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Dynamics of Intracellular Granules with CD63-GFP in Rat Basophilic Leukemia Cells

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CD63 is located on the basophilic granule membranes in resting basophils, mast cells, and platelets, and is also located on the plasma membranes of the cells. We constructed a CD63-GFP (green fluorescent protein) plasmid and introduced it into rat basophilic leukemia (RBL-2H3) cells to observe the movements of CD63 on degranulation. The movements of CD63-GFP were studied in living RBL cells by confocal laser scanning microscopy (CLSM). CD63-GFP, in which GFP was conjugated to the C-terminus of CD63, was located on both the granule membranes and the plasma membranes of RBL cells. The diameter of the fluorescent granules in the cytoplasm varied from 0.5 to 1.5 μm. Before antigen stimulation most granules with CD63-GFP hardly moved in RBL cells. However, after antigen stimulation the plasma membranes ruffled violently and the granules moved dramatically. They reached the plasma membranes in a few minutes and fused with them instantaneously. Analysis of the movement of each granule provided a new insight into the elementary process of degranulation. The velocity of the granule movement toward the plasma membranes on antigen stimulation was calculated to be 0.1 ± 0.02 μm/s. This shows that the granules are able to reach the plasma membranes in 2 or 3 min if the diameter of the cells is 20 μm.

Key words: CD63, confocal laser scanning microscopy, degranulation, green fluorescent protein, RBL-2H3 cell.

The release of histamine and other inflammatory mediators from tissue mast cells and blood basophils is the primary event in a variety of acute allergic and inflammatory conditions (*1*). The release of mediators from these cells is an energy- and calcium-dependent process initiated by the interaction of an antigen with membrane-bound IgE (*2*). RBL-2H3 cells, which are considered to be a model of mucosal mast cells, have high affinity receptors for IgE (FcεRI) and can be stimulated to release histamine through aggregation of the receptors with an antigen (*3*).

Aggregation of FcεRI triggers a cascade of signaling events beginning with tyrosine phosphorylation of the β and γ subunits of FcεRI, and activation of several tyrosine kinases including Lyn and Syk (*4*), with consequent activation of phospholipase Cγ. The latter catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate resulting in the liberation of inositol 1,4,5-trisphosphate and diacylglycerol, which induce the release of intracellular calcium ions from endoplasmic stores and the activation of protein kinase C, respectively (*5*). These secondary messengers are thought to initiate a cascade of biochemical events leading to the release of histamine and other inflammatory mediators from RBL-2H3 cells (*6–10*).

CD63 is located on the basophilic granule membranes in resting basophils, mast cells, and platelets (*11–15*). Activation of basophils or mast cells induces the fusion of cytoplasmic granules with the plasma membrane and the successive release of inflammatory mediators, such as histamine. On activation of human basophilic granulocytes with anti-IgE, the expression of CD63 on the cell surface increased, which was detected by means of flow-cytometry together with anti-CD63 monoclonal antibodies (*16*). Flow-cytometry, however, is not always used to study the expression, especially in the case of the increase in surface expression of CD63 after FcεRI crosslinking being low. In addition, it is not possible to observe the realtime expression of CD63 in individual cells.

To solve these problems we studied the surface expression of CD63 in RBL-2H3 cells by means of CLSM together with mouse anti-CD63 IgG1 (*13, 14*). We observed the realtime surface expression of CD63 in RBL-2H3 cells and showed that the expression of CD63 reflected the degranulation, but the dynamic movements of CD63 in the intracellular granule membranes remained to be elucidated. In the present study we prepared a plasmid, CD63-GFP (or GFP-CD63) and introduced it into RBL-2H3 cells to obtain stable transfectant cells. The results constitute new information for studying the dynamics of intracellular granules in living RBL cells.

MATERIALS AND METHODS

Construction of CD63-GFP—The construction method used for mouse CD63-GFP was as follows: GFP expression vectors (pEGFP-N1) and the mouse CD63 gene were purchased from Clontech (Palo Alto, CA) and ATCC (Rockville, MD), respectively. CD63 cDNA served as a template for

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PCR amplification with appropriate oligonucleotide primers, and GFP was conjugated to the C-terminus of CD63. For the generation of a CD63-GFP chimera protein, CD63 cDNA was amplified with oligonucleotides such that *Sac*II and *Age*I restriction sites were introduced at the 5′ and 3′ ends, respectively. The fusion between GFP and the C-terminus of CD63 was achieved through *Sac*II and *Age*I restriction sites. For comparison, GFP-CD63, in which GFP was fused at the N-terminus of CD63, was constructed.

Transfection of Plasmid DNAs—RBL-2H3 cells were cultured in MEM medium supplemented with 10% FCS from Boehringer. The cells were electroporated in cold K^+-PBS buffer with 20 µg of plasmid DNA at 250 V and 950 µF using Gene Pulser (Bio-Rad). RBL-2H3 cells with CD63-GFP plasmid DNA were cultured in 35 mm dishes for a few days and then stable transfectants were obtained by the selection with the antibiotic G418 (Gibco). After that, a cloned cell line of RBL cells with GFP was obtained.

Western Blot Analysis—Western blot analysis was performed following the previously reported procedure (*17*). To prepare a whole cell lysate, collected RBL cells were suspended in lysis buffer (1% 3-[(3-cholamidopropyl)-dimethylammonio]-propane sulfate (CHAPS), 20 mM Tris-HCl, 10 mM EDTA, 10% glycerol, 8 µg/ml leupeptin, 2 µg/ml pepstatin, and 1 mM PMSF) at pH 7.5. After 30 min incubation at 4°C, the insoluble material was removed by centrifugation at 10,000 rpm for 15 min at 4°C. After centrifugation, the resulting supernatants were solubilized with Laemmli buffer at 100°C for 2 min and then proteins separated by electrophoresis were transferred to PVDF membranes with an electroblotter. After blocking with 0.5% casein, the membranes were probed with a 1:2,000 dilution of a rabbit anti-GFP antibody (Clontech) and then treated with a horseradish peroxidase (PO)–conjugated goat anti-rabbit secondary antibody. Immunoreactivity was visualized by enhanced chemiluminescence with LAS-1000 (Fuji Film).

β-Hexosaminidase Secretion Assay—Degranulation of RBL cells was monitored by measuring the activity of the granule-stored enzyme, β-hexosaminidase, secreted into cell supernatants, as described by Hampe and Pecht (*18*). Briefly, RBL cells were plated on 24-well plates at 5×10^4 cells/well. On the following day, the cells were sensitized with anti-DNP IgE (0.5 µg/ml) for 30 min, and then monolayers were washed in HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.1% glucose, and 0.1% BSA, pH 7.2) and incubated with an average of six DNP groups conjugated to BSA (DNP_6-BSA) at 37°C. Following the incubation, 20 µl samples of the supernatants were transferred to 96-well plates and then 20 µl of the substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 100 mM citrate, pH 4.5) was added. The plates were incubated for 1 h at 37°C. The reaction was stopped with 160 µl of 167 mM Na_2CO_3-$NaHCO_3$ buffer. The color due to the substrate hydrolysis was measured with a microplate reader at 405 nm. The results are expressed as percentages of the total β-hexosaminidase content of the cells (determined from the values for control cells dissolved with 0.1% Triton X-100). Each assay was performed in triplicate.

Confocal Laser Scanning Microscopy—Confocal laser scanning microscopy (CLSM) was performed by the previously reported procedure (*13*, *14*). The transfected RBL cells were harvested from culture dishes and transferred to an observation chamber from Elekon (Chiba). In the experiments we incubated the transfected RBL cells with mouse anti-DNP IgE in MEM medium for 30 min. CLSM images were taken under a confocal scanning microscope (Zeiss; LSM-410) with an argon ion laser (488 nm) (*19*). GFP fluorescence was excited at 488 nm and its emission was observed above 515 nm. The temperature of the observation chamber was maintained at 37°C during the experiments. In order to analyze the movements of secretary granules in living RBL cells, we used NIH image software.

RESULTS

Construction of CD63 Conjugated with GFP—Enhanced GFP, a highly fluorescent form of GFP, has been fused to the mouse CD63, at either its C-terminus or N-terminus. Fusion plasmid CD63-GFP, in which GFP was conjugated to the C-terminus of CD63, was introduced into RBL cells, and then we obtained stable transfectant RBL cells by selection with the antibiotic G418. To develop a highly efficient, fluorescent version of CD63, we prepared CD63-GFP chimeras with a GFP variant fused in frame to the fourth amino acid from the end of the mouse CD63 gene. The GFP variant from jellyfish *Aequorea victoria* contains phenylalanine-to-leucine and serine-to-threonine substitutions at amino acids 64 and 65 (F64L and S65T mutations), which makes the resulting chromophore more fluorescent than the wild-type GFP (*20–22*).

Western blot analysis with a rabbit anti-GFP antibody and PO-conjugated anti-rabbit IgG revealed the molecular weight of the chimera protein (CD63-GFP) was around 80 kDa, which was in good agreement with the sum of the molecular sizes of glycosylated CD63 and GFP (Fig. 1).

β-Hexosaminidase Secretion—Next, we studied the degranulation from RBL cells by measuring the activity of the granule-stored enzyme, β-hexosaminidase, secreted into cell supernatants, as described by Hampe and Pecht (*18*). The β-hexosaminidase secretion from the transfected RBL cells was almost the same as the value for wild type RBL cells (see Fig. 2). This suggested that the expression of CD63-GFP did not affect the exocytotic release in the transfected RBL cells.

CLSM Images of CD63-GFP—As the F64L and S65T

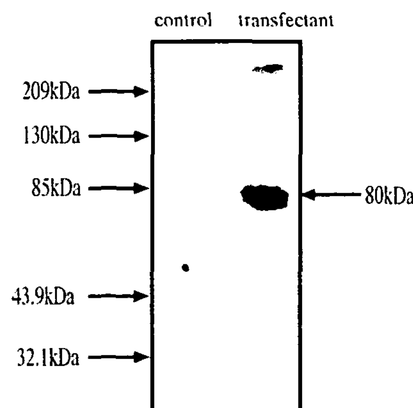

Fig. 1. **Western blot analysis of CD63-GFP using anti-GFP antibodies.** One major band, 80 kDa, was observed, as shown in this figure.

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variant of the GFP fluorophore is a highly fluorescent form and resistant to photobleaching, it can be used to observe the dynamic movements of CD63-GFP during extended periods (~30 min) by means of CLSM. CD63-GFP chimera proteins were located in the granule membranes as well as in the plasma membranes. The fluorescent granules varied from 0.5 to 1.5 μm in diameter (see Figs. 3 and 4). We also examined the distribution of GFP tagged-CD63 at its N-terminus (GFP-CD63). The RBL cells with GFP-CD63 exhibit homogeneous fluorescence in the cytoplasm. This indicated that the N-terminus of CD63 was important for reconstitution into the plasma and granule membranes. So, in the present work we studied the dynamics of the fluorescent granules with GFP tagged-CD63 at the C-terminus (CD63-GFP) in RBL cells.

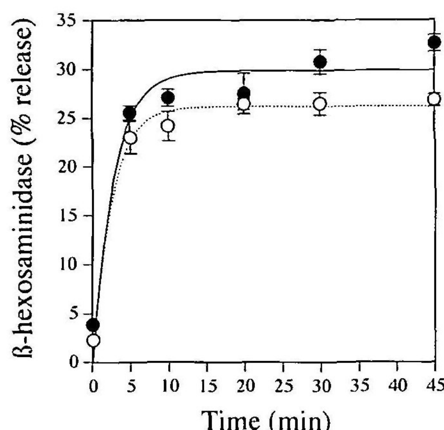

Fig. 2. **The activity of the granule-stored enzyme, β-hexosaminidase, secreted into the RBL cell supernatants was observed, following the method of Hampe and Pecht (*18*).** The level of β-hexosaminidase secretion from the transfected RBL cells (○) was similar to that from wild type RBL-2H3 cells (●). This suggested that the expression of CD63-GFP did not affect the exocytotic release in the transfected RBL cells.

Analysis of the Movements of Secretory Granules—Analysis of the movement of each granule provided a great insight into the elementary process of degranulation. Without an antigen a major population (about 90%) of the fluorescent granules did not move at all, although some of them fluctuated randomly in the local area of the cytoplasm. Typical examples are shown in Fig. 3 (red arrows). In addition, a few granules apparently moved straight along the longitudinal axis of a pseudopodium (see Fig. 3, green arrows). Figure 3 shows a fluorescence image of a typical RBL cell with CD63-GFP (left), and the movements of the granules for 70 s (right).

When an antigen (DNP_6-BSA, 200 ng/ml) was added to the RBL cells with CD63-GFP, the plasma membranes ruffled violently and the granules moved dramatically. In addition to the patterns of granule movements described above (Fig. 3), new movements towards the plasma membranes were observed, as shown in Fig. 4 (right; blue arrows). These kinds of new movements were specific to RBL cells after antigen stimulation (Fig. 3).

Roughly one-third of the granules moved toward the plasma membranes (see Fig. 4, blue arrows). It seemed that they fused with the plasma membranes instanta-

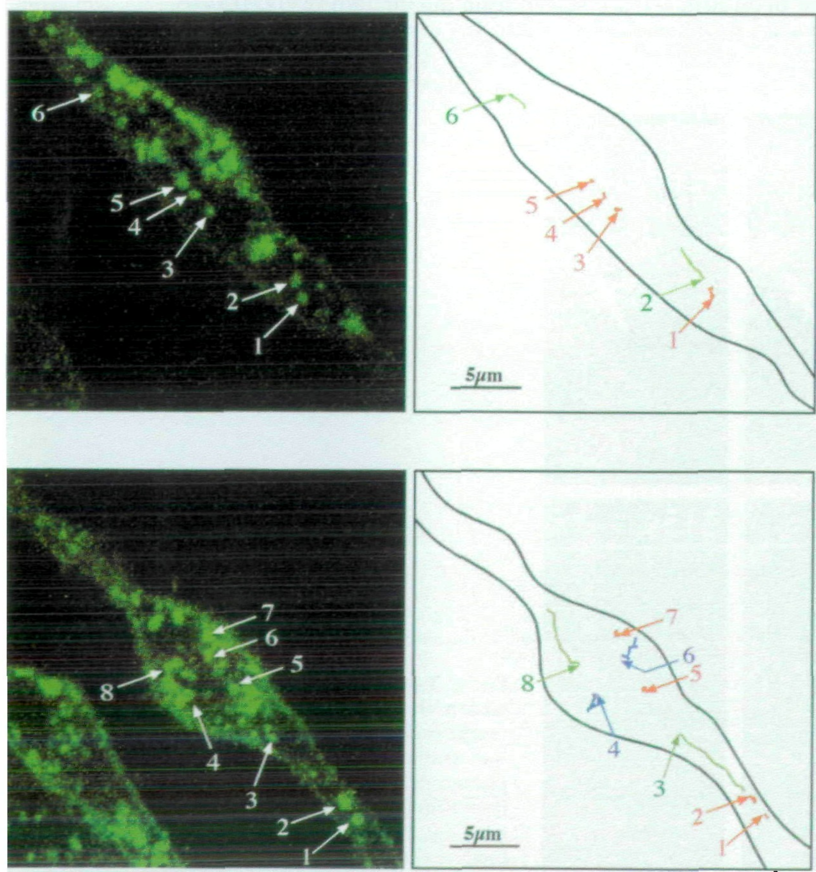

Fig. 3. **Fluorescence images of the granules and plasma membranes in an RBL cell.** CD63-GFP was located in the granule membranes as well as the plasma membranes. (Left) A fluorescence image without antigen stimulation. (Right) A typical example of a trace of the granule movements for 70 s without antigen stimulation. The granules (red) hardly moved in the cytoplasm and the granules (green) moved rather faster (0.16 ± 0.02 μm/s) along the longitudinal axis of the pseudopodium.

Fig. 4. **Fluorescence images of the granules and plasma membranes in an RBL cell after antigen stimulation.** (Left) A fluorescence image after antigen stimulation. (Right) A typical example of a trace of the granule movements for 85 s with stimulation. The granules (red) hardly moved in the cytoplasm, the granules (green) moved rather faster (0.16 ± 0.02 μm/s) along the longitudinal axis of the pseudopodium, and the granules (blue) moved toward the plasma membranes in the RBL cell.

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neously. With antigen stimulation some granules disappeared from the focal plane on CLSM. This was due to the vertical movements of the granules away from the focal plane on CLSM. Such kinds of movements were hardly observed without antigen stimulation. The velocity of the granule movements toward the plasma membranes was calculated to be 0.1 ± 0.02 μm/s. This value indicated that the granules were able to reach the plasma membranes in 2 or 3 min if the diameter of the cells was 20 μm. These times were consistent with the times of secretion for histamine and other inflammatory mediators from RBL cells. Sequential fluorescence images of the fluorescent granules after antigen stimulation are shown in Fig. 5.

Fusion of Granules to the Plasma Membranes—Last, we

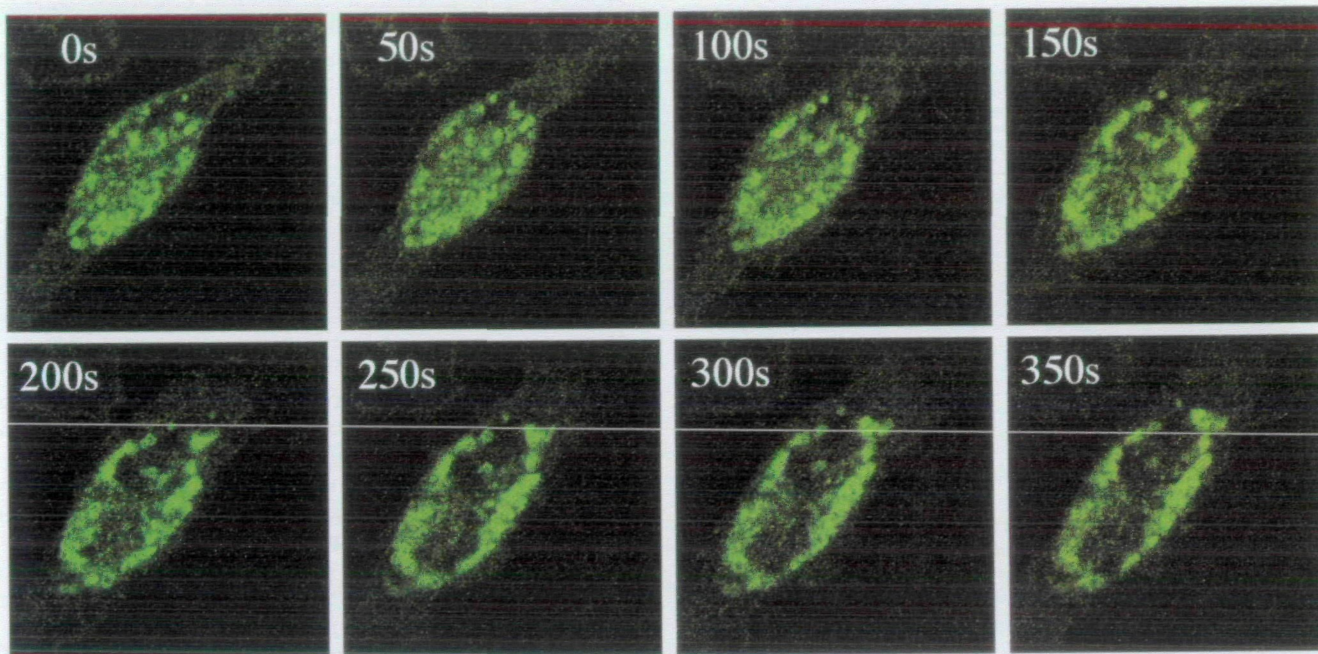

Fig. 5. **Sequential fluorescence images of CD63-GFP in an RBL cell are shown from the top (left to right).** DNP_6-BSA (200 ng/ml) was added to the observation chamber between the first and second frames. Fluorescence images were collected every 50 s at 37°C.

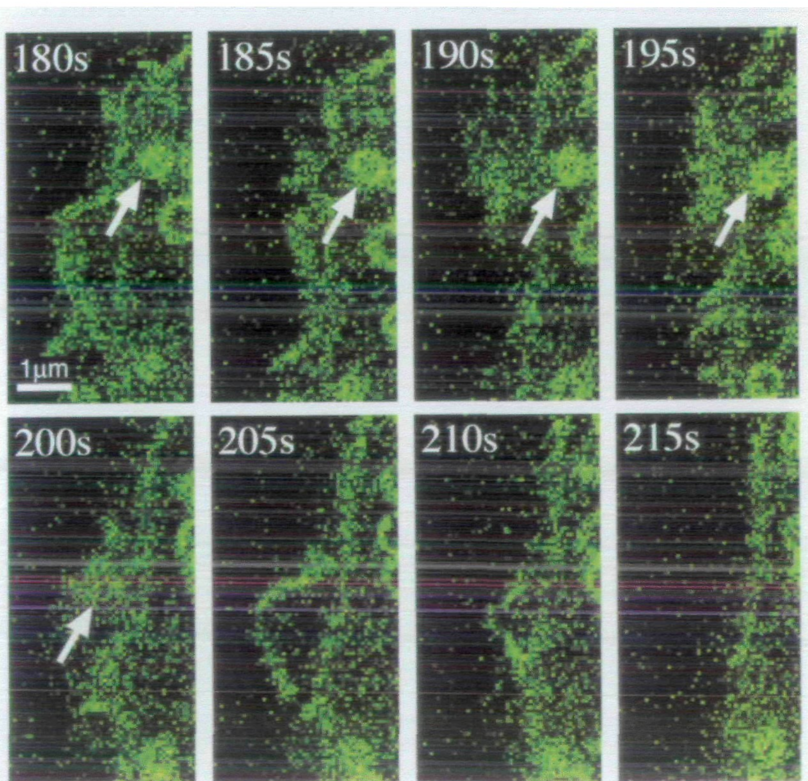

Fig. 6. **Typical sequential fluorescence images, which show a part of the fringes of the plasma membranes, were taken every 5 s.** The fluorescent granule indicated by an arrow moved toward the plasma membrane and disappeared on the fringe of the ruffling membrane after antigen (DNP_6-BSA; 200 ng/ml) stimulation. The time after the addition of antigen is shown in each image.

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checked by means of CLSM whether fusion between granules and the plasma membranes occurred or not. To clarify this point we obtained time-lapse images of the region close to the fringe of the plasma membrane every 5 s for 10 min. Typical images are shown in Fig. 6. The granule indicated by a white arrow disappeared suddenly in the next image on a ruffling plasma membrane. This suggested that the granule fused with the plasma membrane within a few seconds. We traced more than five hundred fluorescent granules in the RBL cells after antigen stimulation. About 14% of the granules fused with the plasma membranes. The fusion between the granules and the plasma membranes occurred only after antigen stimulation. Figure 5 shows an example in which a relatively large number of granules moved towards the plasma membrane after stimulation. Even in such a cell, a considerable number of the granules, however, neither completely fused with the plasma membranes nor disappeared from the cytoplasm. They were still observed along the plasma membranes at 10 min after antigen stimulation (Fig. 5). The fluorescence images shown in Fig. 5 partly explained the previous finding that histamine and other inflammatory mediators were not completely released from RBL cells on antigen stimulation (*3*, *7*).

DISCUSSION

The CD63 antigen is located on the basophilic granule membranes in resting basophils, mast cells, and platelets (*11–15*). We constructed a GFP-conjugated CD63 plasmid and introduced it into RBL-2H3 cells to obtain stable transfectant cells. CD63 is a 30–60 kDa glycoprotein (*11*, *23*, *24*) and belongs to the transmembrane 4 superfamily of proteins (TM4SF). It contains four putative transmembrane domains and three *N*-linked glycosylated sites clustered together on the surface of the lumen side. In addition, surface expression of CD63 on activated basophils and mast cells is correlated with exocytosis of the granule contents (*11–14*). Therefore, expression of CD63 on the cell surface can be explained by fusion of the granule membrane with the plasma membranes. Both the N-terminus and C-terminus of CD63 on the granule membranes face the cytoplasm. Interestingly, the distribution of CD63-GFP in RBL cells was different from that of GFP-CD63. When GFP was conjugated to the C-terminus of CD63 (CD63-GFP), the chimera proteins were expressed on both the granule membranes and plasma membranes of RBL cells as native CD63 proteins. In contrast, GFP-CD63, in which GFP was conjugated to the N-terminus of CD63, was homogeneously distributed in the cytoplasm. It was localized on neither the granules nor the plasma membranes. These results suggested the possibility that the N-terminus of CD63 might play an important role in reconstitution into the bilayer membranes.

We traced more than five hundred granules in RBL cells before and after antigen stimulation. The patterns of granule movements were classified roughly into three groups. In the first group the granules hardly moved, as shown in Figs. 3 and 4 (red arrows). In the second group they moved along the longitudinal axis of a pseudopodium as shown in Figs. 3 and 4 (green arrows). These two patterns of movements were observed even without antigen stimulation. In the third group the movements was toward the plasma membranes. These movements were observed only after antigen stimulation. From the results it was estimated that fluorescent granules reached the plasma membranes within 2 or 3 min after antigen stimulation, if the diameter of the RBL cells was 20 μm. Although CD63 is localized in secretory granules and lysosomes, it is difficult to discriminate between secretory granules and lysosomes. However, it is considered that the granules that moved towards the plasma membranes after antigen stimulation were mostly secretory granules.

Together with the movements of the fluorescent granules, the plasma membranes ruffled violently and some of the granules fused with the ruffling plasma membranes instantaneously. However, a considerable number of them never completely fused with the plasma membranes and many of them remained in the cytoplasm. They gathered along the inside of the plasma membranes at 20 or 30 min after antigen stimulation. These fluorescence images of RBL cells with CD63-GFP were well consistent with the previous finding that histamine and other inflammatory mediators were not completely released from RBL cells on antigen stimulation (*3*, *7*). Anyway, the details of the discharged granules in RBL-2H3 cells were determined by electron microscopy and atomic force microscopy (*9*, *25*), however, the dynamic processes of the degranulation in RBL cells were observed for the first time with the fluorescent granules of CD63-GFP chimera proteins.

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