both β and γ. In addition, densitometric analysis of panel C of this figure indicates that ~25% of the total immunoreactive β species become ubiquitinated after receptor engagement. The six different γ species seen in Figure 6D correspond to the species #1–#6 of Figure 2D. Receptor disengagement results in the disappearance of the ubiquitinated forms of β and γ (compare Figure 6C and D with E and F, respectively). This disappearance applies to the phosphorylated forms of the receptor (containing the γ species #2, #3, #5 and #6), as well as to the non-phosphorylated forms (containing the γ species #1 and #4). Thus, the disappearance of the ubiquitinated forms of the receptor is not simply due to a dephosphorylation.

To rule out that this disappearance could be due to a selective loss of ubiquitinated receptors (~25% of total, see above), we calculated the yield of recovery of surface receptors under the same experimental conditions as described above. In a first type of experiment, surface FcεRI receptors were saturated with [^{125}I]IgE and were engaged (or not) for 30 s with the multihapten antigen DNP[30]–HSA, before being disengaged with the monovalent hapten ε-dinitrophenyl–lysine (DNP–lysine). The receptors were then solubilized and the recovery of the [^{125}I]IgE assessed.

Table I. Recovery of FcεRI-bound [^{125}I]IgE from unstimulated and stimulated RBL-2H3 cells after solubilization

Stimuli		[^{125}I]IgE c.p.m.[a]		% Recovery
DNP–HSA (ng/ml)	DNP–lysine (μM)	Insoluble	Soluble	
–	–	0.83 × 10^3	1.3 × 10^5	99
500	–	1.3 × 10^4	0.87 × 10^5	87
500	50	1.39 × 10^3	1.1 × 10^5	98.7

[a]RBL-2H3 cells were loaded with [^{125}I]IgE, engaged with multivalent antigen and disengaged with monovalent antigen, and then solubilized with 0.5% TX-100. The c.p.m. corresponding to [^{125}I]IgE in the insoluble and soluble fractions were determined using a Hewlett-Packard gamma counter. The results of the experiment shown here are representative of four similar experiments.

Table I shows that the recovery of non-engaged or disengaged receptors is virtually identical. A small portion of the IgE-bound receptors seems to be lost after receptor engagement, which may correspond to receptors becoming insoluble due to an association with the cytoskeleton. Therefore, there is no selective loss of IgE-bound receptors after receptor disengagement. In a second type of experiment, the receptors were biosynthetically labeled with [^{35}S]methionine. The surface receptors were then saturated with IgE, engaged with antigen and disengaged with

Table II. Quantitative analysis of biosynthetically labeled FcεRI α and β subunits from unstimulated and stimulated RBL-2H3 cells

Stimuli		^{35}S c.p.m.[a] (±SD)		Ratio α/β
DNP–HSA (ng/ml)	DNP–lysine (μM)	α	β	
–	–	187.4 ± 1.8	183.1 ± 1.7	1.02
500	–	181.7 ± 1.6	150.2 ± 1.5	1.20
500	50	204.7 ± 1.6	192.7 ± 1.6	1.06
1000	–	174.1 ± 4.1	133.9 ± 1.4	1.30
1000	50	230.5 ± 1.8	213.6 ± 1.6	1.07

[a]RBL-2H3 cells were labeled with a [^{35}S]methionine and [^{35}S]cysteine protein mix, loaded with anti-DNP mouse IgE, and then stimulated, lysed and immunoprecipitated as described in Materials and methods. Quantitative analysis of the bands corresponding to the labeled α and β subunits was performed using a radioanalytic imaging system. The results expressed as c.p.m. ± SD are determined by the radioanalytic imaging system after 24 h of continuous analysis and correction for background.

monovalent hapten as above, before being resolved by SDS–PAGE. The gels were then analyzed directly by a radioanalytic imaging system, and the counts associated with α and β were determined. Table II shows that the ratio between α and β is the same when non-engaged or disengaged receptors are compared, regardless of whether the receptors are engaged with 500 ng or 1 μg of antigen. Thus, there is no selective loss of β after receptor disengagement. Therefore, we can conclude that the disappearance of the ubiquitinated forms of the receptor following receptor disengagement is due to a true deubiquitination.

Discussion

We have shown previously that activated FcεRI receptors associate with at least five distinct phosphopolypeptides (pp180, pp125, pp48, pp42 and pp28). These polypeptides, like the receptor β and γ subunits, become rapidly phosphorylated/dephosphorylated following receptor engagement/disengagement. The physical association or the coupling between the receptor and one of these polypeptides, pp125, increases after receptor engagement and dissociation or uncoupling is observed after receptor disengagement (Paolini *et al.*, 1992).

In this study, we have identified some of these putative receptor-associated proteins as species corresponding to ubiquitinated and multiubiquitinated forms of β (pp42 and pp48) and γ (at least six species >14 kDa). The ubiquitination of β and γ occurs as an early event in response to receptor engagement with antigen and can affect either phosphorylated or non-phosphorylated forms of β and γ. Therefore, ubiquitination and phosphorylation generate a complex variety of activated receptor isoforms.

Although not shown in this paper, we have examined, as recently published (Paolini *et al.*, 1992), a fraction of engaged receptors and a fraction of non-engaged receptors on the same cells and found that the ubiquitination of β and γ affects only activated receptors.

The ubiquitination is a covalent modification occurring when one ubiquitin molecule (8.5 kDa) is linked to a target protein, usually by the formation of an isopeptide bond through the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue in the acceptor protein (Hershko, 1988; Finley and Chau, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992). The biosynthesis of ubiquitin–protein conjugates involves multiple steps requiring different classes of enzymes: activating enzyme (E1), a family of different conjugating enzymes (E2) and ubiquitin–protein ligases (E3) (Hershko *et al.*, 1983). From studies performed *in vitro* (Kong and Chock, 1992), it has been demonstrated that the enzymatic activity of E1 and of the 32 kDa E2 isozyme can be potentiated by their phosphorylation, suggesting that phosphorylation could be a mechanism to activate the ubiquitin enzyme system *in vivo*. However, the mechanism involved in the recognition of the membrane-bound proteins by the ubiquitin system is not known. We have demonstrated that non-phosphorylated forms of γ can be recognized by the ubiquitin system. Therefore, it is unlikely that phosphorylation plays a major role in the mechanism of recognition of γ by the enzymatic system.

Several cell surface receptors, including the MEL-14 leukocyte homing receptor (Siegelman *et al.*, 1986), the PDGF receptor (Yarden *et al.*, 1986) and growth hormones (Leung *et al.*, 1987), are constitutively modified by ubiquitin. Recently, multiubiquitination, in response to ligand activation, has been demonstrated for the PDGF β-receptor (Mori *et al.*, 1992) and the TCR ζ chain and CD3 δ subunit (Cenciarelli *et al.*, 1992). Multiubiquitination plays a fundamental role in mediating intracellular protein degradation (Chau *et al.*, 1989; Gregori *et al.*, 1990). However, the existence of metabolically stable multiubiquitinated proteins (Wu *et al.*, 1981), and the identification of specific isopeptidases able to remove ubiquitin from conjugates (Wilkinson *et al.*, 1989), suggest that multiubiquitination may be reversible and may play other roles than protein degradation.

We have observed the disappearance of phosphorylated and ubiquitinated forms of β and γ after receptor disengagement (Figure 5). We can exclude that the disappearance of these forms is due only to their dephosphorylation. In fact, following receptor disengagement we are not able to detect any ubiquitinated forms of β and γ, whether they are phosphorylated or not (Figure 6). In theory, this disappearance could be explained by a selective degradation of these multiubiquitinated forms of β and γ. However, the disappearance occurs extremely rapidly, within 30 s of receptor disengagement and is, therefore, unlikely to correspond to a process of degradation, moreover complete. In addition, our quantitative analysis with the radiolabeled ligand or with the ^{35}S-labeled α and β subunits clearly indicates that there is no significant loss of α or β chain after receptor disengagement. The only conclusion left to our data is that the disappearance of the ubiquitinated forms of the receptor is due to an enzymatic process which deubiquitinates the disengaged receptors. Therefore, ubiquitination and deubiquitination of the FcεRI receptors appear to be controlled at the cell surface by receptor engagement and disengagement. An intracellular enzymatic event which is controlled extracellularly and is so rapid and reversible does not seem to correspond to a process of degradation. We propose that this dynamic modification of cell surface receptors corresponds to a novel signaling pathway which can be rapidly turned on/off. Prevention by mutation of this ligand-induced receptor ubiquitination will be of great interest and will not only help to identify the receptor ubiquitination

sites, but will hopefully provide some insight into this possibly new transmembrane receptor function.

Materials and methods

Reagents and antibodies

Media and sera were purchased from Biofluids (Rockville, MD); DNP[30]–HSA (30 molecules of DNP/molecule of HSA) and DNP–lysine were from Sigma (St Louis, MO). Radiochemicals were purchased from either Amersham ([^{32}P]orthophosphate, catalog no. PBS.13A) or DuPont/NEN ([^{35}S]protein labeling mixture, catalog no. NEG-072). The chemoluminescent substrate for Western blotting (AMPPD) was purchased from Tropix (Bedford, MA). Anti-DNP monoclonal mouse IgE was purified from the supernatant of hybridoma Hi-DNP ϵ 26.82 (Liu *et al.*, 1980) in a mini flo-path bioreactor (Amicon) by ion-exchange chromatography on diethylaminoethyl-Trisacryl (IBF). The anti-ubiquitin antiserum was purchased from Sigma (No. U-5379); the goat anti-mouse and goat anti-rabbit antibodies conjugated to alkaline phosphatase were from Jackson Immunoresearch (West Grove, PA); anti-FcϵRI β subunit antibody JRK (Rivera *et al.*, 1988) and anti-FcϵRI γ subunit antiserum (Letourneur *et al.*, 1991) were described previously.

Cell culture

A subline of RBL-2H3 cells was obtained from B.Rivnay (Procept, Boston, MA). Cells were maintained as an adherent monolayer in minimal essential medium with Earle's salts (EMEM) with 16% heat-inactivated fetal bovine serum and 1% L-glutamine at 37°C with 5% CO_2.

In vivo phosphorylation, immunoprecipitation, SDS–PAGE and Western blotting

RBL-2H3 cells were incubated in phosphate-free medium for 4 h at 37°C before the addition of 5 μg/ml of monomeric monoclonal anti-DNP mouse IgE for 1 h at 37°C, and then labeled at 3×10^7 cells/ml with 37 MBq/ml [^{32}P]orthophosphate for 2 h at 37°C. The cells (5×10^7/sample) were incubated with medium alone or with DNP–HSA for the time and in the amount indicated for individual experiments. In some experiments, stimulation was followed by the addition of 50 μM DNP–lysine for 30 s. The cells were then immediately washed and lysed as previously described (Paolini *et al.*, 1991). The postnuclear supernatants were precleared with protein A–Sepharose beads (Pharmacia), and then sequentially immunoprecipitated with non-specific rabbit IgG and rabbit polyclonal anti-IgE, each bound to protein A–Sepharose. The samples were analyzed by SDS–PAGE (13.5% gel under reducing conditions) and autoradiography. For Western blotting, the postnuclear supernatants from $0.5-1 \times 10^8$ cells were immunoprecipitated and analyzed by SDS–PAGE (as above) or by NEPHGE–PAGE (as described below) and the proteins were transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA) for 6 h at 50 V. Membranes were then incubated overnight in Tris-buffered saline (TBS) (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 5% bovine serum albumin (BSA) before a 2 h incubation in the same buffer containing 15 μg/ml anti-β antibody, 1:200 dilution of anti-γ serum or 1:10 dilution of anti-ubiquitin antiserum. After two washes in TTBS (TBS containing 0.1% Triton X-100 and 2.5% BSA), the membranes were incubated with 0.2 μg/ml goat anti-mouse or goat anti-rabbit antibodies conjugated to alkaline phosphatase and developed with the chemiluminescence substrate AMPPD according to the manufacturer's instructions (Tropix).

Two-dimensional NEPHGE–PAGE and two-dimensional tryptic phosphopeptide mapping

For two-dimensional analysis, immunoprecipitates from 1×10^8 cell extract postnuclear supernatants were loaded at the anode end of 6% acrylamide, 2% ampholine (pH 3.5–10) tube gels. Separation was performed for 6 h at 500 V. NEPHGE gels were equilibrated for 1 h with 1 × SDS sample buffer and then frozen at −70°C or applied directly on top of the second dimension gels which were run at 20 mA/gel. Tryptic phosphopeptide analysis was performed as described previously (Boyle *et al.*, 1992) with some modifications. Gel slices corresponding to different phosphoproteins were cut and the proteins digested with 50 μg/ml of trypsin in 50 mM NH_4HCO_3 to increase recovery of elution. Extracted proteins were vacuum dried and dissolved in 50 μl thin-layer buffer at pH 1.9 [50 ml formic acid (88%), 156 ml glacial acetic acid, 1794 ml water]. The samples were applied to TLC cellulose plates (100 mm DC-cellulose, EM Separations, Gibbstown, NJ) and electrophoresed in the first dimension for 30 min at 1000 V. The plates were air-dried and the second dimension, consisting of ascending chromatography in phospho-chromatography buffer at pH 8.9 (750 ml *n*-butanol, 150 ml glacial acetic acid, 600 ml water), was performed for 6 h at room temperature. The ^{32}P-labeled phosphopeptides were detected by autoradiography.

Biosynthetic labeling

RBL-2H3 cells were incubated in methionine- and cysteine-free medium for 4 h and labeled for 18 h with 1 mCi/150 cm^3 confluent flask with a [^{35}S]methionine and [^{35}S]cysteine protein mix. Stimulation, lysis and immunoprecipitation were performed as described above. Quantitation of the bands corresponding to labeled α and β chains was achieved using a radioanalytic imaging system (AMBIS Systems, San Diego, CA).

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