Direct visualization of glucagon-like peptide-1 secretion by fluorescent fusion proteins

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ABSTRACT
Live-cell imaging with fluorescent proteins (FPs) is a powerful tool for investigating the exocytosis processes of hormones. However, the secretion process of glucagon-like peptide-1 (GLP-1) has not been visualized by FPs, which might be because tagging FPs inhibits GLP-1 synthesis through the post-translational processing from proglucagon. Here, we have developed FP-tagged GLP-1 by inserting FPs into the middle of GLP-1 and adding the proglucagon signal peptide. Confocal imaging confirmed that GLP-1 fused to FPs with high folding efficiency showed granular structure, in which secretory vesicle markers colocalized. The fluorescence intensity of FP in the culture supernatant from cells treated with KCl or forskolin was significantly increased compared with those from untreated cells. Furthermore, FP-tagged GLP-1 enables direct visualization of stimulation-dependent exocytosis of GLP-1 at a single granule resolution with total internal reflection fluorescence microscopy. FP-tagged GLP-1 might facilitate the screening of GLP-1 secretagogues and the discovery of new antidiabetic drugs.

INTRODUCTION

Incretins are gastrointestinal hormones secreted in response to meal intake and stimulate insulin secretion from pancreatic β-cells[1]. In mammals, two incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), have been reported. GLP-1 has been shown to play a critical role in maintaining glucose tolerance, whereas GIP does not improve blood glucose in type 2 diabetes patients; instead, it antagonizes the glucagon suppressive effect of GLP-1[2]. In intestinal L cells, GLP-1 is synthesized from its precursor, proglucagon, through the cleavage of the signal peptide by signal peptidase and prohormone convertase 1/3-mediated post-translational processing[3] (Figure S1a). The secretion of GLP-1 is triggered by a variety of substances, including glucose; amino acids, such as glutamine; fatty acids; and bile acids[4]. In addition, KCl and forskolin are known to have pharmacological effects on GLP-1 secretion through depolarization and increased cyclic adenosine monophosphate levels, respectively[5,6].

Fluorescent protein (FP) is a powerful tool to track protein dynamics in living cells[7]. Intracellular localization or secretory processes of insulin and growth hormone have been visualized with fluorescent fusion proteins[8,9]. FP-tagged peptide hormones that are colocalized with GLP-1 in the secretory granules, such as human growth hormone, neuropeptide Y and tissue plasminogen activator, have been utilized to analyze GLP-1 exocytosis[5,10,11]. However, to the best of our knowledge, there have been no reports of fluorescent fusion proteins of GLP-1. One of the blockers to generate fluorescent fusion proteins of GLP-1 might be accounted for by its complex post-translational processing steps. Thus, simple tagging of FP can easily compromise the original localization and function of GLP-1. In the present study, by inserting fluorescent protein in the middle of the GLP-1 peptide, we have succeeded in developing the fluorescent fusion protein of GLP-1 and visualizing GLP-1 exocytosis in living cells.

MATERIALS AND METHODS
See Appendix S1 for detailed information.

RESULTS

First, using the molecular structures of liraglutide and dulaglutide as a reference, we carefully determined the site of GLP-1 to which FPs are added (Figure S1). In liraglutide, Lys26 is palmitoylated through a γ-glutamate spacer[12], whereas in dulaglutide, the modified human immunoglobulin G4 Fc region is linked to GLP-1 analog through a peptide linker[13]. Given that the molecular weight of the Fc region (63 kDa) is much larger than that of GLP-1 (3.3 kDa)[13], there might be room for modification or the addition of large molecules to the middle of GLP-1. Therefore, we decided to make FPs inserted between Lys26 and Val27 of GLP-1.

Next, we determined the localization of GLP-1-FPs in GLU-Tag cells. The fluorescent fusion protein of GLP-1 and mCherry was mainly distributed throughout the cytoplasm, as

was mCherry alone (Figure 1a). In contrast, the fusion protein harboring the signal peptide of proglucagon at the N-terminus of GLP-1-mCherry (spGLP-1-mCherry) showed a granular structure. Interestingly, mCherry tagged with the signal peptide (spmCherry) was diffusely localized in the cytoplasm, showing that both the signal peptide and GLP-1 sequence are required for vesicular localization of the fusion protein. To confirm such observed vesicular structures were indeed secretory granules, neuropeptide Y-Venus or tissue plasminogen activator-Venus, well established secretory granule markers, was co-expressed with spGLP-1-mCherry in GLUTag cells. neuropeptide Y and tissue plasminogen activator were colocalized with spGLP-1-mCherry, whereas the early endosomal marker, Rab5, failed to do so (Figure 1b). Thus, spGLP-1-mCherry is localized preferentially to secretory granules. Immunoblot analysis of cell lysate
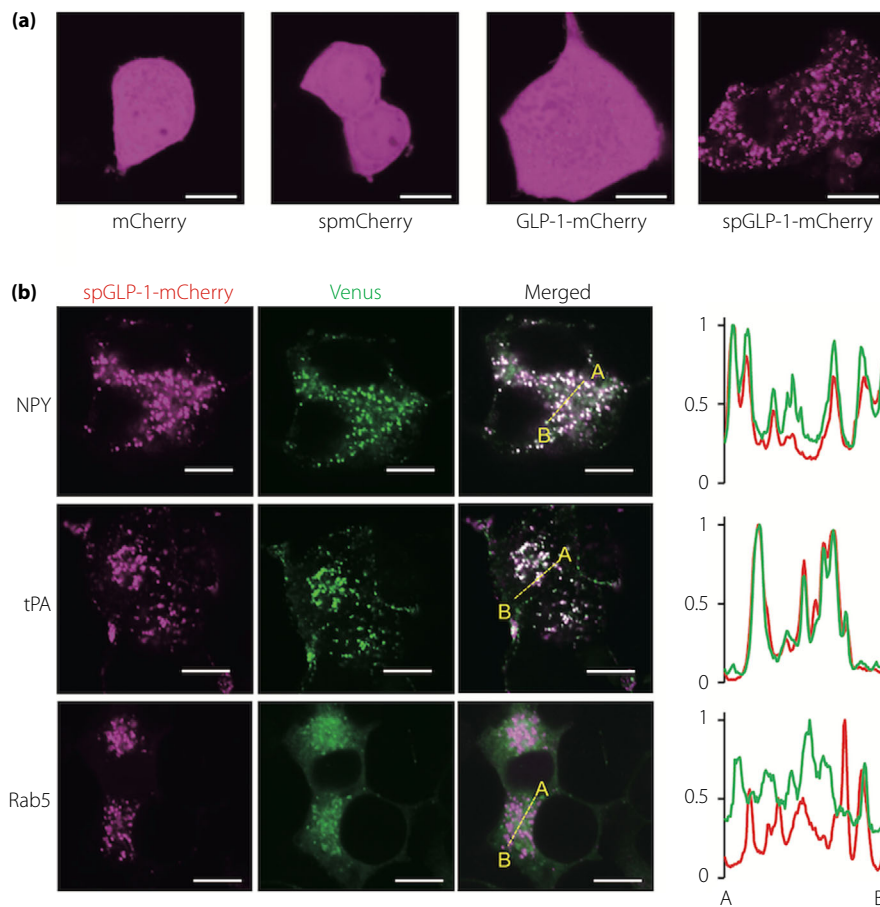

Figure 1 | Establishment of fluorescent fusion proteins of glucagon-like peptide-1 (GLP-1). (a) GLUTag cells were transfected with an expression vector for mCherry, mCherry with signal peptide (spmCherry), GLP-1-mCherry or spGLP-1-mCherry. Confocal images were acquired 48 h after transfection. Representative images are shown (bar, 10 μm). (b) GLUTag cells were co-transfected with expression vectors for spGLP-1-mCherry and a secretory granule marker, neuropeptide Y (NPY) or tissue plasminogen activator (tPA), tagged with Venus. The early endosome marker, Rab5, served as a negative control for cytoplasmic vesicles other than secretory granules. Confocal images were acquired 48 h after transfection. Representative images are shown (left, bar, 10 μm). The fluorescence intensities of signal peptide of proglucagon at the N-terminus of GLP-1 (spGLP-1)-mCherry and secretory granule markers along the yellow line drawn from point A to point B on the image were quantified and plotted (right).
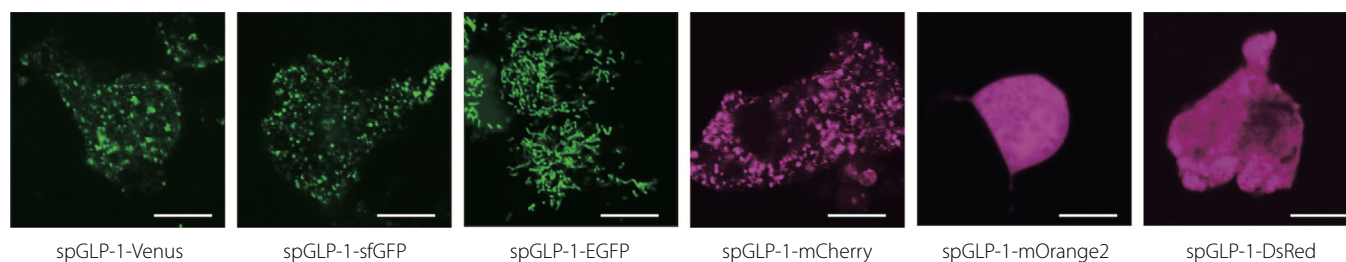
| spGLP-1-Venus | spGLP-1-sfGFP | spGLP-1-EGFP | spGLP-1-mCherry | spGLP-1-mOrange2 | spGLP-1-DsRed |

Figure 2 | Localization of signal peptide of proglucagon at the N-terminus of glucagon-like peptide-1 (spGLP-1)-fluorescent proteins (FPs). GLUTag cells were transfected with an expression vector for spGLP-1-superfolder green fluorescent protein (sfGFP), spGLP-1-Venus, spGLP-1-enhanced green fluorescent protein (EGFP), spGLP-1-mOrange2 or spGLP-1-DsRed. Confocal images were acquired 48 h after transfection. Representative images are shown (bar, 10 μm).

from spGLP-1-mCherry-expressing GLUTag cells showed two bands of ca. 30 and 34 kDa, the smaller and larger ones representing the processed form and unprocessed form, respectively (Figure S1c). In contrast, the only unprocessed form could be observed in HEK293T cells, which do not express prohormone convertase 1/3[14].

We next aimed to expand the color pallet of GLP-1-FPs. Among the expressed fusion proteins of spGLP-1 with superfolder green fluorescent protein (sfGFP) and Venus, in addition to spGLP-1-mCherry, were localized in vesicular compartments (Figure 2). spGLP-1-enhanced GFP showed tubular structure, as reported for neuropeptide Y-enhanced GFP[15]. We noticed that maturation times of FPs, fusion protein with which

localized in vesicular structures, were <20 min (Table S2), showing that higher folding efficiency might be required for spGLP-1-FPs to be sorted to secretory granules.

To assess whether spGLP-1-FPs in secretory granules were secreted from cells, we quantified the fluorescence intensity of spGLP-1-mCherry in culture supernatants. As expected, the fluorescence intensity of the conditioned medium of spGLP-1-mCherry-expressing cells was significantly higher than that of control cells (Figure 3a). Next, we further examined stimulation-induced spGLP-1-mCherry secretion, and confirmed that the known GLP-1 secretagogues KCl and forskolin increased fluorescence intensity of the cultured medium (Figure 3b). Essentially similar results were obtained for
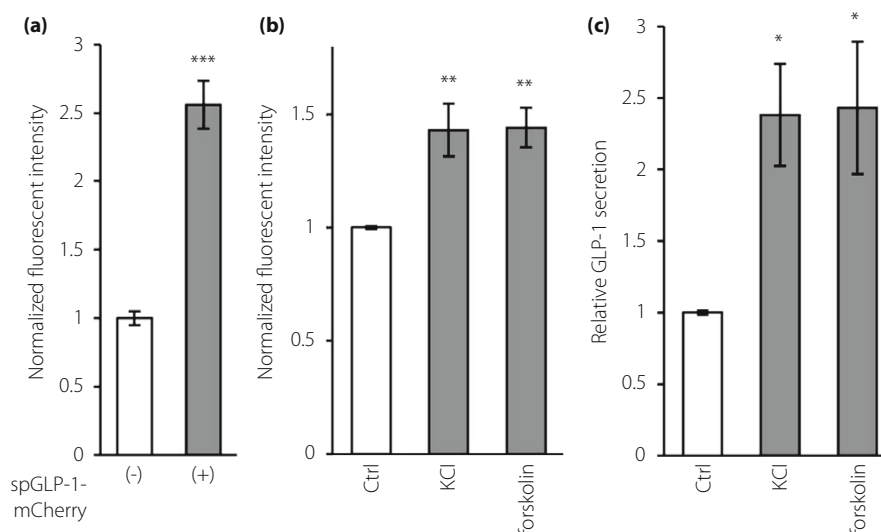

Figure 3 | Evaluation of glucagon-like peptide-1 (GLP-1) secretion by measuring fluorescence intensity in culture supernatants. (a) GLUTag cells were transfected with an expression vector for superfolder green fluorescent protein (sfGFP)-mCherry. Untransfected cells served as a control. A total of 48 h after transfection, the culture supernatants were collected, and their fluorescence intensities were measured by spectrofluorometer. $n = 10$, ***$P < 0.001$. (b) GLUTag cells were transfected with an expression vector for spGLP-1-mCherry. A total of 48 h after transfection, cells were treated with 70 mmol/L KCl or 10 μmol/L forskolin for 2 h. Then, the culture supernatants were collected, and their fluorescence intensities were measured by spectrofluorometer. $n = 6$, **$P < 0.01$. (c) GLUTag cells were stimulated by 70 mmol/L KCl or 10 μmol/L forskolin for 2 h, and the amount of GLP-1 in the cultured medium was determined by enzyme-linked immunosorbent assay. $n = 4$, *$P < 0.05$.

endogenous GLP-1 by enzyme-linked immunosorbent assay (Figure 3c). These data suggested that spGLP-1-mCherry can be utilized to assess both the basal and induced GLP-1 secretion.

Finally, we aimed to visualize GLP-1 secretion in living cells at a single vesicle resolution by total internal reflection fluorescence microscopy. To visually identify exocytosis as a flash event by total internal reflection fluorescence microscopy, the fluorescence intensity of FP needs to be changed substantially at pH 6 (Figure S2). Venus and sfGFP fulfill this requirement (Table S2), and flash events could be observed in spGLP-1-sfGFP-expressing cells (Figure 4a, Video S1 and S2). We used

STC-1 cells in subsequent experiments, because they showed more flash events than GLUTag cells. Flash events and GLP-1 secretion were significantly promoted by the treatment with glucose, glutamine, forskolin and α-linolenic acid (Figure 4b,c). Therefore, spGLP-1-sfGFP deciphered both basal and nutrient-induced GLP-1 secretion.

DISCUSSION

In the present study, visualization of GLP-1 secretion from L cells was accomplished using the newly developed fluorescent fusion protein, spGLP-1-FP. Adding the fluorescent protein in the middle of GLP-1, rather than at the N- or C-terminus,
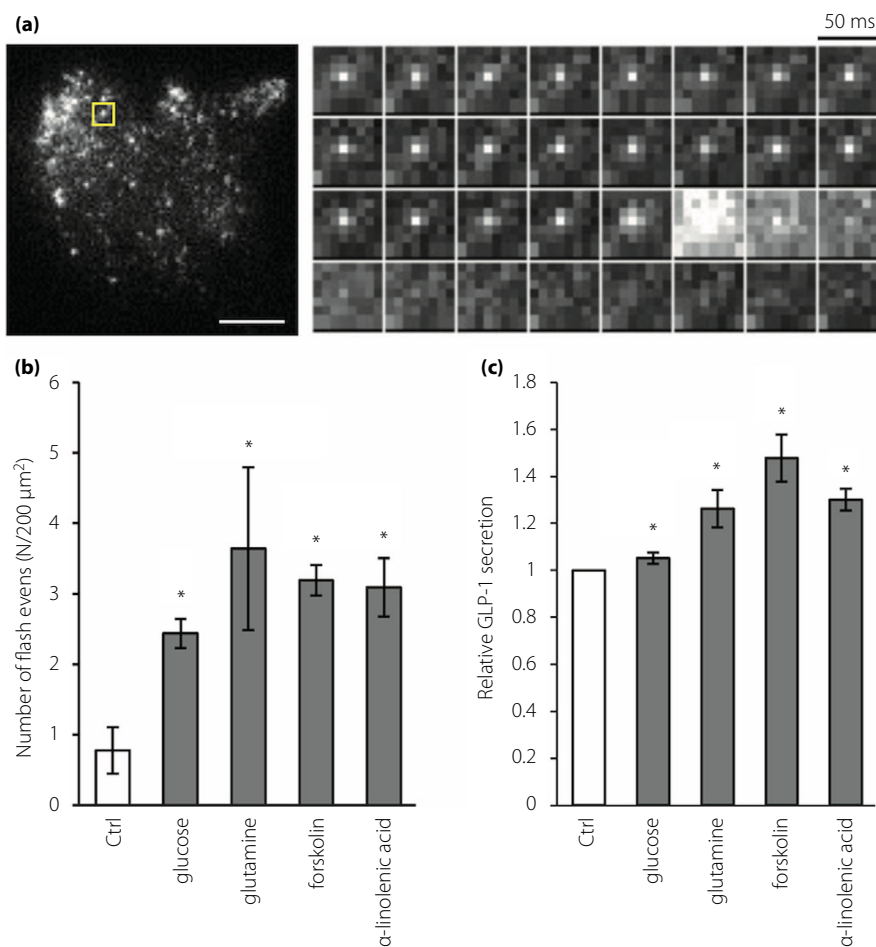

Figure 4 | Observation of glucagon-like peptide-1 (GLP-1) exocytosis by total internal reflection fluorescence microscopy. STC-1 cells were transfected with an expression vector for signal peptide of proglucagon at the N-terminus of GLP-1 (spGLP-1)-superfolder green fluorescent protein (sfGFP) and, after 48 h, were observed by total internal reflection fluorescence microscopy. (a) An example of a typical flash event in STC-1 cells expressing spGLP-1-sfGFP (50-ms intervals). Sequential images of single-vesicle fusion with the plasma membrane were presented in the presence of 17.5 mmol/L glucose and 2.5 mmol/L glutamine (bar, 10 μm). (b) STC-1 cells expressing spGLP-1-sfGFP were stimulated by 20 mmol/L glucose, 10 mmol/L glutamine, 10 μmol/L forskolin and 100 μmol/L α-linolenic acid. The number of flash events (/200 μm^2) for 10 min was counted and plotted, and the control was standardized as 1. $n = 5$, *$P < 0.05$. (c) STC-1 cells were stimulated by 20 mmol/L glucose, 10 mmol/L glutamine, 10 μmol/L forskolin and 100 μmol/L α-linolenic acid for 2 h, and the amount of GLP-1 in the cultured medium was determined by enzyme-linked immunosorbent assay. $n = 4$, *$P < 0.05$.

might be crucial in preserving the GLP-1 function and ensuring transfer to secretory granules. Nevertheless, only limited FPs with high folding efficiency could be recruited to these fusion proteins. pH sensitivity of FPs is also crucial to show flash events in total internal reflection fluorescence microscopy.

The GLP-1 fusion proteins developed in the present study will facilitate gaining more mechanistic insight into GLP-1 secretion. Whereas enzyme-linked immunosorbent assay, widely used to determine the amount of hormone secretion, analyzes the sum of hormone synthesis and its secretion, spGLP-1-FPs can specifically dictate the secretion process. In combination with enzyme-linked immunosorbent assay, spGLP-1-FPs will provide full access to the underlying mechanism for GLP-1 secretion. Quick and straightforward determination of the secretion amount by measuring the fluorescence intensity of the culture media is also promising to accelerate drug screening.

DISCLOSURE

The authors declare no conflict of interest.

Approval of the research protocol: N/A.

Informed consent: N/A.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1| Experimental procedures

Figure S1| Construction of signal peptide of proglucagon at the N-terminus of glucagon-like peptide-1-mCherry plasmid.

Figure S2| Schematic illustration of flash events.

Table S1| Sequence of primers.

Table S2| Properties of fluorescent proteins used in this study.

Video S1| Visualization of signal peptide of proglucagon at the N-terminus of glucagon-like peptide-1-superfolder green fluorescent protein exocytosis in STC-1 cells.

Video S2| Visualization of signal peptide of proglucagon at the N-terminus of glucagon-like peptide-1-superfolder green fluorescent protein exocytosis in GLUTag cells.