

p28, a Novel IgE Receptor-associated Protein, Is a Sensor of Receptor Occupation by Its Ligand in Mast Cells*

Received for publication, August 26, 2003, and in revised form, December 28, 2003
Published, JBC Papers in Press, January 8, 2004, DOI 10.1074/jbc.M309456200

Nicolas Charles, Renato C. Monteiro, and Marc Benhamou†

From INSERM EMI-0225, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75870 Paris Cedex 18, France

Mast cells express the high affinity receptor for IgE (FcεRI). Aggregation of this receptor by IgE and antigen leads to a signaling cascade resulting in the secretion of histamine, in the synthesis of other pro-inflammatory mediators such as leukotrienes and prostaglandins, and in the production of various cytokines, all of which participate in the development of the allergic reaction. In the last years, growing evidence accumulated that binding of IgEs to FcεRI in itself induces active signals leading to mast cell survival, increased expression of FcεRI, transient induction of histidine decarboxylase synthesis, and increased cell adhesion. The mechanisms underlying monomeric IgE signaling in the absence of receptor aggregation are still poorly understood. Here, we show that a protein of 28 kDa (p28) is physically and constitutively associated with FcεRI in mast cells. Co-immunoprecipitation studies from ¹²⁵I surface-labeled cells demonstrated that this association involves at least 50% of membrane-expressed FcεRI. After the addition of monomeric IgE to the cells, the p28-FcεRI complex dissociates almost completely in less than 2 min. This dissociation is temperature-sensitive and is not due to the recruitment of additional proteins to the complex. Stripping bound IgE from the cells by acidic treatment promotes a rapid reassociation between p28 and FcεRI. Altogether, these data are consistent with a conformational regulation of the complex. Thus, p28 is a sensor for FcεRI occupation by IgE on mast cells, and its dissociation from the receptor could represent an early step of monomeric IgE signaling.

Mast cells are now recognized as important players in innate and adaptive immunity (1). Indeed mast cells have the capacity to phagocytose bacteria (2) and to internalize antigen through fluid phase (3), to recruit immunocompetent cells (4), to migrate to lymph nodes (5), and to activate lymphocytes both specifically through antigen presentation and nonspecifically (6, 7).

In addition to these physiologic roles, mast cells are involved in allergic reactions. They express the high affinity receptor for IgE (FcεRI), a tetrameric receptor composed of an αβγ₂ complex (8). The α chain is responsible for the high affinity binding of IgE ($K_D = 10^{-9}$ to 10^{-10} M), which represents the sensitization step, whereas the β and γ₂ subunits are involved in signal transduction (9). Aggregation of this receptor by IgE and antigen leads to a signaling cascade that involves kinases, phosphatases, G proteins, and calcium mobilization (9). This results

in the secretion of histamine, in the synthesis of other pro-inflammatory mediators such as leukotrienes and prostaglandins, and in the production of various cytokines, all of which participate in the development of the allergic reaction and of innate inflammatory responses to invading pathogens.

Constitutive associations between a fraction of FcεRI and various signaling molecules have been reported. These signaling effectors include the tyrosine kinases Lyn (10) and Fyn (11), the tyrosine phosphatase SHP-2 (12), and the serine/threonine kinase protein kinase Cδ (13), all of which show increased association after receptor aggregation. Other effectors, such as the tyrosine kinase Syk, are recruited to the aggregated and tyrosine-phosphorylated receptor after stimulation (14–17).

Until recently, an opinion prevailed that cell sensitization by IgE is a passive step that does not lead to any cell activation signal, whereas IgE-dependent cell stimulation would be achieved only after FcεRI aggregation by the antigen in the presence of its specific IgE. However, in the last years, growing evidence accumulated that binding of IgE to FcεRI in itself induces active signals leading to increased expression of FcεRI (18–21), mast cell survival (22, 23), transient induction of histidine decarboxylase synthesis (24), and increased cell adhesion (25). The mechanisms involved in these signals are poorly understood. Indeed, whereas some authors have reported that monomeric IgEs are capable of inducing significant receptor phosphorylation, synthesis of cytokines, and protection of Bcl-X_L from degradation (23), others found no such signaling events (22), although both groups agreed that monomeric IgEs induce mast cell survival through binding to FcεRI (22, 23). Therefore, the critical question of the mechanisms leading from monomeric IgEs binding to cell activation is still an unresolved controversy.

Here, we show that a protein of 28 kDa (p28) is physically and constitutively associated with a majority of membrane-expressed FcεRI in the absence of IgEs in the RBL-2H3 mast cell line. After binding of monomeric IgEs, p28 dissociates very rapidly from FcεRI. These data were confirmed in nontumoral rat bone marrow-derived mast cells. Further, we provide indirect evidence that this association/dissociation mechanism is regulated by the conformational state of the receptor. Thus, p28 is a sensor of FcεRI occupation by IgEs, and its dissociation from the receptor could represent an early step of the monomeric IgE signaling.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal anti-rat phospholipid scramblase (rPLSCR)¹ antibody 129.2, monoclonal anti-rPLSCR and anti-p28 antibody 17.3, and monoclonal anti-FcεRIβ antibody 30.9 have been produced in the laboratory as described (26, 27). Anti-FcεRIα mono-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 33-1-44-85-62-64; Fax: 33-1-44-85-62-60; E-mail: benhamou@bichat.inserm.fr.

¹ The abbreviations used are: rPLSCR, rat phospholipid scramblase; HBT, Hanks' buffer saline solution supplemented with bovine serum albumin and Tris; IP, immunoprecipitation; IB, immunoblotting; mAb, monoclonal antibody; HPLC, high pressure liquid chromatography; IL, interleukin.

clonal antibody BC4 was kindly provided by Dr. R. P. Siraganian (National Institutes of Health, Bethesda, MD) (28). Rabbit polyclonal anti-FcεRIγ antibody was kindly provided by Dr. U. Blank (Institut Pasteur, Paris, France). Murine dinitrophenyl-specific clone SPE-7 IgE, horseradish peroxidase-conjugated antibodies to mouse IgG Fc, and horseradish peroxidase-conjugated protein A were from Sigma-Aldrich. Murine dinitrophenyl-specific clone DNP-48 IgE (29) was kindly provided by Dr. R. P. Siraganian (National Institutes of Health) and produced as described (30). For monomeric IgE DNP-48 preparation, IgE was diluted in phosphate-buffered saline, filtered, and separated by gel filtration through an S-200 Superdex™ column (Amersham Biosciences) connected to an HPLC ÄKTA-Basic automated liquid chromatography system (Amersham Biosciences). Murine dinitrophenyl-specific clone 26.82 IgE was kindly provided by Dr. F.-T. Liu (La Jolla Institute for Allergy and Immunology, San Diego, CA) (30).

Cell culture medium (Dulbecco's modified Eagle's medium), fetal calf serum and other culture reagents were from Invitrogen. Triton X-100, SDS, bovine serum albumin, proteases, and phosphatases inhibitors (leupeptin and aprotinin, NaF, and Na₃VO₄) were from Sigma-Aldrich.

Cells and Cell Sensitization—The rat basophilic leukemia mast cell line RBL-2H3 (31) was cultured as an adherent monolayer in Dulbecco's modified Eagle's medium with Glutamax, without sodium pyruvate, supplemented with 10% fetal calf serum, streptomycin, and penicillin. In sensitization experiments, the cells were washed twice in 37 °C Hanks' buffer saline solution supplemented with 50 mM Tris-HCl, pH 7.2, and 0.1% bovine serum albumin (HBT pH 7.2), and incubated at 37 °C in the same medium for the time indicated with or without IgE.

For rat bone marrow-derived mast cells, femurs and tibias from Wistar rats (6–13 weeks old) were flushed, and myeloid cells were cultured for 3 weeks in RPMI 1640 medium supplemented with 10 ng/ml recombinant rat IL-3β (Peprotech, Rocky Hill, NJ), 20% fetal calf serum, 50 μM 2-mercaptoethanol, nonessential amino acids, streptomycin, and penicillin. Over 90% cells were positive for FcεRI by fluorescence-activated cell sorter analysis.

Cell Lysis, Immunoprecipitation, and Immunoblotting—Sensitized or unsensitized cells were washed twice with ice-cold phosphate-buffered saline solution and lysed in 200 μl of lysis buffer per million cells (50 mM HEPES or Tris-HCl, pH 7.2, 50 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, 0.1% SDS, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 10 min on ice. Then the cells were scraped, and the soluble cell lysates were recovered after centrifugation at 14,000 × *g* for 10 min at 4 °C.

For immunoprecipitation, the cell lysates were incubated for 2 h at 4 °C on a rotating wheel with the indicated antibodies directly coupled to Sepharose-4B beads prepared following the manufacturer's instructions (Amersham Biosciences) for mAbs 17.3, 129.2, BC4, and 30.9 and for mouse serum IgG. In some instances, effluent proteins were subjected to a second round of immunoprecipitation as described above. In all cases after immunoprecipitation, the beads were washed six times in lysis buffer, and the proteins were eluted by boiling in Laemmli sample buffer (32). The proteins were resolved by SDS-PAGE in 10% or 12% polyacrylamide gels and electrophoretically transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The polyvinylidene difluoride membranes were blocked in 4% bovine serum albumin in Tris-buffered saline solution containing 0.05% Tween 20. The membranes were then successively probed with the desired primary antibody and an appropriate secondary horseradish peroxidase-conjugated antibody in the same buffer and visualized by enhanced chemoluminescence and exposure to X-Omat films (Eastman Kodak Co.).

Dissociation of IgE from RBL-2H3 Cells—Bound IgEs were removed from sensitized RBL-2H3 cells as described before (33). Briefly, the cells were sensitized as described above. After sensitization, the cells were washed twice in HBT pH 7.2, then washed twice in HBT pH 6.0, washed once more in 50 mM glycine, pH 2.9, containing 150 mM NaCl, and incubated in the same buffer for 4 min. Then the cells were washed again in HBT pH 6.0, followed by HBT pH 7.2. After incubation in HBT pH 7.2 for various time lengths, the cells were washed with ice-cold phosphate-buffered saline and lysed in the lysis buffer.

Iodine Cell Surface Labeling and Quantification of FcεRIα—Twenty million RBL-2H3 cells were surface-labeled with Na¹²⁵I (1 mCi; Amersham Biosciences) using the lactoperoxidase method. After labeling, the cells were lysed, and immunoprecipitations with mAbs 17.3, BC4, and 30.9 and with irrelevant mouse IgG were performed on lysates from 5 × 10⁶ cells each. After 2 h of immunoprecipitation, the effluents were subjected to a second round of immunoprecipitation with mAb BC4-coupled beads to collect the residual FcεRIα. The first and second immunoprecipitations were washed six times in lysis buffer and eluted

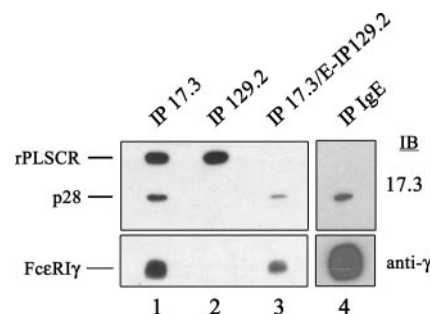


FIG. 1. p28, but not rPLSCR, is constitutively associated with the FcεRIγ. Upper panel, lane 1, rPLSCR and p28 immunoprecipitated by mAb 17.3. Lane 2, rPLSCR immunoprecipitated by mAb 129.2. Lane 3, the effluent of the lane 2, depleted of rPLSCR, was subjected to a second round of immunoprecipitation with mAb 17.3. Lane 4, immunoprecipitation with monoclonal IgE DNP-48 beads. IPs are analyzed by 17.3 IB. Lower panel, same samples as in upper panel but immunoblotted with a polyclonal anti-FcεRIγ. The gel was 12% polyacrylamide. This experiment is representative of three.

by boiling in Laemmli's sample buffer. After resolution by SDS-PAGE, autoradiographies were performed with BioMax MR-1 films (Eastman Kodak Co.). Some gels were electrophoretically transferred onto polyvinylidene difluoride membranes and blotted with mAb 30.9, as described above, to examine the presence of FcεRIβ. After autoradiography, FcεRIα gel spots were cut, and the radioactivity was counted in a γ counter. For each experimental point, 100% corresponds to the addition of FcεRIα counts recovered in the two rounds of immunoprecipitations. The percentage of FcεRIα precipitated in each first round is calculated by comparison with the corresponding 100%.

All surface-expressed rat FcεRIα are associated with FcεRIβ (8). Therefore, FcεRIα recovered in anti-FcεRIβ immunoprecipitation is a marker of the efficiency of co-immunoprecipitation of FcεRIα with receptor-associated proteins in our stringent experimental conditions. Thus, additional calculations were made taking FcεRIα co-precipitated with anti-FcεRIβ as a new 100% to obtain a corrected value for FcεRIα co-precipitated with mAb 17.3.

Statistical Analysis—The results of Fig. 5B were analyzed by independent sample two-tailed Student's test. The results are presented as the means ± S.D. (*, *p* < 0.003; **, *p* < 0.0005; NS, not significant).

RESULTS

FcεRI Is Constitutively Associated with a 28-kDa Protein—As we described in a previous report, two monoclonal antibodies, 129.2 and 17.3, recognize the rPLSCR (26). Phospholipid scramblase activity results in a redistribution of phospholipids between the two leaflets of the plasma membrane when cells are activated or enter apoptosis (34, 35). The rPLSCR is tyrosine-phosphorylated after FcεRI engagement (26). Reassessment of the specificity of the anti-rPLSCR antibodies confirmed that both mAbs 129.2 and 17.3 bind the 37-kDa rPLSCR. However, it appeared that mAb 17.3 recognizes by immunoprecipitation (IP) and immunoblotting (IB) an additional protein of 28 kDa (p28) (Fig. 1).

Our initial aim was to determine whether rPLSCR was physically associated with FcεRI. To that end, immunoprecipitations with mAb 17.3 or mAb 129.2 (IP 17.3 or IP 129.2) were carried out on RBL-2H3 cell lysates under stringent conditions (lysis buffer containing 1% Triton X-100 and 0.1% SDS). Immunoblots revealed the presence of the FcεRIγ chain in mAb 17.3, but not in mAb 129.2, immunoprecipitates. This was not due to direct interaction between mAb 17.3 and FcεRIγ, because mAb 17.3 did not bind FcεRIγ by immunoblotting nor immunoprecipitated it when lysates depleted of both p28 and rPLSCR were used (data not shown). These observations indicated that FcεRIγ was co-precipitated by virtue of an association with either p28 or rPLSCR. To determine which of these two proteins was involved in this interaction, the cell lysates were first depleted of rPLSCR by an IP 129.2. FcεRIγ was still recovered in 17.3 immunoprecipitates from these depleted ly-

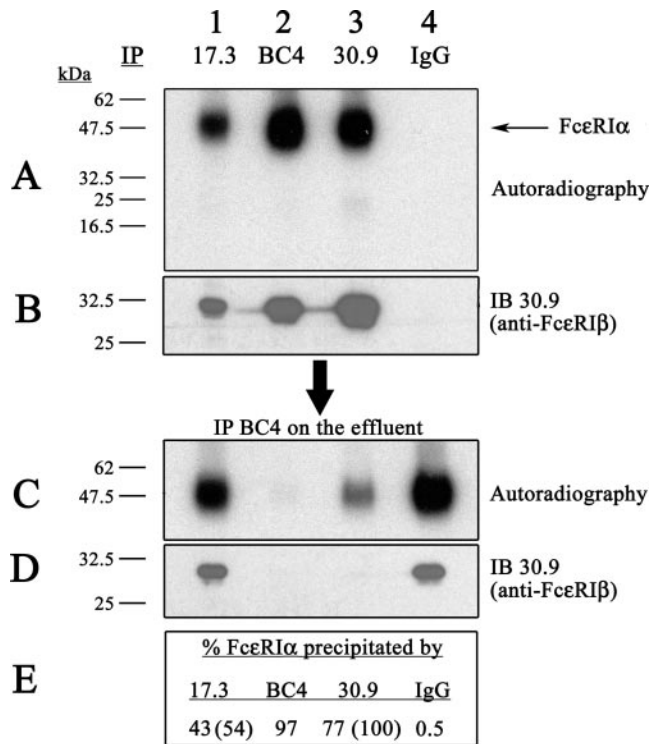


FIG. 2. More than 50% of the membrane-expressed FcεRI are associated with p28. A, autoradiography of the surface expressed ^{125} I-iodinated proteins precipitated or co-precipitated with the monoclonal antibodies indicated (lanes 1–3, where BC4 is an anti-FcεRIα and 30.9, an anti-FcεRIβ) and with mouse irrelevant polyclonal IgG (lane 4). B, anti-FcεRIβ immunoblot (mAb 30.9) analysis of the samples analyzed in A. C, effluents of the immunoprecipitations analyzed in A and B were subjected to immunoprecipitation with the anti-FcεRIα mAb BC4 and resolved in SDS-PAGE, and the gel was autoradiographed. D, anti-FcεRIβ immunoblot analysis of the samples of C. All of the gels were 12% polyacrylamide. E, quantitative analysis of the immunoprecipitations. The bands corresponding to iodinated FcεRIα were counted in a γ counter. For each lane, the 100% is the addition of counts recovered in A and C. The percentage of FcεRIα precipitated in each lane of A is indicated and determined by comparison with the corresponding 100%. The numbers in parentheses represent the corrected values, taking the values obtained with anti-FcεRIβ immunoprecipitation (lane 3) as a new 100%.

sates (Fig. 1), suggesting an association between p28 and FcεRI. To confirm this association, the FcεRI was purified by immunoprecipitation with IgE-coupled beads. p28, but not rPLSCR, was recovered together with FcεRIγ (Fig. 1) and FcεRIβ (data not shown) in the immunoprecipitated material. Therefore, p28 is part of a complex that includes FcεRIγ and FcεRIβ. In the figures that will follow, although rPLSCR is present in each IP 17.3, it will not be represented to allow for a better comprehension.

To examine whether p28 was associated with the complete $\alpha\beta\gamma_2$ FcεRI complex, the membrane proteins of RBL-2H3 cells were radiolabeled with ^{125} I. Immunoprecipitations with mAb 17.3, anti-FcεRIα chain mAb BC4, anti-FcεRIβ chain mAb 30.9, and control IgG were performed. Radiolabeled material comigrating with FcεRIα in SDS-PAGE (as seen with mAb BC4 immunoprecipitates) was recovered together with FcεRIβ in mAb 17.3 and mAb 30.9, but not IgG, immunoprecipitates (Fig. 2). Therefore, p28 is constitutively associated with membrane-expressed FcεRI complex. No other radiolabeled protein was detected in the immunoprecipitates. More precisely, no band corresponding to p28 was observed, suggesting that this protein did not contain an extended extracellular domain that could be radiolabeled with iodine and that its association with FcεRI took place in the transmembrane or in the intracellular

compartment. To determine the fraction of FcεRI that was associated with p28, the effluent of each immunoprecipitation was subjected to a second round of immunoprecipitation with anti-FcεRIα mAb BC4, and the recovered radiolabeled FcεRIα chain was counted in a γ counter. The total counts recovered in each set of two successive immunoprecipitations were comparable with one another. Calculations indicated that 43% of membrane FcεRIα had been recovered in mAb 17.3 immunoprecipitates (Fig. 2). 77% of FcεRIα were co-immunoprecipitated with FcεRIβ. Because every membrane-expressed FcεRIα chain is associated with FcεRIβ (8, 9), the latter data show that under our stringent experimental conditions, no more than 77% of the α chain was co-immunoprecipitable with a protein that is constitutively associated with it. Taking this value as the 100% and correcting the data to take into account this parameter, gave an estimate of 54% of membrane FcεRIα that were associated with p28. Under the same conditions, no FcεRIα chain was detectable after an IP 129.2 (data not shown), and less than 0.5% was detectable after an IP performed with mouse irrelevant IgG as a negative control (Fig. 2). In another experiment, the calculated percentages of FcεRIα co-immunoprecipitated with p28 were of 37 and 46%, before and after correction, respectively. Therefore, despite our stringent experimental conditions, nearly 50% of membrane FcεRI were found to be constitutively associated with p28.

p28 Dissociates from FcεRI after IgE Binding on Its Receptor—The association between p28 and FcεRI after FcεRI sensitization was examined next. RBL-2H3 cells were incubated with different concentrations of the monoclonal IgE DNP-48. As shown in Fig. 3 (A and B), the association between p28 and the FcεRI was abolished after sensitization with IgE in a dose-dependent manner. At 1 μ g/ml of IgE, the association was strongly reduced, and at 10 μ g/ml of IgE, the association was barely detectable. An identical dissociation was induced by the anti-FcεRIα mAb BC4 (data not shown). These data indicate that the association of p28 with the receptor is dependent of the occupation state of the FcεRI at the cell surface. When RBL-2H3 cells were sensitized with an ascitis dilution titrated to less than 0.5 μ g/ml of anti-DNP IgE, no detectable dissociation of the complex was observed (data not shown). Under these conditions, when RBL-2H3 cells were stimulated with antigen (dinitrophenyl-coupled human serum albumin), 80% of β -hexosaminidase were released, and the association remained identical to that observed after or before sensitization alone (data not shown). Therefore, the association between p28 and FcεRI is not significantly modulated by the IgE-induced activation of the cell. In sensitization time course with 10 μ g/ml IgE, the dissociation was detected in less than 30 s and complete within 2 min after the addition of IgE (Fig. 3, C and D). Therefore, this dissociation is an early event following IgE binding to FcεRI.

The p28-FcεRI Dissociation Is Not Due to the Recruitment of Competing Effectors—To examine whether the IgE-induced dissociation was temperature-sensitive in the cells, sensitization experiments were performed on RBL-2H3 cells at 4 and 37 $^{\circ}$ C (Fig. 4). At 37 $^{\circ}$ C, the IgE-induced dissociation was complete as shown above, whereas at 4 $^{\circ}$ C a significant proportion of this association was still detected. To examine whether the recruitment of another protein to the complex was responsible for the temperature sensitivity of the IgE-induced p28 dissociation from FcεRI, a series of experiments was performed next. The p28-FcεRI complex was immunoprecipitated with mAb 17.3, and after washings, the beads were incubated for 5 min at 4 or 37 $^{\circ}$ C in the presence or absence of 15 μ g/ml of IgE (Fig. 5A). Under these conditions, the dissociation occurred at 37 $^{\circ}$ C but not at 4 $^{\circ}$ C. The fraction of eluted FcεRI from eight separate experiments was quantified through FcεRIβ with National In-

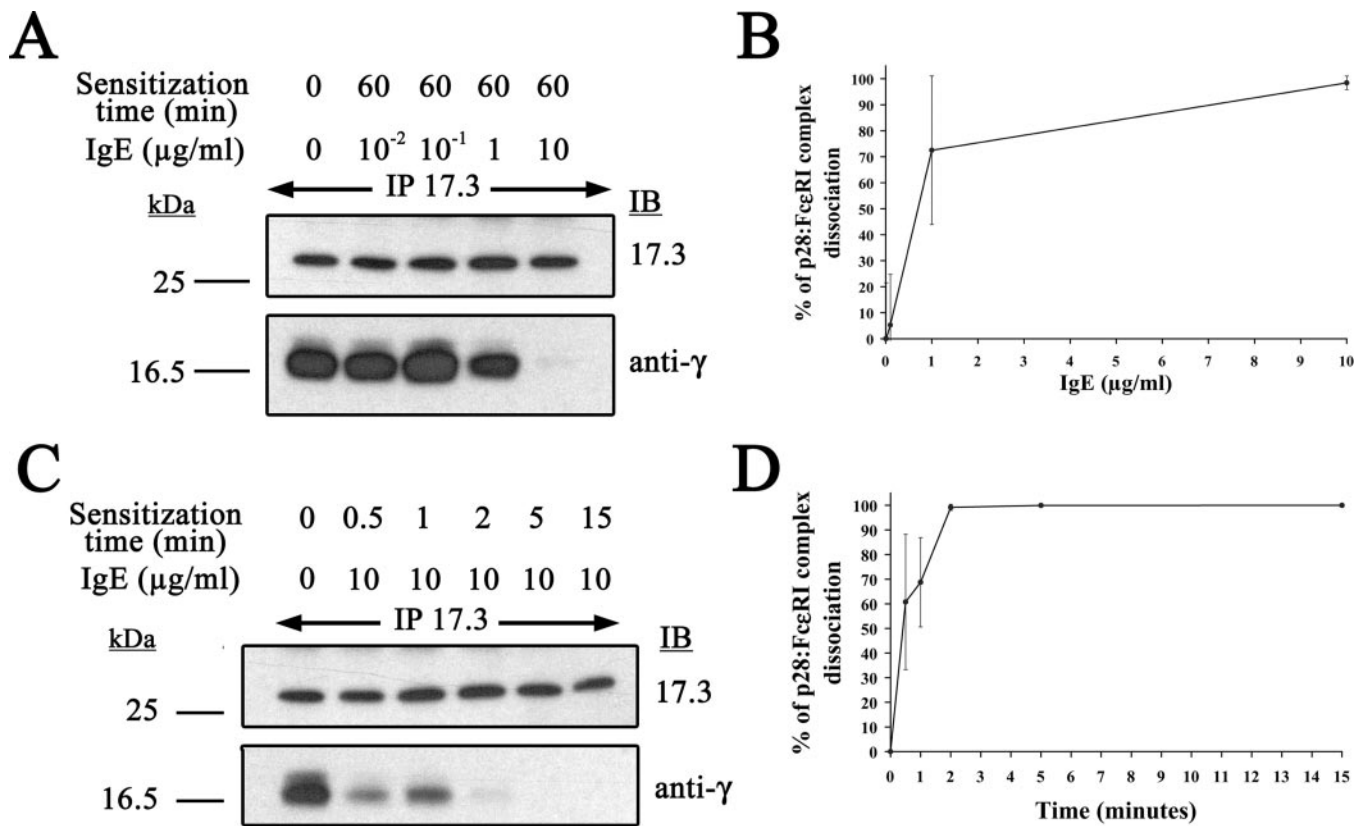


FIG. 3. IgE sensitization of the receptor induces p28-FcεRI dissociation with a time- and dose-dependent manner. A, dose response of IgE. RBL-2H3 cells were incubated for the indicated time length with increasing concentrations of IgE DNP-48, and proteins in the lysates were immunoprecipitated with mAb 17.3. The presence of p28-FcεRI complexes was examined by mAb 17.3 (upper panel) and by anti-FcεRIγ (lower panel) IB. The gel was 12% polyacrylamide. This experiment is representative of three. B, quantification of IgE-induced FcεRI dissociation from p28. Blots from three dose-response experiments conducted as in A were scanned, and the bands were quantified with National Institutes of Health Image software. The data are the means ± S.D. C, time course of sensitization. RBL-2H3 cells were incubated for various time lengths with the indicated concentrations of IgE DNP-48, and proteins in the lysates were immunoprecipitated with mAb 17.3. The presence of p28-FcεRI complexes was examined by mAb 17.3 (upper panel) and by anti-FcεRIγ (lower panel) IB. The gel was 12% polyacrylamide. This experiment is representative of three. D, quantification of IgE-induced FcεRI dissociation from p28. Blots from three time course experiments conducted as in C were scanned, and the bands were quantified with National Institutes of Health Image software. The data are the means ± S.D.

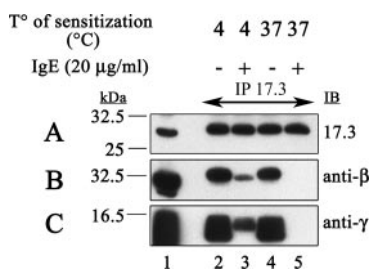


FIG. 4. p28-FcεRI dissociation is dependent on the temperature. RBL-2H3 cells were incubated for 30 min in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 20 μg/ml of IgE DNP-48 at the temperature (T°) indicated. After these treatments, the cell lysates were immunoprecipitated with mAb 17.3 (lanes 2–5), resolved by SDS-PAGE, and analyzed by IB with mAb 17.3 (A), anti-FcεRIβ mAb 30.9 (B), and polyclonal anti-FcεRIγ (C). Lane 1, whole cell lysate positive control. The gel was 12% polyacrylamide. This experiment is representative of three.

stitutes of Health Image software (version 1.62) (Fig. 5B). Whereas less than 20% of FcεRIβ was dissociated by IgE at 4 °C, over 80% were eluted by IgE at 37 °C ($p < 0.0005$). Altogether, these data demonstrate that the association/dissociation is temperature-sensitive and explain why p28 can be co-precipitated with FcεRI using IgE-coupled beads at 4 °C (cf. Fig. 1). Furthermore, these data indicate that the dissociation is not dependent on the recruitment of additional cell proteins and that it involves only the complex containing FcεRI and p28.

The Dissociation Is Induced by Monomeric IgE—To deter-

mine whether the dissociation was due to the FcεRI aggregation by IgE aggregates contaminating the IgE preparation, the DNP-48 IgE was further purified by gel filtration through HPLC. Although no degranulation of the cells was ever observed with the DNP-48 IgE preparation routinely used in this study, a minor fraction of the preparation was indeed made of aggregates (Fig. 6A). The major peak corresponding to monomeric IgE was selected to perform sensitization experiments. Again, IgEs induced a complete dissociation of the p28-FcεRI complex, and no difference was seen between HPLC-purified monomeric IgE and the original IgE preparation (Fig. 6B). To rule out the possibility that the dissociation of p28 and FcεRI was an artifact associated with the DNP-48 IgE, we tested the ability of two other IgEs (SPE-7 and 26.82) to induce dissociation of the complex. As shown in Fig. 6B, the dissociation was complete with 26.82 and nearly complete with SPE-7. Therefore, the IgE-induced dissociation of the p28-FcεRI complex is not restricted to a specific clone of IgE.

The Dissociation Is a Reversible Event—A series of experiments was performed next to determine whether the dissociation involved an irreversible alteration of FcεRI and/or p28. Thus, after a cell sensitization of 30 min with 20 μg/ml of IgE (conditions under which dissociation of the complex is complete), IgEs were eluted from the cells by an acidic treatment with glycine. Previous studies have shown that under these conditions over 90% bound IgE were removed from membrane-expressed FcεRI (33). As shown in Fig. 7, glycine treatment alone induced a minor decrease in the association between p28

FIG. 5. *In vitro* dissociation of the p28-FcεRI complex. A, proteins from RBL-2H3 cell lysates were immunoprecipitated with mAb 17.3. After immunoprecipitation, the beads were subjected to various treatments. *Lane 1*, RBL-2H3 total cell lysate. *Lane 2*, after immunoprecipitation, 17.3 beads were washed and incubated 5 min with 10 μg/ml IgE at 4 °C, and the proteins thus eluted were recovered. *Lane 3*, proteins remaining associated with the 17.3 beads after the incubation analyzed in *lane 2* were recovered by boiling in Laemmli sample buffer. *Lane 4*, same as *lane 2* but during a 5-min incubation at 37 °C without IgE. *Lane 5*, proteins remaining associated with the 17.3 beads after the incubation analyzed in *lane 4* were recovered by boiling in Laemmli sample buffer. *Lane 6*, same as *lanes 2* and *4*, but during a 5-min incubation at 37 °C with 10 μg/ml IgE. *Lane 7*, proteins remaining associated with the 17.3 beads after the incubation analyzed in *lane 6* were recovered by boiling in Laemmli sample buffer. *Upper panel*, IB with mAb 17.3. *Lower panel*, immunoblot with anti-FcεRIβ mAb 30.9. B, quantification of eluted FcεRIβ. Blots from eight experiments conducted as for A were scanned, and the bands were quantified with National Institutes of Health Image software. The data are the means ± S.D. (*, $p < 0.003$; **, $p < 0.0005$; NS, not significant).

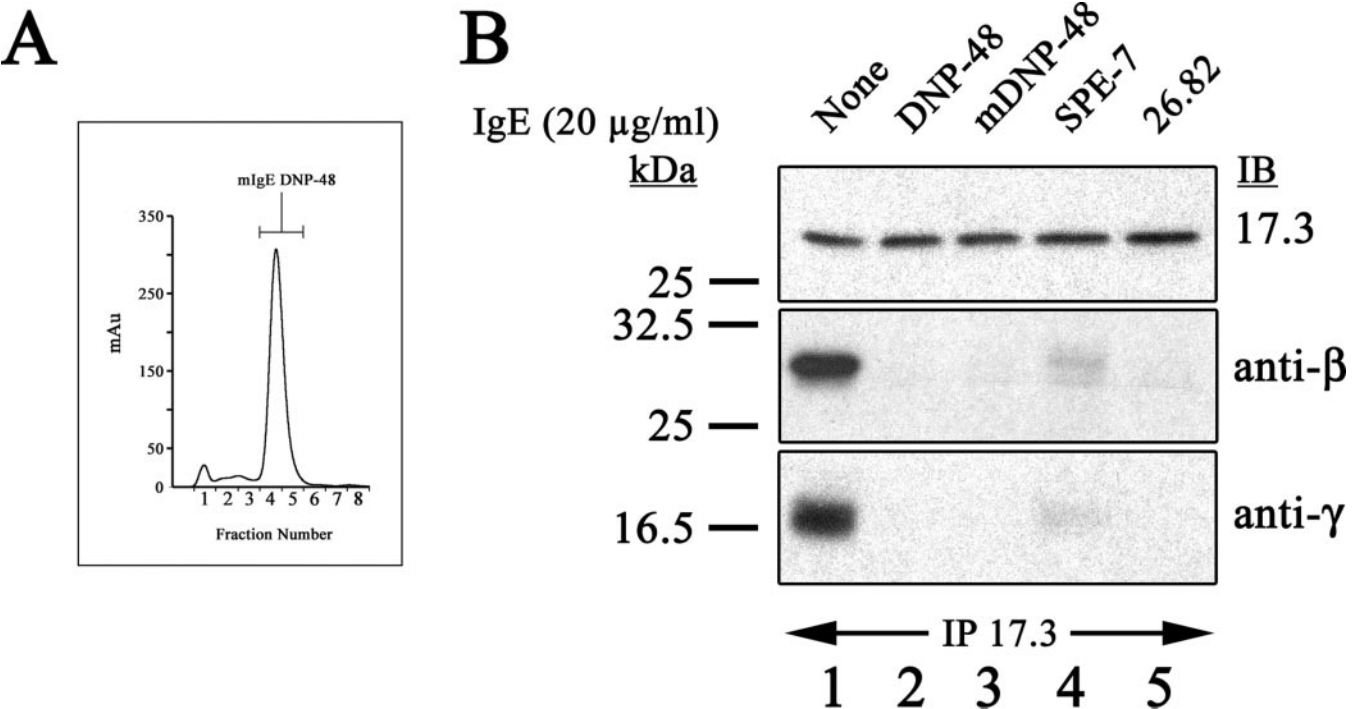
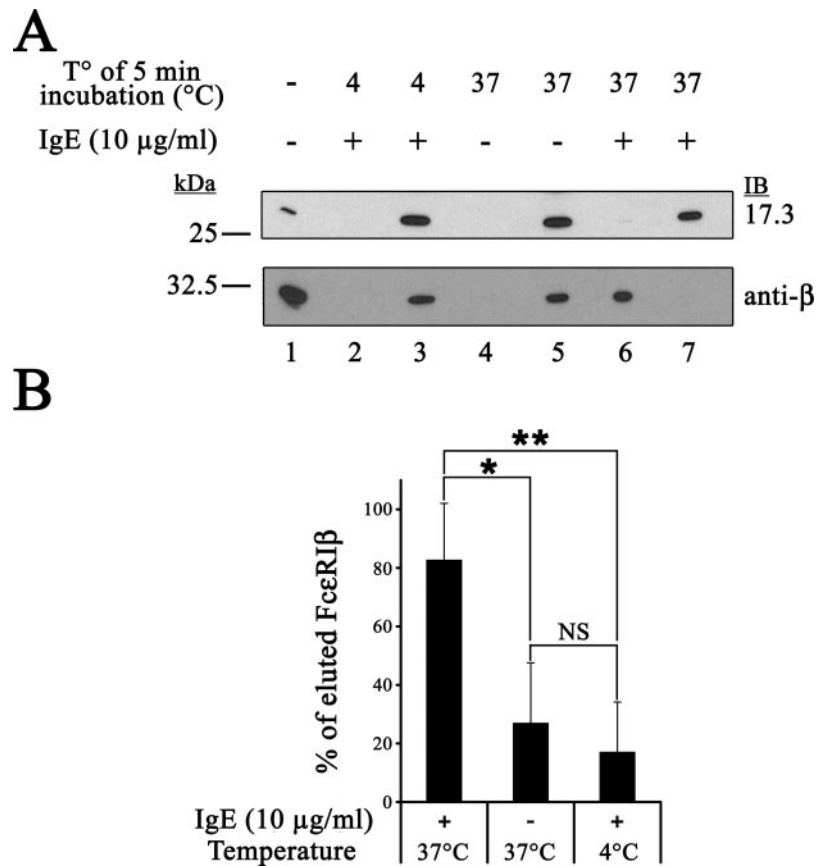


FIG. 6. p28-FcεRI dissociation is induced by monomeric IgE and does not depend on the clone of IgE used to sensitize the cells. A, monomeric IgE purification. The optical density at 280 nm (mAu) of IgE resolved by HPLC gel filtration is shown. *Fractions 1–3* correspond to polymeric IgE, and *fractions 4 and 5* correspond to the monomeric IgE (mIgE DNP-48) used in B. B, RBL-2H3 cells were incubated for 30 min at 37 °C with sensitization buffer alone (*lane 1*), 20 μg/ml of routine IgE DNP-48 preparation (*lane 2*), 20 μg/ml of the pooled monomeric fractions of IgE DNP-48 shown in A (mDNP-48) (*lane 3*), 20 μg/ml of monoclonal IgE clone SPE-7 (*lane 4*), or 20 μg/ml of monoclonal IgE clone 26.82 (*lane 5*). After these treatments, RBL-2H3 cell lysates were subjected to IP with mAb 17.3, resolved by SDS-PAGE, and analyzed by IB with mAb 17.3 (*top panel*), anti-FcεRIβ mAb 30.9 (*middle panel*), and polyclonal anti-FcεRIγ (*bottom panel*). The gel was 12% polyacrylamide. This experiment is representative of three. mAu, milliabsorbance unit.

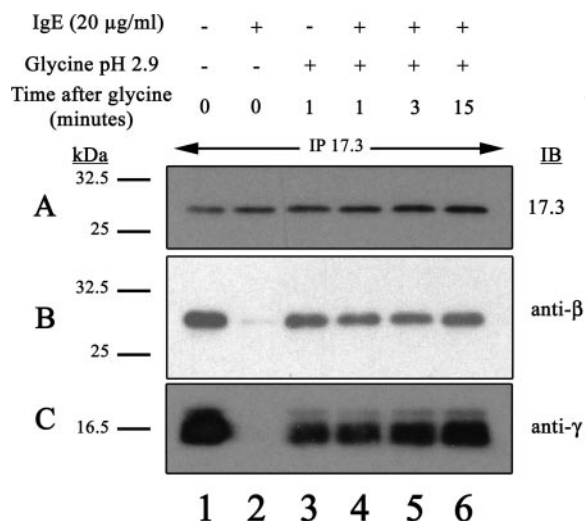


FIG. 7. Removing IgE induces rapid p28-FcεRI reassociation. RBL-2H3 cells were first sensitized (lanes 2 and 4–6) or not (lanes 1 and 3) for 30 min at 37 °C as indicated. Then IgE were removed where indicated, by an acidic treatment of the cells for 4 min (as described under “Experimental Procedures”), and the cells were further incubated in HBT medium in the absence of IgE at 37 °C for the indicated time length (lanes 4–6). Lane 1, nonsensitized control; lane 2, sensitized and non-acid-treated control; lane 3, unsensitized and acid-treated control. After these treatments, RBL-2H3 cell lysates were subjected to IP with mAb 17.3, resolved by SDS-PAGE, and analyzed by IB with mAb 17.3 (A), anti-FcεRIβ mAb 30.9 (B), and polyclonal anti-FcεRIγ (C). The gel was 12% polyacrylamide. The experiment is representative of three.

and FcεRI in nonsensitized cells. By contrast, in IgE-sensitized cells, a 1-min treatment with glycine was sufficient to allow for the complete reassociation between p28 and FcεRI.

Aggregation of FcεRI has been reported so far to promote association between effector molecules and FcεRI. Therefore, finally, we examined whether receptor aggregation could promote reassociation of p28 with FcεRI. Thus, cells were incubated for 1 h with 20 μg/ml IgE to induce a complete dissociation of the complex, washed, and stimulated for 30 min with 1:10 serial dilutions of antigen between 10^{-2} and 10^2 μg/ml. No reassociation of p28 to FcεRI was observed (data not shown).

Association and Dissociation of the Complex Is Observed in Nontumoral Mast Cells—To confirm these observations in nontumoral mast cells, experiments were conducted in rat bone marrow-derived mast cells. p28 was detected in these cells as it had been in RBL-2H3 cells. Immunoprecipitation with mAb 17.3 on nonsensitized rat bone marrow-derived mast cells allowed the recovery of FcεRIβ (Fig. 8) and γ (data not shown) together with p28. Sensitization of the cells with IgE induced complete dissociation of the complex as it had in the RBL-2H3 mast cell line (Fig. 8).

DISCUSSION

The present work describes a new FcεRI signaling effector that is a sensor of FcεRI occupation by its ligand. Indeed, p28, a 28-kDa protein, is constitutively associated with at least 50% of the membrane-expressed FcεRI. Upon binding of IgE to its receptor, p28 rapidly dissociates from the latter with a kinetics that is comparable with that observed with IgE binding (31). This dissociation was not due to aggregation of the receptor because HPLC-purified monomeric IgE were fully effective to induce it and was not the result of an artifact associated with the DNP-48 IgE used throughout the study because the same result was obtained with two other IgE. Cell activation did not modulate p28-FcεRI association whether positively or negatively, and the dissociation was not the result of protein recruitment or of an irreversible protein alteration but most

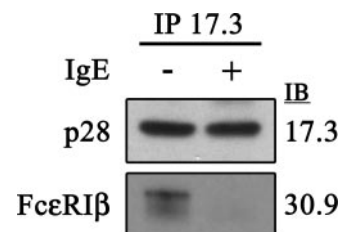


FIG. 8. Association/dissociation of p28-FcεRI complex in rat bone marrow-derived mast cells. Recombinant rat IL-3β-dependent rat bone marrow-derived mast cells were incubated for 30 min with or without 15 μg/ml of IgE, and the proteins in the lysates were immunoprecipitated with mAb 17.3. The presence of p28-FcεRI complexes was examined by mAb 17.3 (upper panel) and by mAb 30.9 anti-FcεRIβ (lower panel) immunoblotting. The gel was 12% polyacrylamide. This experiment is representative of three.

likely of a protein conformational change. Furthermore, when the bound IgE is stripped from FcεRI by an acidic treatment of the cells, p28 reassociates immediately with the receptor, revealing that it senses whether the receptor is in an occupied or in a free state.

The identity of this p28 sensor protein is still unknown. mAb 17.3 recognizes the 37-kDa rPLSCR and p28, demonstrating the existence of a common epitope between these two proteins. By screening an RBL-2H3 λ-ZAP cDNA expression library with mAb 17.3, more than 200 positive clones were obtained. None of these clones were coding for a protein other than the rPLSCR (data not shown), suggesting either that p28 was not produced by the cDNA expression library or that it is an isoform of rPLSCR. Analyses by flow cytometry and confocal microscopy of RBL-2H3 cells with mAb 17.3 did not reveal any extracellular staining of the cells (data not shown). After permeabilization, however, cytosolic and membrane staining were observed, but because mAb 17.3 binds both rPLSCR and p28, it was impossible to determine the contribution of the latter to the overall staining (data not shown). Nevertheless, the absence of the p28 epitope recognized by mAb 17.3 in the extracellular compartment, together with the absence of p28 radiolabeling in cells that have been surface-labeled with ^{125} I, indicate that p28 does not contain a large extracellular domain. Interestingly, the PLSCR family includes members with a small or no extracellular domain (36). Therefore, it is possible that p28 could be a spliced isoform or a post-translation processing product of rPLSCR.

The association of the p28 sensor protein with FcεRI is most likely regulated by changes in the conformation of the receptor. The evidence collected in this study supports this conclusion. Indeed, p28 dissociation from FcεRI can be induced by IgE *in vitro* after purification of the complex, demonstrating that no recruitment of additional protein was required. This also demonstrated that the dissociation was not dependent on additive molecular alterations, such as phosphorylation or palmitoylation, of p28 or FcεRI. Additional indirect evidence is that this dissociation is temperature-sensitive. Finally, p28 dissociates from FcεRI immediately following IgE binding, and this dissociation is immediately reversed following stripping of the bound IgE from its receptor. Altogether, although not exclusive of other explanations, these data are in agreement with a conformation-regulated association of p28 with FcεRI. Reported crystallographic studies of the receptor revealed regions of conformation flexibility in the extracellular domain of the FcεRIα chain (37), as well as changes in the conformation of IgE after binding to the receptor (38, 39). However, for obvious reasons, no data are available concerning IgE-induced potential changes in the conformation of the intracytoplasmic domain of FcεRIα or of both FcεRIβ and FcεRIγ that have almost no extracellular domain and that are involved in IgE-dependent FcεRI signal transduction (9).

Although the identity of the chain with which p28 is associated is still an unresolved question, the simplest model would involve the FcεRIα chain itself, particularly in regard of the conformation hypothesis described above. This would be the first signaling function ascribed to the cytoplasmic tail of FcεRIα.

A minor fraction (3–20%) of FcεRI is constitutively associated with the tyrosine kinase p53/56^{lyn} (40), a protein involved in the early steps of IgE/antigen-mediated cell activation. This observation is in agreement with the receptor aggregation paradigm. Indeed, one p53/56^{lyn} molecule can phosphorylate nearly all the receptors engaged in a receptor aggregate, thereby initiating an early amplification of the signal (40). By contrast, a major fraction (≥50%) of FcεRI is associated with p28. The difference between these two situations could lie in the fact that the signal generated by p28 is independent of receptor aggregation; binding of one IgE to one FcεRI results in the release of p28.

The molecular events of downstream monomeric IgE binding to its receptor have been explored and include tyrosine phosphorylation of the receptor and of Shc and SHIP, as well as activation of phosphatidylinositol-3 kinase (22, 23, 41). Yet there are contradictory reports on the phosphorylation of several molecular effectors such as extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase, and protein kinase B, and the involvement of the Bcl2 family of apoptosis regulating factors (22, 23). The signaling pathway, and thus the molecular partners, of p28 are actively under investigation and will help understand the cellular function(s) activated, or inactivated, by p28. Indeed, recent works have reported that the sensitization of mast cells by monomeric IgE induces many cellular responses including up-regulation of FcεRI membrane expression, cell survival, increased transcription of the gene for histidine decarboxylase, and adhesion (18–25). Yet, conflicting observations have been reported that sensitization induces the production of leukotriene C4 and of IL-6, and the transcription of the genes for IL-4, IL-13, and tumor necrosis factor α (22, 23). The production of cells deficient in p28 after its identification should help recognize the cellular function(s) activated downstream p28-FcεRI dissociation. Whatever these functions, our report demonstrates that p28 is a new IgE receptor-associated protein that is involved in an early step of signal transduction downstream IgE binding and that serves as a sensor of FcεRI sensitization, a function that has been unsuspected so far.

Acknowledgments—We are indebted to Dr. Reuben P. Siraganian (National Institutes of Health, Bethesda, MD), Dr. Fu-Tong Liu (La Jolla Institute for Allergy and Immunology, San Diego, CA), and Dr. Ulrich Blank (Institut Pasteur, Paris, France) for the generous gift of reagents. We also thank Professor B. Boris Vargaftig and his lab at the Pasteur Institute (Paris, France) for support throughout the initial steps of this study and Dr. Ivan C. Moura for mIgE purification.

REFERENCES

- Mekori, Y. A., and Metcalfe, D. D. (2000) *Immunol. Rev.* **173**, 131–140
- Malaviya, R., Ross, E. A., MacGregor, J. I., Ikeda, T., Little, J. R., Jakischik, B. A., and Abraham, S. N. (1994) *J. Immunol.* **152**, 1907–1914
- Frandji, P., Tkaczky, C., Oskeritzian, C., Lapeyre, J., Peronet, R., David, B., Guillet, J. G., and Mecheri, S. (1995) *Cell Immunol.* **163**, 37–46
- Malaviya, R., Ikeda, T., Ross, E., and Abraham, S. N. (1996) *Nature* **381**, 77–80
- Wang, H. W., Tedla, N., Lloyd, A. R., Wakefield, D., and McNeil, P. H. (1998) *J. Clin. Invest.* **102**, 1617–1626
- Tkaczky, C., Villa, I., Peronet, R., David, B., Chouaib, S., and Mecheri, S. (2000) *J. Allergy Clin. Immunol.* **105**, 134–142
- Mecheri, S., and David, B. (1997) *Immunol. Today* **18**, 212–215
- Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J. P. (1989) *Nature* **337**, 187–189
- Nadler, M. J., Matthews, S. A., Turner, H., and Kinet, J. P. (2000) *Adv. Immunol.* **76**, 325–355
- Eiseman, E., and Bolen, J. B. (1992) *Nature* **355**, 78–80
- Parravicini, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L. E., O'Shea, J. J., and Rivera, J. (2002) *Nat. Immunol.* **3**, 741–748
- Kimura, T., Zhang, J., Sagawa, K., Sakaguchi, K., Appella, E., and Siraganian, R. P. (1997) *J. Immunol.* **159**, 4426–4434
- Germano, P., Gomez, J., Kazanietz, M. G., Blumberg, P. M., and Rivera, J. (1994) *J. Biol. Chem.* **269**, 23102–23107
- Benhamou, M., Ryba, N. J., Kihara, H., Nishikata, H., and Siraganian, R. P. (1993) *J. Biol. Chem.* **268**, 23318–23324
- Shiue, L., Zoller, M. J., and Brugge, J. S. (1995) *J. Biol. Chem.* **270**, 10498–10502
- Kihara, H., and Siraganian, R. P. (1994) *J. Biol. Chem.* **269**, 22427–22432
- Hutchcroft, J. E., Geahlen, R. L., Deanin, G. G., and Oliver, J. M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9107–9111
- Furuichi, K., Rivera, J., and Isersky, C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1522–1525
- Hsu, C., and MacGlashan, D., Jr. (1996) *Immunol. Lett.* **52**, 129–134
- Yano, K., Yamaguchi, M., de Mora, F., Lantz, C. S., Butterfield, J. H., Costa, J. J., and Galli, S. J. (1997) *Lab. Invest.* **77**, 185–193
- Kubo, S., Matsuoka, K., Taya, C., Kitamura, F., Takai, T., Yonekawa, H., and Karasuyama, H. (2001) *J. Immunol.* **167**, 3427–3434
- Asai, K., Kitaura, J., Kawakami, Y., Yamagata, N., Tsai, M., Carbone, D. P., Liu, F. T., Galli, S. J., and Kawakami, T. (2001) *Immunity* **14**, 791–800
- Kalesnikoff, J., Huber, M., Lam, V., Damen, J. E., Zhang, J., Siraganian, R. P., and Krystal, G. (2001) *Immunity* **14**, 801–811
- Tanaka, S., Takasu, Y., Mikura, S., Satoh, N., and Ichikawa, A. (2002) *J. Exp. Med.* **196**, 229–235
- Lam, V., Kalesnikoff, J., Lee, C. W., Hernandez-Hansen, V., Wilson, B. S., Oliver, J. M., and Krystal, G. (2003) *Blood* **102**, 1405–1413
- Pastorelli, C., Veiga, J., Charles, N., Voignier, E., Moussu, H., Monteiro, R. C., and Benhamou, M. (2001) *J. Biol. Chem.* **276**, 20407–20412
- Pastorelli, C., Veiga, J., Charles, N., Voignier, E., Moussu, H., Monteiro, R., and Benhamou, M. (2002) *Mol. Immunol.* **38**, 1235–1238
- Basciano, L. K., Berenstein, E. H., Kmak, L., and Siraganian, R. P. (1986) *J. Biol. Chem.* **261**, 11823–11831
- Stracke, M. L., Basciano, L. K., Fischler, C., Berenstein, E. H., and Siraganian, R. P. (1987) *Mol. Immunol.* **24**, 347–356
- Liu, F. T., Bohn, J. W., Ferry, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R., and Katz, D. H. (1980) *J. Immunol.* **124**, 2728–2737
- Barsumian, E. L., Isersky, C., Petrino, M. G., and Siraganian, R. P. (1981) *Eur. J. Immunol.* **11**, 317–323
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Isersky, C., Rivera, J., Mims, S., and Triche, T. J. (1979) *J. Immunol.* **122**, 1926–1936
- Sims, P. J., and Wiedmer, T. (2001) *Thromb. Haemostasis* **86**, 266–275
- Williamson, P., and Schlegel, R. A. (2002) *Biochim. Biophys. Acta* **1585**, 53–63
- Wiedmer, T., Zhou, Q., Kwok, D. Y., and Sims, P. J. (2000) *Biochim. Biophys. Acta* **1467**, 244–253
- Garman, S. C., Sechi, S., Kinet, J. P., and Jardetzky, T. S. (2001) *J. Mol. Biol.* **311**, 1049–1062
- Wurzberg, B. A., and Jardetzky, T. S. (2002) *Mol. Immunol.* **38**, 1063–1072
- Wan, T., Beavil, R. L., Fabiane, S. M., Beavil, A. J., Sohi, M. K., Keown, M., Young, R. J., Henry, A. J., Owens, R. J., Gould, H. J., and Sutton, B. J. (2002) *Nat. Immunol.* **3**, 681–686
- Yamashita, T., Mao, S. Y., and Metzger, H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11251–11255
- Huber, M., Helgason, C. D., Damen, J. E., Liu, L., Humphries, R. K., and Krystal, G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11330–11335