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# SNARE Proteins Are Critical for Regulated Exocytosis of ECP from Human Eosinophils

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**The SNARE hypothesis, describing a protein assembly–disassembly pathway, was recently proposed for the sequential steps of synaptic vesicle docking, activation and fusion. To determine if SNARE proteins are involved in regulated exocytosis in eosinophils, the presence and functional role of SNAREs was examined in human blood eosinophils. Immunoblotting, subcellular fractionation, and immunocytochemistry documented that vesicle-associated membrane protein-2 (VAMP-2), a vesicle-SNARE, was expressed in human eosinophils. Syntaxin 4 and SNAP-25 were also detected. Sequencing of cloned RT-PCR products amplified from a domain conserved among VAMP isoforms revealed identity only to VAMP-2 but not to VAMP-1 or cellubrevin. Functional experiments revealed that tetanus toxin pretreatment, which cleaved VAMP-2 in eosinophils, significantly inhibited both IgE receptor- and phorbol ester-mediated exocytosis of eosinophil cationic protein (ECP) from streptolysin-O-permeabilized eosinophils. Thus, these results strongly suggest a critical role of SNAREs in regulated exocytosis in eosinophils.** 

***Key Words:*** **human; eosinophils; regulated exocytosis; allergy; cell activation; Fc receptors.**

The association between eosinophilia and asthma was observed shortly after eosinophils were first described, and it is well known that eosinophils are predominant among the cells involved in the inflammatory response to parasites (1), in asthma (2), and in allergic rhinitis (3). It is well established that eosinophils release toxic proteins and other mediators that are important contributors to the pathogenesis in allergic diseases. Regulated membrane trafficking and exocytosis play a vital role in intercellular signalling and release of mediators by cells of the immune system. However, the molecular apparatus involved in regulated exocytosis in immunocompetent cells, including eosinophils, is currently unknown. Neurons release neurotransmitters at synapses via a highly regulated process involving the fusion of synaptic vesicles with the presynaptic plasma membrane (4, 5). Substantial evidence supports the view that the sequential steps of synaptic vesicle docking, activation and fusion involve vesicle-targeting receptors as described in the SNARE hypothesis (6). Although the distribution of v-SNARE to vesicular membrane and t-SNARE to target membrane appears to be less specific than originally proposed, the assembly of vesicle (v-SNARE) and target-localized (t-SNARE) SNARE proteins is necessary and sufficient critical for the docking and fusion of transport vesicles to target membranes (4, 7). In the case of synaptic vesicle exocytosis, synaptobrevin/VAMP is a v-SNARE, and syntaxins and SNAP25/23 are t-SNAREs. Multiple mammalian isoforms of v-SNAREs and t-SNAREs have been identified and sequenced (8). VAMP-1 and -2 were first identified as synaptic vesicle proteins, and their functional characterization has been restricted to the nervous system. However, several recent papers have identified VAMP-2 in cells and tissues outside the central nervous system (9–11) and in some instances exocytosis or translocation of vesicular markers to the cell surface have been shown to be dependent on SNARE complex components (12–20). Here, compelling evidence is presented for the essential role of SNARE proteins in regulated exocytosis of ECP from human eosinophils.

Abbreviations used: SNARE, soluble NSF attachment protein receptor; VAMP, vesicle-associated membrane protein; TeTx, tetanus toxin; FcεRI, high affinity receptor for IgE.

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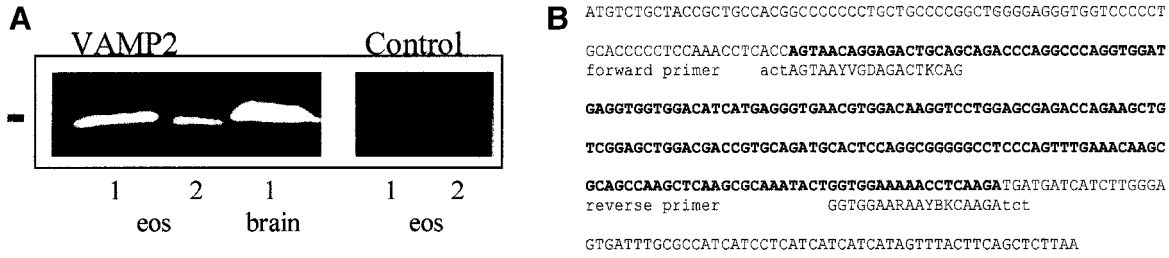


**FIG. 1.** Expression of VAMP-2 protein in eosinophil homogenates and brain synaptic vesicles. (A) Immunoblotting reveals that vesicle associated membrane protein-2 (VAMP-2), a vesicle-SNARE, is expressed in human eosinophils and comigrates with the corresponding 18-kDa VAMP-2 band from brain synaptic vesicles. The negative control using preimmune serum was blank. Western blots of eosinophil cell lysates from two donors (lane 1 and 2) and purified synaptic vesicles (lane 3) were probed with an anti-VAMP-2 polyclonal antibody (12) and developed with the ECL system (Amersham International, U.K.). The bar indicates a molecular weight of 18 kilodaltons. Immunolabeling control: Immunoblot with eosinophil cell lysates (lane 1) and brain synaptic vesicles (lane 2) probed with preimmune serum. (B) cDNA sequences of VAMP-2 from eosinophils and brain are identical. The sequence of the conserved PCR fragment cloned from RT-PCR of eosinophil RNA was identical to bases 82–283 of the coding sequence of VAMP-2 (GenBank Accession No. M36201). The conserved sequence is in bold print, and the primers used to amplify VAMP-2 from eosinophil RNA are printed below the sequence.

## MATERIALS AND METHODS

*Donors.* All donors met the American Thoracic Society definition of asthma (21) or the international consensus on the diagnosis of rhinitis (22). Human blood eosinophils were purified (>98% purity) from allergic donors as described (23).

*Preparation of rat brain extracts.* Whole rat brains (250-gram Wistar rats) were homogenized in dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, 8.5 $\mu$M leupeptin, 1 mM PMSF) (13). The homogenate was centrifuged at 4000*g* for 15 min at 4°C. The pellet was rehomogenized and recentrifuged to increase yields. The pellets were resuspended in dissecting buffer and assayed for protein concentration by Lowry's method. Synaptic vesicles were prepared by gradient centrifugation as described (24).

*Molecular biology.* RNA (1 $\mu$g), prepared from $2 \times 10^7$ eosinophils was reverse transcribed with Expand RT (Boehringer Mannheim) and a $T_{17}$ primer. The cDNA was amplified with primers for VAMP and human $\beta$-actin as a positive control (30 cycles). The PCR product for VAMP was cloned into pBluescript II SK+. Independent clones were sequenced in both directions.

*Gradient centrifugation.* Purified eosinophils ($3 \times 10^6$ cells/ml) from normal individuals were suspended in HBSS with 0.1% HSA and 0.11% $Ca^{2+}$ were incubated for 20 min at 37°C, and were diluted by adding ice cold PBS. The cells were centrifuged and resuspended in 2 ml 6% sucrose in 10 mM Pipes, pH 7.4 and after 15 min on ice disrupted by ultrasound. The intact cells and nuclei were pelleted by centrifugation (5 min, 4°C, 1000*g*). The postnuclear supernatant was layered on top of a sucrose gradient (60, 55, 46, 43, 37, 34, 32, 30, 25, 20% (w/w) in 10 mM Pipes, pH 7.4). The sucrose gradients were centrifuged over night at 4°C, at 25000 rpm in an SW 28,1 rotor (Beckman Instruments, Inc., CA). Fractions (425 $\mu$l) were collected from top of the tube. Sucrose density in fractions was determined by estimation of the refractive index.

*Immunohistochemistry.* Cryosections (0.8–1 $\mu$m) of purified human eosinophils fixed with 4% paraformaldehyde in 0.1 M Cacodylate buffer (pH 7.2–7.4) were subjected to immunolabeling using affinity purified anti-VAMP-2 antibody or serum as described previously (13). The label was visualised using secondary antibodies conjugated to alkaline phosphatase.

*Cell biology.* Purified eosinophils ($5 \times 10^5$ cells/200 $\mu$l) were incubated overnight on fibronectin-coated plates (fibronectin had previously been shown to up regulate functional FcεRI on eosinophils (data not shown)). Prior to stimulation, all cells were permeabilized with streptolysin-O (50 units/ml, Life Technologies) in RPMI (GIBCO) for 20 min at 37°C and 5% $CO_2$. The eosinophils were then pretreated by incubation with TeTx light chain for 30 min, centrifuged and washed in RPMI. Degranulation was induced by incubation for 2 h with medium (Fig. 3B, lanes labelled medium), with 5 $\mu$g/ml IgE (from the cell line U266) and stimulation with anti-IgE (50 ng/ml rabbit anti-human antibody, Dako, Denmark) for 3 h (lanes labelled IgE/$\alpha$-IgE) and PMA ($10^{-8}$ M, Sigma, USA) for 3 h (lanes labelled PMA). ECP concentration was measured by specific RIA (Pharmacia Upjohn, Uppsala, Sweden).

TeTx light chains were concentrated by ultracentrifugation (Centricon-3 concentrators; Amicon, Inc., Beverly, MA) before use. Protein concentrations were determined by Bradford's assay. The batch of toxin samples used in the present study contained 16 $\mu$M TeTx light chain, 100 mM NaCl, 10 mM Hepes pH 7.6, 15 mM $NaPO_4$, 0.5 mM PMSF, 40 mM imidazole, 1 mM EDTA and 1 mM DTT. Purified TeTx light chain was activated by addition of DTT to a final concentration of 10 mM and 2 mM $ZnCl_2$ to ensure that the toxins are recharged. The toxin was then incubated at room temperature for 1 h to reduce disulfide bonds. TeTx light chain was added to crude cell lysates or brain vesicles in 100 mM NaCl, 20 mM Hepes, pH 7.4 and 1.0% Triton X-100. The samples were incubated for 1 h at 37°C and the reaction was stopped by adding 5× SDS sample buffer. Purified *Clostridium* tetanus toxin (lot 60) was kindly provided by Statens Seruminstitut (Copenhagen, Denmark) and recombinant tetanus toxin light chain was prepared as described previously (13). Both toxins gave similar results.

## RESULTS AND DISCUSSION

*Human eosinophils express VAMP-2.* To examine the molecular apparatus involved in eosinophil degranulation, we have tested whether human eosinophils express VAMP proteins as key components of the SNARE complex. Whole cell lysates of human peripheral blood eosinophils from 10 allergic donors were analysed by Western blotting. Figure 1A illustrates that the anti-VAMP-2 antibody recognized a molecule of approximately 18 kD which comigrated with VAMP-2 from rat brain extracts. Other components of the SNARE complex were detected by Western blotting (SNAP-25) and by flow cytometry (syntaxin 4, SNAP 23/25; data not shown). Anti-VAMP-1 antibodies did not detect any VAMP-1 expression in eosinophils.

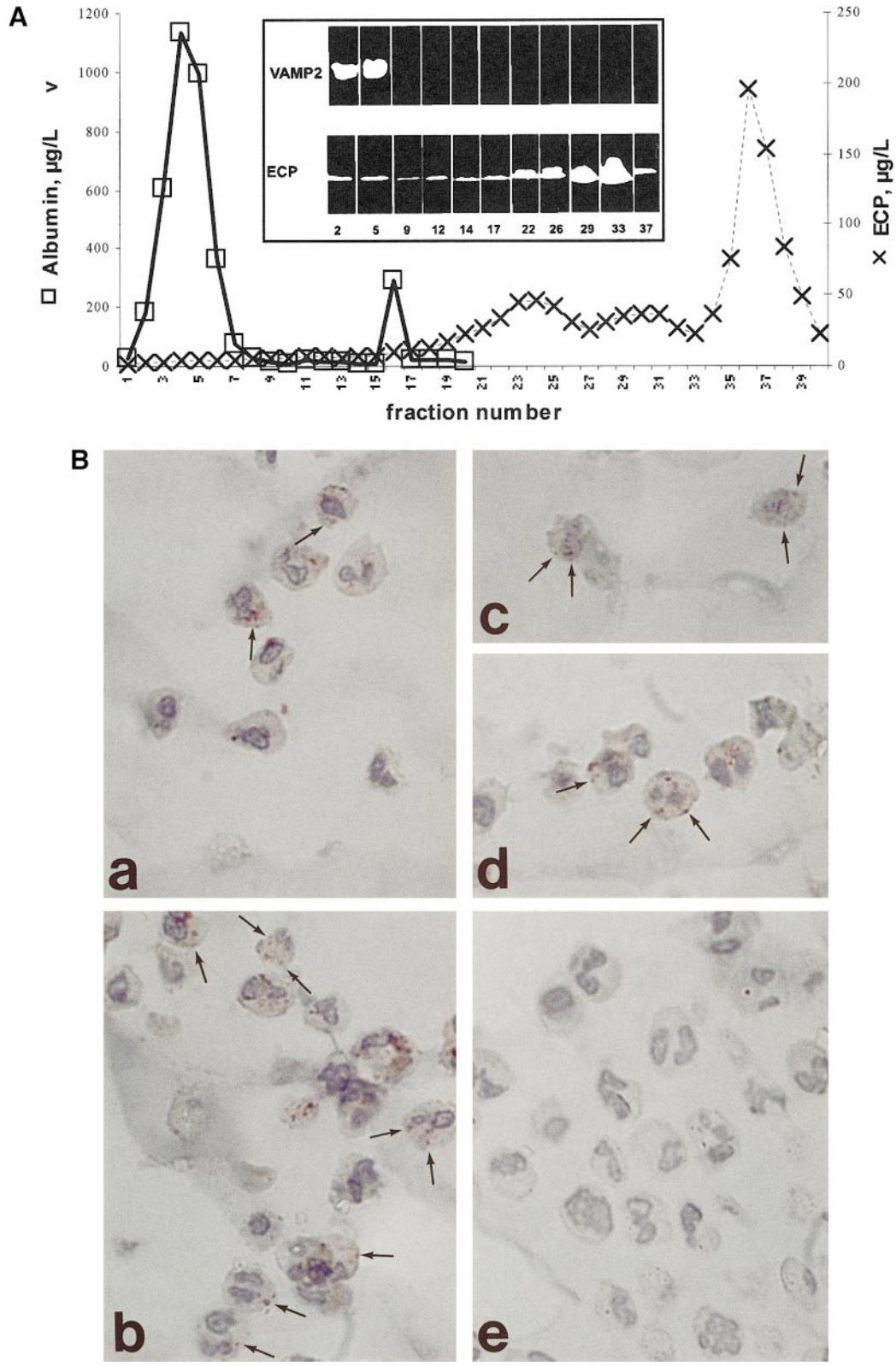


**FIG. 2.** Subcellular identification of VAMP-2 in human eosinophils. (A) VAMP-2 and eosinophil cationic protein are found in fractions 2 and 5 of a sucrose gradient of human eosinophils on a Western blot. VAMP-2 was only found in factions 2 and 5, whereas ECP was found in all fractions, with highest abundance in the eosinophil granules in fractions 29–33. ECP concentration per million cells (stippled line) was measured by specific RIA (Pharmacia Upjohn, Uppsala, Sweden). Total albumin content (solid line) was determined by RIA. In fractions 21 to 40, the albumin concentration was below the detection limit. Western blotting was performed as described (13) with anti-VAMP-2 antibody

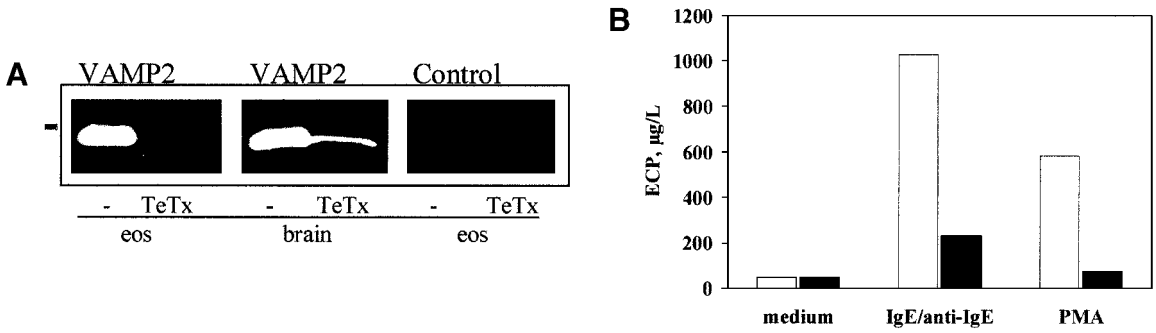


**FIG. 3.** TeTx light chain cleaves VAMP-2 and inhibits ECP release from eosinophils. (A) TeTx degrades VAMP-2 in human eosinophil lysates (Eos) and rat brain vesicles (Brain). The negative control with preimmune serum was blank. Crude eosinophil lysates (Eos) and membrane fractions from brain (Brain) were divided into equal fractions and analyzed by Western blotting with the anti-VAMP-2 antibody (13), which revealed that tetanus toxin degraded VAMP-2 in both eosinophil lysates and vesicular controls. (B) TeTx pretreatment inhibits eosinophil degranulation mediated by high affinity IgE receptor (FcεRI) stimulation or directly by phorbol ester stimulation (PMA). To study the functional relevance of VAMP-2 in eosinophils, permeabilized cells were pretreated with recombinant TeTx light chain (or TeTx, not shown) and the regulated release of ECP was measured in response to stimulation with medium, IgE/anti-IgE or PMA. The average of two independent experiments is shown; black bars represent samples treated with TeTx, white bars represent untreated samples.

To further document that eosinophils express VAMP-2, we amplified, cloned and sequenced VAMP-2 from eosinophils using primers corresponding to the most conserved domain of the VAMP family of proteins using standard techniques. The results revealed complete identity of the sequence in eosinophils with that of human neuron VAMP-2 (Fig. 1B). This supports the hypothesis that eosinophils use the same SNARE proteins for regulated exocytosis that are described in neurons.

*VAMP-2 is associated with secretory vesicles of human eosinophils.* Next we investigated the subcellular localization of VAMP-2 in eosinophils by subcellular fractionation and immunocytochemistry of unstimulated normal eosinophils. By subcellular fractionation of blood eosinophils on sucrose gradients we found VAMP-2 and ECP in the light fractions 2 and 5 that also contain albumin (Fig. 2A). Albumin is a marker for a rapidly mobilisable pool of secretory vesicles of granulocytes that also contain CD11b and cytochrome b558 (25). ECP was found in all tested fractions, with the highest levels detected in fractions, which contained the secretory eosinophil granules. Only about 2% of the total cellular content of ECP was found in fractions that also contained VAMP-2. It is presently unclear whether this surprisingly small fraction is due to a very dynamic process of regulated exocytosis that is preceded by transport of ECP to secretory vesicles, or whether presently uncharacterised SNARE proteins participate in regulated exocytosis of ECP. Consistent with the subcellular fractionations anti-VAMP-2 antiserum primarily labelled vesicles, but not granules, of eosinophils by immunocytochemistry using semithin cryosections of purified cells (Fig. 2B, a–d). Immunolabeling controls were negative (Fig. 2B, e).

*Cleavage of VAMP-2 significantly reduces degranulation by eosinophils.* Tetanus toxin exerts its effect by cleaving VAMP-2 in neuronal synapses, and purified or recombinant forms of the toxin have been used widely to document a role of SNAREs in regulated exocytosis in cell types (12–20). As shown in Fig. 3A treatment with TeTx light chain efficiently degraded both synaptic vesicle and eosinophil VAMP-2 protein.

To determine whether this cleavage of VAMP-2 could modify regulated exocytosis in eosinophils, TeTx was introduced into streptolysin-O-permeabilized, eosinophils before stimulating them with medium, IgE/anti-IgE or phorbol 12-myristate 13-acetate (PMA). PMA mediates cellular activation through protein kinase C (26), and IgE/anti-IgE is a specific stimulus known to elicit degranulation of ECP by eosinophils (27), and may cause deposition of ECP in the bronchi. Deposition of eosinophil granule proteins is thought to be a primary cause of the pathological airway remodelling observed in asthma (28). As shown in Fig. 3B, pretreatment of eosinophils with TeTx markedly inhibited release of ECP from eosinophils following stimulation with either IgE/anti-IgE (>80% inhibition) or PMA (>90% inhibition) when compared to permeabilized cells not treated with toxin. In control experiments heat-inactivated TeTx did not inhibit degranulation

and with EG1 to detect ECP. (B) Detection of VAMP-2 in eosinophils by immunocytochemistry (13, 29). Cryosections were labeled with antibodies to VAMP-2 using serum (a and b), affinity purified antibodies (c and d) or preimmune serum (e). Arrows indicate VAMP-2 labeled vesicular structures beneath the plasma membrane present in most cells probed with anti-VAMP-2. Large granules were generally unlabeled. Magnification is 1100×.

(not shown). Thus TeTx degraded VAMP-2 identified in total eosinophil extracts, and significantly inhibited exocytosis of ECP following stimulation through IgE receptors or activation of the protein kinase C pathway. This strongly suggests that VAMP-2, and hence SNAREs, play important roles in exocytosis of ECP following these stimuli.

Presence of v- and t-SNAREs was recently shown to be both necessary and sufficient for membrane fusion (7). Here, the v-SNARE VAMP-2 is shown to be present in human eosinophils and functional evidence for a role of SNARE components is provided in eosinophil degranulation induced by both IgE and the protein kinase C pathway. This supports the notion that the SNARE hypothesis describes a general mechanism for regulated exocytosis, which is not only found in neuronal cells but also in cells outside the nervous systems (12–20) including the immune system. The molecular interactions resulting from these stimuli that lead to regulated exocytosis at the eosinophil cell membrane are currently being investigated.

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## REFERENCE

1. Gleich, G. J., and Adolphson, C. R. (1986) *Adv. Immunol.* **39,** 177–253.
2. Bousquet, J., Chanez, P., Lacoste, J. Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J., and Godard, P. (1990) *N. Engl. J. Med.* **323,** 1033–1039.
3. Kay, A. B. (1985) *Clin. Exp. Immunol.* **62,** 1–12.
4. Rothman, J. E. (1994) *Nature* **372,** 55–63.
5. Hanson, P. I., Heuser, J. E., and Jahn, R. (1997) *Curr. Opin. Neurobiol.* **7,** 310–315.
6. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) *Nature* **362,** 318–324.
7. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998) *Cell* **92,** 759–772.
8. Advani, R. J., Bae, H. R., Bock, J. B., Chao, D. S., Doung, Y. C., Prekeris, R., Yoo, J. S., and Scheller, R. H. (1998) *J. Biol. Chem.* **273,** 10317–10324.
9. Brumell, J. H., Volchuk, A., Sengelov, H., Borregaard, N., Cieutat, A. M., Bainton, D. F., Grinstein, S., and Klip, A. (1995) *J. Immunol.* **155,** 5750–5759.
10. Pitzurra, L., Rossetto, O., Chimienti, A. R., Blasi, E., and Bistoni, F. (1996) *Cell Immunol.* **169,** 113–116.
11. Majo, G., Aguado, F., Blasi, J., and Marsal, J. (1998) *Life Sci.* **62,** 607–616.
12. Gaisano, H. Y., Sheu, L., Foskett, J. K., and Trimble, W. S. (1994) *J. Biol. Chem.* **269,** 17062–17066.
13. Nielsen, S., Marples, D., Birn, H., Mohtashami, M., Dalby, N. O., Trimble, W., and Knepper, M. (1995) *J. Clin. Invest.* **96,** 1834–1844.
14. Alexander, E. A., Shih, T., and Schwartz, J. H. (1997) *Am. J. Physiol.* **273,** F1054–F1057.
15. Olson, A. L., Knight, J. B., and Pessin, J. E. (1997) *Mol. Cell Biol.* **17,** 2425–2435.
16. Jacobsson, G., Hakansson, M. L., Hulting, A. L., and Meister, B. (1997) *Regul. Pept.* **71,** 37–44.
17. Macaulay, S. L., Hewish, D. R., Gough, K. H., Stoichevska, V., MacPherson, S. F., Jagadish, M., and Ward, C. W. (1997) *Biochem. J.* **324,** 217–224.
18. Pitzurra, L., Adami, C., Sevilla, M., Polonelli, L., Bistoni, F., and Blasi, E. (1999) *Cell Immunol.* **191,** 20–25.
19. Baram, D., Adachi, R., Medalia, O., Tuvim, M., Dickey, B. F., Mekori, Y. A., and Sagi-Eisenberg, R. (1999) *J. Exp. Med.* **189,** 1649–1658.
20. Chen, D., Bernstein, A. M., Lemons, P. P., and Whiteheart, S. W. (2000) *Blood* **95,** 921–929.
21. (1992) *Allergy* **47,** 1–61.
22. (1994) *Allergy* **49,** 1–34.
23. Hansel, T. T., De, V. I., Iff, T., Rihs, S., Wandzilak, M., Betz, S., Blaser, K., and Walker, C. (1991) *J. Immunol. Methods* **145,** 105–110.
24. Huttner, W. B., Meyermann, R., Neuhoff, V., and Althaus, H. H. (1979) *Brain Res.* **171,** 225–237.
25. Calafat, J., Kuijpers, T. W., Janssen, H., Borregaard, N., Verhoeven, A. J., and Roos, D. (1993) *Blood* **81,** 3122–3129.
26. Kato, M., Abraham, R. T., and Kita, H. (1995) *J. Immunol.* **155,** 357–366.
27. Capron, M., Soussi, G. A., Morita, M., Truong, M. J., Prin, L., Kinet, J. P., and Capron, A. (1995) *Allergy* **50,** 20–23.
28. Gleich, G. J., Motojima, S., Frigas, E., Kephart, G. M., Fujisawa, T., and Kravis, L. P. (1987) *J. Allergy Clin. Immunol.* **80,** 412–415.
29. Nielsen, S., Smith, B. L., Christensen, E. I., Knepper, M. A., and Agre, P. (1993) *J. Cell Biol.* **120,** 371–383.