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Real-time monitoring of pH-dependent intracellular trafficking of ovarian cancer G protein-coupled receptor 1 in living leukocytes

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G-protein coupled receptors (GPCRs) are involved in many diseases and important biological phenomena; elucidating the mechanisms underlying regulation of their signal transduction potentially provides both novel targets for drug discovery and insight into living systems. A proton-sensing GPCR, ovarian cancer G protein-coupled receptor 1 (OGR1), has been reported to be related to acidosis and diseases that cause tissue acidification, but the mechanism of proton-induced activation of OGR1-mediated signal transduction in acidic conditions remains unclear. Here, pH-dependent intracellular trafficking of OGR1 was visualized in living leukocytes by a real-time fluorescence microscopic method based on sortase A-mediated pulse labeling of OGR1. OGR1 labeled on the cell surface with a small fluorescent dye was clearly observed to remain in the plasma membrane during incubation in mildly acidic medium (pH 6.6) and to be internalized to the intracellular compartments on changing the medium to slightly basic pH (7.7). Quantitative single-cell image analysis showed that most of the internalized OGR1s were then recycled to the plasma membrane for signal transduction if the extracellular pH was returned to the mildly acidic state. However, in a minor population of cells (40%), the internalized OGR1s were retained in endosomes or transported to lysosomes and degraded, leading to low efficiency of their recycling to the plasma membrane. Thus, the present live-cell monitoring strongly suggests that the signal transduction activity of OGR1 is regulated by pH-dependent internalization and recycling to the plasma membrane.

[**Key words:** G-protein coupling receptors; Molecular imaging; Intracellular trafficking; Fluorescent labeling; Single-cell image analysis]

G-protein coupled receptors (GPCRs) are a superfamily of cell surface signaling proteins that play an important role in many diseases, including inflammation and cancer development (1). Accordingly, GPCRs have attracted attention as targets for drug discovery—in fact, 40% of drugs used in clinical practice were reported to be screened by targeting GPCRs (2). Ovarian cancer G protein-coupled receptor 1 (OGR1) is one such disease-related receptor, first found as a transcript in ovarian cancer cells, and now known to be expressed in tissues including spleen, testis, small intestine, peripheral blood leukocytes, brain, heart, lung and kidney (3). Almost 15 years ago, OGR1 was reported to be a proton-sensing GPCR which is activated in extracellular acidic conditions (4). This proton-induced activation of OGR1 was shown to be involved in a variety of important biological phenomena such as pH homeostasis in osteoblasts and osteocytes (4), suppression of the growth and motility of smooth muscle cells in ischemic heart disease and atherosclerosis (5), and airway inflammation and dysfunction in asthma (6). In addition, it is well known that a decrease in pH can occur locally in cancer tissues (7). Therefore, elucidating the mechanism of proton-induced OGR1 activation potentially provides a novel target for medical treatment, diagnosis and drug discovery. However, the mechanism underlying the pH-responsive control of OGR1-mediated signal transduction remains unclear.

The amount of GPCRs on the cell surface is well known to be controlled by internalization in response to extracellular conditions so as to regulate signal transduction (8). Most ligand-activatable receptors are internalized through agonist-induced conformational changes, resulting in either degradation in lysosomes or recycling onto the cell surface after ligand dissociation (9). Alternatively, constitutively-active GPCRs are spontaneously internalized in the absence of any ligands, and this constitutive internalization can be regulated by binding to a ligand (10). Therefore, it is easily assumed that the activity of proton-sensing GPCRs is also regulated through a proton-dependent increase or decrease in internalization from the cell surface. Among the proton-sensing GPCR superfamily (the OGR1 family), which includes OGR1, G-protein-coupled receptor 4 (GPR4), T-cell death-associated gene 8 (TDAG8) and G2 accumulation receptor (G2A), the intracellular distribution of G2A was first investigated by using a fluorescent protein-G2A fusion construct (11). In this pioneer study, the constitutive internalization of G2A was observed to be suppressed by binding to a lipid mediator but was not influenced by extracellular pH. However, in our previous studies using an

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enzymatic pulse labeling method, fluorescent-labelled G2A, which was modified on the cell surface with a small molecular dye, was observed to remain on the plasma membrane in acidic conditions and to internalize into the intracellular compartments if the extracellular pH was changed from mildly acidic to slightly basic (12,13). Recently, proton-induced redistribution of OGR1 family receptors monitored by immunostaining on fixed and permeabilized cells was reported (14). In this report (14), G2A was similarly confirmed to remain on the plasma membrane at mildly acidic pH, but OGR1 and TDAG8 were distributed in the cytosol at pH 6.4. However, such static observations on permeabilized cells may not accurately reflect the real dynamic distribution changes of receptors in living cells.

Therefore, in this study, we examined pH-dependent intracellular trafficking of OGR1 in living cells by our real-time GPCR monitoring method based on a sortase-mediated pulse labeling technique (15). In this method, a receptor protein, which is tagged with a short substrate peptide sequence at the N-terminus, is simply labeled on cell surfaces with a fluorescent dye-modified peptide by two-step sortase A (SrtA)-mediated transpeptidation. As reported for G2A (12,13), a model leukocyte expressing the tagged OGR1 was prepared by retroviral gene-transfer and immobilized on a poly (ethylene glycol)-lipid-modified substrate to allow us to accurately observe the OGR1 distribution without influence from drift, floatation and rotation of cells. The tagged OGR1 was successfully labeled on the cell surface in a pulsed manner and microscopically monitored in mildly acidic and slightly basic extracellular conditions. The present live-cell quantitative observation of labeled OGR1 elucidated that OGR1 remains on the plasma membrane at mildly acidic pH and internalizes to the intracellular compartments at slightly basic pH, as observed for G2A.

MATERIALS AND METHODS

Plasmid construction and retroviral gene transduction We constructed a plasmid for expression of human OGR1 tagged with both the SrtA-recognition sequence (LPETGGGGG, $LPETG_5$) and the hemagglutinin (HA) epitope sequence at its extracellular N-terminus ($LPETG_5$-HA-OGR1). The DNA fragment encoding $LPETG_5$-HA-OGR1 was cloned into the upstream region of the internal ribosomal entry site (IRES) (between the *Eco*RI and *Bam*HI cleavage sites) of the pMK-IRES-puro retrovirus vector to generate pMK-$LPETG_5$-HA-OGR1-IRES-puro.

Retroviral packaging Plat-E cells were transfected with pMK-$LPETG_5$-HA- OGR1-IRES-puro using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Tokyo, Japan) according to the manufacturer's protocol and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1 μg/ml puromycin and 10 μg/ml blasticidin (Gibco, Tokyo, Japan) for 2 days. The culture medium supernatant (2 ml/well) was placed onto a 24-well plate that was precoated with RetroNectin (Takara-Bio, Shiga, Japan). After incubation for 4 h, the supernatant was discarded, and a suspension of murine pro-B cells (Ba/F3 cells; 1×10^4 cells/well) in a culture medium (RPMI 1640 medium supplemented with 10% FBS and 1 ng/ml IL-3 [from Gibco, Tokyo, Japan]) was added to the wells for retroviral transduction. After growing to 80% confluence, the culture medium was changed to the selection medium containing 2 μg/ml puromycin. After incubation for 2 days for selection, the puromycin-resistant cells obtained were Ba/F3 cells expressing tagged OGR1 (OGR1-Ba/F3).

Immunostaining and microscopic observation For immunostaining, OGR1-Ba/F3 cells were washed with Dulbecco's phosphate-buffered saline (PBS) and then treated with fluorescein isothiocyanate (FITC)-conjugated anti-HA-antibody (Bethyl Laboratories, Tokyo, Japan; 1:1000 dilution) for 30 min at room temperature, followed by rinsing with PBS. The stained cells were observed with a confocal laser scanning microscope (LSM510, Carl Zeiss Inc., Oberkochen, Germany) equipped with a 63 × oil immersion objective lens.

Double staining by SrtA-mediated labeling and immunostaining OGR1-Ba/F3 cells were incubated in cleavage medium (RPMI 1640 medium supplemented with 0.1% BSA, 30 μM SrtA and 1 mM triglycine) for 1 h at 37°C in a humidified incubator under 5% CO_2. After rinsing with RPMI 1640 medium supplemented with 0.1% BSA (RPMI 1640-BSA), cells were incubated in labeling medium (RPMI 1640 medium supplemented with 0.1% BSA, 30 μM SrtA and 10 μM Alexa Fluor488 (AF488)-labeled DLPETGG peptide) for 1 h in the incubator as described above. Here, AF488-labeled peptide was prepared by mixing the synthesized DLPETGG peptide (Toray Research Center, Inc., Otsu, Shiga) and AF488 succinimidyl ester (Thermo Fisher Scientific Inc., Yokohama, Japan). The enzymatically stained cells were rinsed with RPMI 1640-BSA and incubated in the mildly acidic medium (RPMI 1640-BSA adjusted to pH 6.6 with 5 mM HEM [HEPES:EPPS:MES = 1:1:1, adjusted to pH 6.6]) for 45 min. After removal of the medium by centrifugation and aspiration, the cell pellet was suspended in 4% paraformaldehyde-PBS solution and incubated for 1 h at room temperature for cell fixation. The fixed cells were rinsed with PBS and then incubated in 0.5% Triton X-100 solution (in PBS) for 15 min at room temperature. The permeabilized cells were rinsed with PBS twice, and, without any blocking steps, directly treated with Dylight 405-conjugated anti-HA-antibody (Rockland Immunochemicals Inc., Limerick, PA; 1:1000 dilution) for 50 min at room temperature. After rinsing with PBS twice, the double-stained cells were observed by confocal microscopy as described above. The green and blue fluorescences at each pixel on the original images were subtracted from the mean autofluorescence values, which were determined from images of the intact cells (Supplemental Fig. S1).

Cell immobilization on poly(ethylene glycol)-lipid-coated substrates A cell immobilization reagent-coated substrate was prepared by a slightly modified procedure from that reported in our previous work (16). A 35-mm glass-base dish with a diameter of 12 mm (Iwaki Glass, Chiba, Japan) was dipped in a collagen solution, prepared by diluting a commercially available collagen solution (Cellmatrix type I-A; Kurabo Co., Osaka, Japan) with an HCl solution (pH 3.0) ten-fold. After incubation overnight, the surface of the dish was washed three times with MilliQ water (Millipore Corp., Billerica, MA, USA). The collagen-coated surface was modified with poly(ethylene glycol) (PEG) monooleyl ester (PEG-lipid): a solution of Sunbright OE-080CS (NOF Corp., Tokyo, Japan) in PBS (20 μM) was applied to the collagen-coated surface and incubated at 37°C for 1 h. The PEG-lipid-modified surface was rinsed with MilliQ water six times. A suspension of CellTracker Blue-stained OGR1-Ba/F3 cells in PBS was put on the surface. After incubation for 15 min, free cells were removed from the surface by rinsing with PBS.

SrtA-mediated pulse labeling and time-lapse observation Before labeling and time-lapse observation, the immobilized OGR1-Ba/F3 cells were incubated in RPMI 1640-BSA for 30 min. As described above, the tagged OGR1 was labeled by the two-step SrtA-mediated labeling method in the cleavage and labeling media. After labeling, the cells were washed with RPMI 1640-BSA three times to remove nonspecifically adsorbed AF488-DLPETGG peptide from the cell surface, and then the medium was replaced with the mildly acidic medium. The intracellular distribution of the fluorescently-labeled OGR1 was observed under mildly acidic conditions with a confocal microscope. The pH of the medium was increased to 7.7 by adding 14 μl of a 10% $NaHCO_3$ solution to 1 ml of the mildly acidic medium. Immediately after the pH jump, time-lapse observation was performed for 15 min with 1-min intervals. For re-acidification of the medium, the slightly basic medium was replaced with the mildly acidic one. Just after re-acidification, time-lapse observation was restarted and performed for 1 h with 5-min intervals.

Image analysis The acquired time-lapse images were analyzed using a modified custom software based on a previously established image analysis program (Supplemental Fig. S2) (12,13). The two-color fluorescent images of individual cells were cut out from the original time-lapse fluorescent image series of the AF488-labeled OGR1 (green) and CellTracker Blue (blue) images. The blue fluorescent image was used for preparing the mask of the cytosol region, and the green one was used for both preparation of the mask of the cell surface region and quantification of intracellular OGR1. The brightness of the fluorescent image at all the pixels representing the cell surface or the intracellular compartments was summed as the amount of labeled OGR1 in each region. To cancel out the influence of photobleaching, the relative brightness of each region was normalized with the total average brightness of two regions, and normalized brightness of each region was plotted against the observation time of the analyzed image.

Model fitting A model for the intracellular OGR1 trafficking on leukocytes, which was based on a previously reported two-region distribution model (12), was applied to fit the image analysis data. In order to be simplified, this model is based on the following assumptions; the only deprotonated OGR1 can be internalized; the OGR1 concentrations at the membrane [$OGR1_{mem}$] and in the interior compartments [$OGR1_{int}$] are spatially uniform; the measured fluorescence intensity is proportional to the concentration of labeled OGR1. In addition, to apply the model to the image analysis date obtained from the confocal images of spherical leukocytes, the ratio of visible membrane volume to whole membrane volume, c_1 was newly employed (Supplemental Table S1). In this study, it is presumed that most of the interior region is seen in the confocal slice of observation by the confocal laser-scanning microscope, but a large part of the membrane region is out of the confocal slice (invisible) due to the spherical shape of Ba/F3 cells. In our previous model for adherent cells, the whole

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