Imaging pancreatic β-cells in the intact pancreas

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Hara, Manami, Restituto F. Dizon, Benjamin S. Glick, Catherine S. Lee, Klaus H. Kaestner, David W. Piston, and Vytautas P. Bindokas. Imaging pancreatic β-cells in the intact pancreas. Am J Physiol Endocrinol Metab 290: E1041–E1047, 2006. First published December 20, 2005; doi:10.1152/ajpendo.00365.2005.—We have developed a method to visualize fluorescent protein-labeled β-cells in the intact pancreas through combined reflection and confocal imaging. This method provides a 3-D view of the β-cells in situ. Imaging of the pancreas from mouse insulin I promoter (MIP)-green (GFP) and red fluorescent protein (RFP) transgenic mice shows that islets, β-cell clusters, and single β-cells are not evenly distributed but are aligned along the large blood vessels. We also observe the solitary β-cells in both fetal and adult mice and along the pancreatic and common bile ducts. We have imaged the developing endocrine cells in the embryos using neurogenin-3 (Ngn3)-GFP mice crossed with MIP-RFP mice. The dual-color-coded pancreas from embryos (E15.5) shows a large number of green Ngn3-expressing proendocrine cells with a smaller number of red β-cells. The imaging technique that we have developed, coupled with the transgenic mice in which β-cells and β-cell progenitors are labeled with different fluorescent proteins, will be useful for studying pancreatic development and function in normal and disease states.

development of pancreas; islet formation

THE MOUSE HAS EMERGED as an important model for studying the development and function of the insulin-producing pancreatic β-cells because of the relative ease of genetically modifying the mouse genome (7, 26). This includes procedures for genetically tagging β-cells and β-cell progenitors with reporter genes such as fluorescent proteins (9, 12). The fluorescent reporter transgenes can be used to monitor β-cell development as well as physiological and pathological changes in normal and disease states (10, 14, 25). Moreover, the availability of different colored fluorescent proteins makes it possible to mark different cell types simultaneously and to monitor their interactions.

The pancreas is a unique organ where the endocrine micro-organs are embedded in the exocrine tissue. Our current understanding of the structure of the pancreas is dominated by two-dimensional analyses. The ability to visualize the spatial distribution of pancreatic cells (endocrine and exocrine) in situ and in three dimensions could provide a better understanding of the overall organization of endocrine cells and the relationship between different structures in the pancreas.

Here, we report a method for visualizing fluorescent protein-labeled pancreatic β-cells in intact pancreas. We show the overall spatial organization of β-cells and islets in the pancreas, including their uneven distribution throughout the pancreas and their dynamic relationship with the large blood vessels. The three-dimensional reconstruction of β-cells in situ may provide new insights into β-cell development and migration and also into the formation of islets.

RESEARCH DESIGN AND METHODS

Mouse models. The generation and characterization of the mouse insulin I promoter-green fluorescent protein (MIP-GFP) transgenic mice has been described (12). We generated the MIP-RFP (red fluorescent protein) and MIP-CFP (cyan fluorescent protein) transgenic mice in a similar manner. Briefly, the MIP-RFP and MIP-CFP transgenic constructs were assembled using an 8.3-kb fragment of the MIP that includes a region from −8.3 kb to +12 bp (relative to the transcriptional start site), the coding region of DsRed.T4 (a rapidly maturing variant of Discosoma red FP; 0.7 kb) (1) or cerulean (an improved variant of CFP; 0.7 kb) (22), respectively, and the 2.1-kb human growth hormone (hGH) gene cassette (11, 20). The 11.1-kb MIP-RFP-hGH or MIP-CFP-hGH fragment was isolated from the vector by digestion of the plasmid construct with SfiI and HindIII and agarose gel electrophoresis. The fragment was further purified using an Elutip-D column (Schleicher & Schuell, Keene, NH). The purified transgene DNA was microinjected into the pronuclei of CD-1 mice by the Transgenic Mouse/ES Core Facility of the University of Chicago Diabetes Research and Training Center. Tail DNA from potential founder mice was screened for the presence of each transgene by PCR using forward and reverse primers for MIP-RFP (5′-CCCATGGTCT-TCTTCTGCAT-3′ and 5′-AAGGTGTACGTGAAGCACCC-3′, respectively) and MIP-CFP (5′-TGGAAACTGCAGCTTCAG-3′, respectively) and MIP-CFP (5′-TGAAAATGCAGCTTCAG-3′, respectively). The derivations of the neurogenin-3 (Ngn3)-GFP mice is described in Lee et al. (17). In these mice, the entire coding region of the Ngn3 gene has been replaced with enhanced GFP (Clontech). The heterozygous knock-in mice appear normal and were used exclusively in our studies. We crossed Ngn3-GFP and MIP-RFP mice. The age of the embryo was defined according to the standard convention. The day on which the vaginal plug was found was day 1 of pregnancy, and at noon the embryos were 0.5 days postcoitum (dpc). The expression of both GFP and RFP in the fetal pancreas was confirmed visually under the dissection microscope. The parental lines were heterozygous for the knock-in allele or transgene, and one-fourth of the offspring were expected to be Ngn3-GFP+ and MIP-RFP+.

All procedures involving mice were approved by the University of Chicago Institutional Animal Care and Use Committee.

Glucose tolerance testing. Intraperitoneal glucose tolerance tests (IPGTTs) were performed after a 4-h fast. Blood was sampled from the tail vein before and 30, 60, 90, and 120 min after intraperitoneal injection of 2 mg/g dextrose. Glucose levels were measured using a Precision Q.I.D. Glucometer (MediSense, Waltham, MA).

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Wholemount immunostaining. Pancreata from Ngn3-GFP knock-in mice at E15.5 were fixed in 4% paraformaldehyde at 4°C for 40 min. Tissues were washed in PBS-0.1% Triton X (PT) for 30 min at room temperature and blocked in PT-5% BSA at 4°C overnight. Rabbit anti-Ngn3 antibody [a gift from Dr. D. A. Stoffers (8)] was added at 1:250 in PT-5% BSA, and the tissues were incubated overnight at 4°C. Tissues were washed in PT-1% BSA for 1.5 h at room temperature. Goat anti-rabbit-Alexa 568 (Invitrogen, Carlsbad, CA) was added at 1:400 in PT-5% BSA for 2 h at room temperature. Embryos were washed in PT-5% BSA for 1.5 h at room temperature, sectioned with a vibratome, and examined using confocal microscopy.

Tissue preparation. Tissues were removed, fixed in 4% paraformaldehyde at 4°C overnight, permeabilized in PT overnight at room temperature, and treated with the tissue-clearing reagent FocusClear (Pacgen Biopharmaceuticals, Burnaby, BC, Canada) for 2 days at room temperature. The bulk tissue was observed in 35-mm culture dishes with coverslip windows forming the bottom of the dish.

Optical imaging. Stereomicroscopic images of mouse tissue were taken using an Olympus SZX12 microscope (Olympus, Melville, NY). Wide-field microscopic images were taken using an Olympus IX81 (Olympus). Confocal laser-scanning optical slice images of bulk tissue were captured every 3–7 μm using an Olympus Fluoview IX70 microscope, 488-nm (argon, 6%), 543-nm (HeNe), and/or 633-nm (HeNe) lasers, ×20 (NA 0.5) or ×60 (oil; NA 1.4) objectives (Olympus). For reflected laser light imaging (11), images were simultaneously captured with GFP signals using a red laser (633 nm) with no emission filter, and images were pseudocolored with red. RFP was excited with a 543-nm line and collected in the 605/45-nm emission bandpass.

Image analysis. Image analysis and postprocessing were performed using ImageJ (http://rsb.info.nih.gov/ij/). Voxel Counter (ImageJ plugin) was used to quantify each volume fraction (i.e., ratio of thresholded voxels to all voxels) in a stack of images. The threshold was set to highlight GFP content of cytoplasm. Volume fractions of GFP-expressing β-cells and non-β-cells (e.g., exocrine tissue) were measured by counting the thresholded voxels in the green and red channels, respectively. The reflected light images allowed the volume fraction to account for the diffuse nature of the pancreas and tissue voids. Alternatively, the threshold could be set to highlight only the brighter signal from nuclei to enable automated cell counting using the 3D Object Counter ImageJ plugin. Numbers of dual-color-labeled cells were counted using functions in ImageJ (colocalization, watershed, and analyze particles). Three-dimensional reconstructions were created with Voxx software (5).

Statistical analysis. Data are expressed as means ± SE. Differences were considered to be significant at \( P < 0.05 \).

RESULTS

Generation of transgenic mice with RFP-labeled pancreatic β-cells. RFP is an ideal reporter for fluorescent imaging, in particular for multicolor experiments with fluorescent proteins such as GFP, because its shift toward longer wavelengths minimizes the overlap with the excitation-emission curve of GFP. The wild-type Discosoma RFP (DsRed) has a slow maturation time with a half-time of >24 h at room temperature, which is a hindrance for some types of experiments such as cell-lineage tracing. We used DsRed.T4, which matures rapidly with a half-time of ~0.7 h (1). The MIP-RFP construct was injected into the fertilized eggs of CD-1 mice. We obtained ten founders, of which five showed detectable pancreatic expression of RFP: two females (lines 14 and 16) and three males (lines 22, 31, and 37). RFP was expressed in the islets of all five founders, and expression levels were similar. The expression pattern within the pancreas was similar to that of our MIP-GFP mice (12). We tested for expression of RFP in pancreas and other tissues at 6 wk of age by Western blotting (Supplemental Fig. S1; see AJP Endocrinol Metab web site).1 We detected RFP expression only in pancreas and not in any of the other tissues examined: brain, heart, lung, liver, kidney, spleen, intestine, muscle, fat, and testis. Expression of the MIP-RFP transgene appears to be specific to pancreatic β-cells. The MIP-RFP mice developed normally. At 6 wk of age, there were no differences in body weight and fasting blood glucose between transgenic and nontransgenic CD-1 male mice [24.0 ± 0.9 (n = 12) and 23.8 ± 1.0 (n = 13) g; 147.8 ± 8.4 and 155.8 ± 7.8 mg/dl, respectively]. IPGTTs at 6 wk showed no statistically significant difference in the response between transgenic and nontransgenic male animals (Supplemental Fig. S2).

1 The Supplemental Material for this article (Supplemental Figs. S1–S2 and Suplemental Videos V1–V8) is available online at http://ajpendo.physiology.org/cgi/content/full/00365.2005/DC1.
Generation of transgenic mice with CFP-labeled pancreatic β-cells. We used Cerulean, an improved CFP variant that has both a higher extinction coefficient, and an improved quantum yield, to generate MIP-CFP transgenic mice. Thus Cerulean is 2.5-fold brighter than ECFP (22). The MIP-CFP construct was injected into the fertilized eggs of CD-1 mice. We obtained five founders: two females (lines 13 and 26) and three males (lines 21, 22, and 24). Neither female founder bred well for unknown reasons. Offspring from all three male founders expressed CFP in the islets, and expression levels were similar. The expression pattern within the pancreas was similar to that of MIP-GFP and MIP-RFP.

Transgenic mice expressing fluorescent proteins in their pancreatic β-cells. The expression of GFP, RFP, and CFP in the pancreas can be imaged through the skin in the developing embryo.}

Fig. 2. Imaging of pancreatic β-cells in the intact whole pancreas. A: pancreas from a newborn (P0) MIP-RFP mouse was removed with surrounding tissues attached (D, duodenum; St, stomach; Sp, spleen). Fluorescent image shows entire distribution of β-cells in the pancreas. Note that there appear to be 2 major streaks of β-cells, one from the pylorus of the stomach to the spleen and another to the duodenum, which likely represent their origin of dorsal and ventral pancreas, respectively. Scale bar, 3 mm (shown in μm). B: merged images of red fluorescence and transmitted light (pseudo-colored with green) captured in intact pancreas in A. A stack of 40 planes with a 10-μm increment (see Supplemental Videos 1 and 2). Note that there are substantial numbers of single or clusters of β-cells. These cells appear to be lined up together with islet-forming β-cells. Scale bar, 50 μm.

Fig. 3. β-Cells found in close association with large vessels. A: left: stereoscopic view of pancreas from a newborn MIP-RFP mouse. Note the vascular network throughout the pancreas. Scale bar, 1 mm (shown in μm). Middle: fluorescent image of the pancreas in left showing RFP-expressing β-cells. Right: fluorescent image captured using the GFP filter set showing signals from RFP that bled through the green channel excited at 488 nm wavelength (white) and the vasculature from the background. Note the striking correlation of the distribution of β-cells and vasculature. B: left: high magnification of bright-field image showing the pancreas from a P2 mouse; the vasculature is prominent. Scale bar, 250 μm. Middle: fluorescent image showing β-cell distribution. Right: fluorescent image (excited at 488 nm) shows β-cells in close proximity to blood vessels. C: left: bright-field image showing a small capillary (arrow) and a larger blood vessel (transverse). Scale bar, 500 μm. Middle: fluorescent image showing a string of β-cells in the transverse and single or clusters of cells aligned. Right: a larger blood vessel appears to attract more β-cells, whereas small clusters of β-cells are found around the capillary (arrow). D: left: pancreas from an adult (4-mo) MIP-GFP mouse. Scale bar, 250 μm. Middle: fluorescent image showing GFP-expressing β-cells. Right: islet-forming β-cells are found along the blood vessel.
fetus by means of a stereomicroscope (e.g., MIP-GFP embryo at E15.5 in Fig. 1A). Pancreata showing β-cells labeled with GFP, RFP, and CFP are shown in Fig. 1, B–D. The β-cell expression of these fluorescent proteins is evident from E13.5 through adult.

Pancreatic β-cells are not evenly distributed throughout the pancreas. To capture β-cell distribution in the intact pancreas, the pancreas from a newborn (P0) MIP-RFP mouse was removed with surrounding tissues, including duodenum, stomach, and spleen (Fig. 2A). The fluorescent image of the pancreas in situ shows the entire distribution of β-cells. Note that the β-cells are not evenly scattered throughout the organ but are arranged in two major streaks, one from the pylorus of the stomach to the spleen, the other to the duodenum. In addition, there is yet another streak from the junction of the other two streaks toward the bile duct. The first two streaks likely correspond to the dorsal and ventral pancreas, respectively. We captured a stack of merged images of RFP (red) and transmitted light (pseudo-colored with green) (10-μm steps, total 400 μm in thickness) in the same pancreas and generated a 3-D reconstruction of the β-cells in situ (Fig. 2B). This reconstruction shows that single β-cells or β-cell clusters together with islets apparently are aligned together. (A top-down view of this reconstruction is shown in Supplemental Video 1 and animation in Supplemental Video 2).

Pancreatic β-cells are found in close association with the large blood vessels. In the pancreas from neonatal MIP-RFP mice, the vascular network throughout the pancreas can be seen in the bright-field images (Fig. 3, A–C, left). The fluorescent image shows RFP-expressing β-cells in red (Fig. 3, A–C, middle). The optimal excitation wavelength for RFP is 558 nm. It can also be excited by a standard 488-nm laser that allows the intrinsic autofluorescence from pancreatic tissue to be visualized. The fluorescent image was captured using the GFP filter set so that the vasculature in the pancreas could be captured in the background (Fig. 3, A–C, right). The simultaneous visualization of the RFP-expressing β-cells and the pancreas shows that the β-cells are in close proximity to the large blood vessels, and there is a striking correlation of the distribution of β-cells and the vascular network. There appear to be more islets associated with large blood vessels, whereas small clusters of β-cells or small islets are found along the smaller blood vessels (Fig. 3C, arrows). This close association between β-cells and blood vessels is also seen in adult MIP-GFP mice (Fig. 3D), where islets are present along the large blood vessels.

Imaging pancreatic development in tissues labeled with two fluorescent proteins. We examined endocrine cell development in Ngn3-GFP/MIP-RFP mice (Fig. 4). In Ngn3-GFP knock-in mice, 90 ± 6.2% (n = 3) of cells were coexpressing Ngn3 and...
GFP at E15.5 (See DISCUSSION). The proendocrine Ngn3-GFP-expressing cells in the dorsal and ventral pancreas formed a branched structure. Ngn3-GFP is also strongly expressed in the duodenum in these animals (17). The red insulin-expressing cells (MIP-RFP) are also found in a similar branched structure, although there are fewer insulin-positive cells at this stage than Ngn3 positive cells. Confocal microscopic analysis showed that some cells coexpress GFP and RFP and thus appear yellow in the merged image (Fig. 4B, right). Coexpression of GFP and RFP may be the result of the long in vivo half-life of GFP compared with Ngn3 and may mark the immediate β-cell descendents from the Ngn3-positive precursors. Images captured at higher magnification show that the majority of cells express only Ngn3-GFP (Fig. 4C, left) or insulin-RFP (Fig. 4C, middle). For example, the numbers of GFP+, RFP+, and GFP+−RFP+ cells (Fig. 4C) were 139, 25, and 22, respectively.

Small clusters of β-cells are found around the main pancreatic and bile ducts. A stereomicroscopic bright-field image of the gut from a newborn MIP-GFP mouse shows the main pancreatic duct and its junction with the bile duct (Fig. 5, A, left). There are small clusters of GFP-labeled β-cells along both the pancreatic and bile ducts (Fig. 5). GFP-labeled cells are also found in association with both ducts in adult animals (8 mo of age studied). These β-cells appear not to be in direct contact with the duct (arrowheads in Fig. 5C) but are within a thin surrounding layer of adipose tissue, which is seen in old adult mice (>8 wk).

In the newborn pancreas, β-cells cluster into a contiguous mass. We captured images from the intact pancreas from a newborn MIP-GFP mouse by use of reflected light confocal imaging to visualize exocrine pancreatic structures including ducts and fluorescence imaging of GFP to visualize β-cells. Figure 6A shows a montage of optical sections from a top-down view of the pancreas. These data illustrate a β-cell mass that appears to be multiple islets around a duct (Fig. 6A, D). In several optical sections, the islets are virtually all interconnected (Fig. 6A and Supplemental Video 3). Three-dimensional reconstruction of a stack of images shows that, at this stage of development, β-cells form a contiguous mass completely surrounding the pancreatic duct (Fig. 6B and Supplemental Video 4). One optical section from the 3-D reconstruction that is shown in Fig. 6B clearly illustrates the duct within this β-cell mass (Fig. 6C and Supplemental Video 5). In addition, single β-cells and small clusters of β-cells were also present (Fig. 6D). A 3-D reconstruction of 38 sections (3-μm interval) suggests that these apparently isolated cells and clusters based on 2-D analysis may be interconnected, a train of β-cells and β-cell cluster not associated with islets (Fig. 6D, right, and Supplemental Video 6). Fig. 6E shows another example of a large, irregularly shaped mass of β-cells embedded in the surrounding exocrine tissue (Fig. 6E and Supplemental Video 7) in close proximity to the duct (Fig. 6F). We rarely observed a single β-cell in the ductal epithelium (Fig. 6F and Supplemental Video 8).

Quantification of GFP-expressing β-cell mass and number in intact pancreas. We determined the feasibility of automated quantitation of β-cell mass and number for each stack of images shown in Fig. 6, D and E. GFP fluorescence was used for β-cell volume quantitation, and the bulk tissue reflection images were used to account for the diffuse nature of the pancreas and tissue voids. In the (optical) tissue block of Fig. 6D, the exocrine pancreas was 48.3% of the total volume of the

Fig. 5. Small clusters of β-cells around the main pancreatic and bile duct. A: left: bright-field image showing the gut from a newborn MIP-GFP mouse (L, liver; PV, portal vein; D, duodenum). Scale bar, 250 μm. Middle: corresponding fluorescent image shows GFP-expressing β-cells. Right: these β-cells appear to be small clusters and are found around the main pancreatic duct and continuously along the bile duct. B: left: bright-field image showing the gut from a 4-mo-old MIP-GFP mouse (L, liver; PV, portal vein; D, duodenum; P, pancreas; G, gall bladder). Scale bar, 1 mm (shown in μm). Middle: corresponding fluorescent image. Note that bile in the gall bladder strongly autofluoresces. Right: these β-cells are found throughout adulthood, although the number of β-cells decreases as they age. C: left: bright-field image of the gut in B at high magnification (B, bile duct; arrowheads, PV). Scale bar, 500 μm. Middle: fluorescent image showing GFP-expressing β-cells. Right: these β-cells reside in close proximity to the duct and appear to be embedded within a thin layer of fat tissue surrounding the duct.
Fig. 6. Three-dimensional reconstruction of β-cell mass formation in intact pancreas. A: montage of optical sectioning showing top-down view of GFP-expressing β-cells (green) and surrounding exocrine tissue (red) captured by reflected light imaging. Note that the detailed morphology of the surrounding exocrine tissue including the duct (d) is visualized. β-Cells that appear to form multiple islets in each panel in 2-dimensional context are virtually interconnected. Scale bar, 30 μm (see Supplemental Video 3). B: 3-D reconstruction using a stack of images shown in A reveals large β-cell mass surrounding a duct. Left: 3-D presentation used merged images of fluorescent and reflected light. Right: fluorescent images showing only β-cells. Scale bar, 50 μm (see Supplemental Video 4). C: 1 plane from 3-D reconstruction in B clearly shows the duct epithelium within the β-cell mass. Scale bar, 50 μm (see Supplemental Video 5). D: left: small clusters and single β-cells (green) appear to be scattered in the exocrine pancreas (red). Scale bar, 50 μm. Right: 3-D reconstruction of β-cells suggests that there could be interconnections among them (see Supplemental Video 6). E: large β-cell mass embedded in the exocrine pancreas. Left panels show the entire block of tissue from each different angle. Rectangles indicate orientation and scale (90 μm). Embedded β-cells are exclusively shown in right panels (see Supplemental Video 7). F: panel from the β-cell mass in E showing a single GFP-expressing β-cell within the duct epithelium. Scale bar, 50 μm (see Supplemental Video 8).

imaged tissue block. The number of β-cells was 86, which comprised 3.0% of the total volume and 6.1% of the exocrine pancreas (reflected light image). In a block of tissue in Fig. 6E, 71.8% was the exocrine pancreas, and the volume of β-cell mass was 11.4%. The β-cells numbered 248 and constituted 15.8% of the exocrine pancreas in this view. These data demonstrate that β-cell mass and numbers can be readily extracted from 3-D optical volumes by using freely available software.

DISCUSSION

**Macroscopic view of β-cells in the entire pancreas.** We have generated transgenic mice in which pancreatic β-cells are genetically tagged with GFP, RFP, and CFP (Fig. 1). The MIP-GFP mice have been used to study β-cell development and physiology (10, 14, 24, 25) and intrahepatic islet transplantation in mice (13). The new lines of transgenic mice with RFP- and CFP-labeled β-cells described herein broaden the availability of research tools to generate multicolor islets/pancreas. Using these lines of mice, we have shown macroscopic views of β-cell distribution in situ. We have confirmed observations made mainly by standard histological studies (2–4, 6, 15, 16, 19, 20, 23) in the intact pancreas and/or in a 3-D context: uneven distribution of β-cells (Fig. 2) and the close association of β-cells and the large blood vessels (Fig. 3; note the scale bars. Islet capillary supplies are not visualized at the macroscopic scale used.). Small clusters of β-cells were observed around the main pancreatic and common bile ducts in all three MIP-GFP, -RFP, and -CFP transgenic mice in both embryos and adults (Fig. 5), which has not been reported before. These β-cells are in close proximity to the duct but not within the duct epithelium.

“Rainbow mice.” Mouse models in which β-cells and β-cell progenitors are labeled with different fluorescent proteins are useful tools for studying pancreatic development at the structural and molecular levels. We described a first generation of “rainbow mouse” for this purpose: the Ngn3-GFP/MIP-RFP mice. These fluorescent protein-labeled pancreatic cells can be purified by fluorescence-activated cell sorters (12, 18) and used for various types of studies including functional studies as well as global analyses of gene expression to identify specific markers of each stage of development (9). We have used GFP with a long half-life to increase chances of detecting transient gene activation; however, destabilized versions of GFP would offer shorter half-lives (to 1 h) to avoid overestimation of gene activity when desired.

**Three-dimensional reconstruction of β-cells embedded in the pancreas.** The methods that we have described here have allowed us to obtain a 3-D view of β-cells in the context of the surrounding tissues and to extend the description of the exocrine compartment in vivo. Optical sectioning and reconstruction of bulk tissue are simple and rapid. Volume and number of...
β-cells within a block of tissue can be automatically quantified using freely available software. This feature would further lead to the development of a simple and unbiased measurement of β-cell mass. The method we have employed here using fixed tissues represents a significant step forward, and we anticipate that similar data will be obtained in fresh or live tissue using two-photon confocal microscopy.

GRANTS

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