Membrane-Labeled MDCK Cells and Confocal Microscopy for the Analyses of Cellular Volume and Morphology

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A clone of Madin-Darby canine kidney (MDCK) cells whose cell membrane was stably labeled with expressed cyan fluorescent protein (CFP) was established, and changes in their volume and shape induced by hyposmotic stress were analyzed with confocal microscopy. The membrane-targeted CFP was present not only on the cell membrane but also in the endoplasmic reticulum and Golgi apparatus, but was excluded from the mitochondria and cell nucleus. During hyposmosis, the initial swelling and the following regulatory volume decrease could be accurately measured by summation of the cellular volume in every confocal slice crossing the cell at different heights. Changes in the cellular height roughly paralleled the changes in cellular volume when the mean value was compared, but dissociation as much as 30% was observed for individual cells due to changes in cell shape. The present experimental system, which enables direct measurement of cell volume and simultaneous observation of various intracellular phenomena, would be useful for further investigation of cellular volume regulation.

Key words Madin-Darby canine kidney cell; confocal microscopy; regulatory volume decrease; hyposmosis

The composition of the blood plasma is strictly regulated under physiological conditions so that most cells in the body do not experience transmembrane osmotic gradients that would cause them to swell or shrink. Exceptions are blood cells which experience anisotonic conditions as they pass through the renal medulla, and renal epithelial cells. Other cells in the body may also experience anisotonic conditions during pathological conditions such as ischemia, septic shock and diabetic coma. The ability of cells to restore cell volume under such condition may be crucial to their survival. Cells of the heart, brain, kidney, bladder, liver and skeletal muscle possess mechanisms for restoring cell volume under osmotic challenge and can reverse the swelling effect of hyposmosis and the shrinking effect of hyperosmosis.^{1–3)}

To study the mechanism of cell volume regulation, experimental systems which allow accurate measurement of cell volume is indispensable. Indirect measurement of cell volume changes has been performed by loading cells with membrane impermeable fluorescent dyes and measuring cytoplasmic dye concentration.⁴⁾ This method has disadvantages such as transmembrane dye leakage and dye compartmentalization in cellular organelles. Thus, direct measurement of the total cell volume would be of value in certain experiments.

The Madin-Darby canine kidney (MDCK) cell originates from the renal distal tubular epithelium. In this cell line, the key players of water and ion transport such as aquaporins and sodium channels are expressed^{5,6)} and responsiveness to humoral regulatory substances such as vasopressin is well maintained.⁷⁾ Thus, the MDCK cell has been widely used as a model to study volume regulation of renal epithelial cells.

In the present study, we developed a method for accurate measurement of cell volume using laser scanning confocal microscopy and a membrane-targeted cyan fluorescent protein (CFP) with a signal for posttranslational palmitoylation. We established a clone of MDCK cells whose membrane was stably labeled with expressed CFP and the cell volume could be directly measured through summation of the volumes in

each confocal slice.

MATERIALS AND METHODS

Preparation of MDCK Cells Stably Expressing Membrane Targeted CFP MDCK cells were cultured in Eagles modified essential medium (EMEM) supplemented with 1% penicillin, 0.4% streptomycin and 5% fetal bovine serum (FBS). To obtain cells stably expressing membrane targeted CFP, a vector coding CFP containing a signal for posttranslational palmitoylation (pECFP-Mem; Clontech) was introduced by lipofection. For each 9.4 cm² dish with cells grown to 70% confluence, 1.5 µg of the vector DNA was applied together with 20 µl of the lipofection reagent according to the manufacturers protocol (Lipofectamine; Gibco Invitrogen). Stable transformants were obtained by clone culture in the presence of 500 µg/ml G418, a neomycin analogue (Geneticin; Gibco Invitrogen). A transformant clone with a strong fluorescence on the cell membrane was chosen for the experiments. They were plated on glass coverslips 48 to 72 h before the experiments.

Confocal Microscopy Two- or three-dimensional imaging of MDCK cells expressing membrane targeted CFP was performed with LSM510-META (Carl Zeiss, Jena, Germany), as in our previous studies.^{8–10)} Coverslips with cells were placed in a chamber on the stage of the inverted microscope and perfused with the normal medium for confocal microscopy, which was made by adding 25 mM HEPES to the EMEM. The pH of this normal medium was adjusted to be 7.4 and the osmolarity was 300 mOsm as determined by a freezing point based osmometer. The hyposmotic media, which was made by reducing the NaCl concentration by 100 mM, had an osmolarity of 100 mOsm. After the cells were equilibrated for about 30 min in the normal medium, the perfusate was changed to the hyposmotic medium and cell volume was measured at 0, 1, 5, 15 and 30 min. Experiments were performed at 37 °C. The cells were excited at

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458 nm, 488 nm or 514 nm by an Ar⁺ laser or at 543 nm by a HeNe laser. The emission were detected by photomultipliers and organized into two- or three-dimensional images. The objective used was C Apocromat 40x/1.2 NA water immersion or Plan-Apocromat 63x/1.4 oil immersion. Horizontal (x-y) planes were scanned in 512×512 pixels at a speed of line/960 μ s. To obtain the total cell volume, the cells were excited at 458 nm and about 60 focal planes were obtained for each cell at different height from the glass coverslip. As the pinhole size was 70–190 μ m, the thickness of the plane recognized by a single horizontal scan was 0.43 μ m. The cell volume was obtained by summation of the cellular volume in every confocal slice crossing the cell at different heights. The height of each cell was calculated from the number of horizontal scans crossing the cell.

Co-localization Experiments The MDCK clone expressing the membrane-targeted CFP was further stained with fluorescent probes for other intracellular organelles, and the fluorescence of each probe was separately assembled into two dimensional images. In the case of tetramethylrhodamineethyl ester (TMRE), the cells were treated with the probe at a concentration of 500 nM for 30 min at 37°C in the recording chamber and washed away before the experiments. The cells were excited simultaneously at 458 nm and 543 nm, and the emission at 480 to 520 nm and 565 to 615 nm were measured as CFP and TMRE fluorescence, respectively. The endoplasmic reticulum-targeted yellow fluorescent protein (YFP) was expressed in the MDCK clone expressing membrane-targeted CFP by further transfection with an expression vector EYFP-ER (Clontech). The double transfectant was excited at 458 nm and the emission ranging from 462 to 591 nm were separated based on the standard emission spectra of the two fluorescence proteins with the linear unmixing function of the confocal microscope. In the case of BODIPYTM FL C₅-ceramide, the cells were treated with the probe at a concentration of 500 ng/ml for 30 min at 4°C, and then were placed in the recording chamber and incubated at 37°C for 30 min before the experiments. The cells were excited at 458 nm and the emission in the range of 462 to 548 nm was separated with linear unmixing. The cell nucleus-targeted DsRed was expressed in the MDCK clones expressing membrane-targeted CFP by further transfection with an expression vector DsRed-Nuc (Clontech). The emission in the range of 480 to 520 nm on excitation at 458 nm was detected as CFP fluorescence, and the emission above 560 nm on excitation at 543 nm was detected as DsRed fluorescence.

RESULTS

We succeeded to establish a clone of MDCK cells which stably express CFP on the cell membrane (Fig. 1A). Although the growth speed of the transformants was slower than that of wild type MDCK cells, they could be well maintained by culturing in high cell density. The whole cell membrane including that on the bottom side was clearly stained which enabled confocal measurement of cell volume. Some staining of intracellular membranes was also observed, a strong staining at a focal region close to the center of the cell and a weaker broad staining occupying about half of the intracellular area.

After hypotonic shock, cell volume increased during the

Fig. 1. Cell Volume Measurements with MDCK Cells Expressing Membrane-Targeted CFP

(A and B) Typical orthogonal two-dimensional images before (A) and 5 min after (B) the application of hypotonic shock. Bars in the x-y panel indicate 10 μ m. (C) Time course of the changes in cell volume (open circles) and cell height (closed circles) following hypotonic shock expressed as percentages of the corresponding value under normal osmolarity. Hypotonic shock was applied just after measurement under normal osmolarity at time zero. Values are the mean $\pm$ S.E.M. from 14 cells. The volume of the MDCK cells before the application of osmotic shock was $10.7 \times 10^3 \pm 1.4 \times 10^3 \mu\text{m}^3$. (D) Correlation of increase in cell volume (ordinate) and cell height (abscissa) in the experiment shown in (C). Data at 1, 5, 15 and 30 min after hypotonic shock were indicated with circles, triangles, squares and diamonds, respectively. The linear regression curve had a slope of 0.97. (E) x-z section of cells before (a) and 5 min after (b) the hypotonic shock. Note the space between the cell in the center and the glass coverslip (arrows). Vertical and horizontal scale bars indicate 10 μ m.

first 5 min and then, decreased towards initial value (Figs. 1B, C). The photobleaching of the membrane dye was not prominent during the measurement and the cell volume could be accurately measured before and during the swelling and shrinking. To evaluate whether the cell height could be used as a simple index of cell volume, the percent increase in cell height was plotted together with (Fig. 1C) and against (Fig. 1D) the percent increase in cell volume. The results indicate that the mean percent increase in cell height roughly parallels the mean percent increase in cell volume. When compared at 5 min after hypotonic shock, the mean percent increase in cell height was $95.1 \pm 3.7\%$ of the mean percent increase in cell volume. However, some cells show dissociation between increase in cell volume and cell height, which may be as large as 30% (Fig. 1D). The boundary between cells was not simple and was changed during the cellular volume changes. In some cases, water was secreted into the space between the basal membrane of the cell and the glass coverslip (Fig. 1E).

To clarify the type of intracellular organelle stained by the membrane-targeted CFP, we analyzed the colocalization of the fluorescence of membrane-targeted CFP and of probes against several types of intracellular organelle (Fig. 2). The

Fig. 2. Colocalization of Membrane-Targeted CFP with Other Fluorescent Probes

(A) Membrane-targeted CFP (a), TMRE (b), and their merged image. (B) Membrane-targeted CFP (a), endoplasmic reticulum-targeted YFP (b), and their merged image (c). (C) Membrane-targeted CFP (a), BODIPYTM FL C₅-ceramide, a probe for the Golgi apparatus (b), and their merge image (c). (D) Membrane-targeted CFP (a), nucleus-targeted DsRed (b), and their merged image. The details of the staining and imaging procedures are indicated in the methods. Horizontal bars indicate 10 μ m. The white region in panel c indicate the region stained by both of the probes in a and b.

excitation-emission wavelength range of CFP enabled simultaneous staining with fluorescent probes which function in wavelength ranges above 500 nm. Simultaneous staining of the CFP-expressing MDCK cells with TMRE revealed that the cells were rich of mitochondria (Fig. 2A). These two types of fluorescence did not co-localize with each other suggesting that membrane-targeted CFP does not localize in the mitochondria. The CFP-expressing MDCK cells were further transfected with a vector coding an endoplasmic reticulum-targeted YFP (Fig. 2B). Some of the focal intracellular staining, but not the broad staining, by membrane-targeted CFP overlapped with the staining by endoplasmic reticulum-targeted YFP. Simultaneous staining of the CFP-expressing MDCK cells with BODIPYTM FL C₅-ceramide, a probe for the Golgi apparatus, revealed that some of the focal staining by membrane-targeted CFP corresponded to the Golgi apparatus (Fig. 2C). The CFP-expressing MDCK cells were further transfected with a vector coding a nucleus-targeted DsRed (Fig. 2D). The intracellular region unstained by CFP coincided with the staining by DsRed indicating that the membrane-targeted CFP is excluded from the cell nucleus.

DISCUSSION

Membrane-targeted CFP stably expressed in MDCK cells stained the cell membrane both on the top and bottom of the

cell and also at the cell boundary (Fig. 1A). Changes in cell volume in response to osmotic shock could be quantitatively analyzed with this cell and confocal microscopy. The initial swelling in response to hyposmotic shock can be explained by passive transmembrane influx of water through aquaporins. This fraction of water was extruded from the cell in a short period and the cell volume returned towards the value under normoxic conditions. This process, known as the regulatory volume decrease, is considered to be the result of activation of various ion channels and transporters.^{10–14} The membrane-labeled MDCK cells established in the present study would be a useful model for the study of such mechanisms. The changes in relative cell volume could be also demonstrated when the cell height was used as an index (Fig. 1C). Although such a simplified method may be useful for certain purposes, the present results showed that the magnitude of the changes in mean cell volume could be underestimated by about 5% (Fig. 1D). Further, dissociation between cell volume and cellular height could be as large as 30%. This is probably because the cell boundary is not simple and is often affected by interaction with the adjacent cells, and sometimes because of the water secreted in the space between the cell and the glass coverslip (Fig. 1E). Thus, for accurate measurement of cell volume, direct measurement is required.

Simultaneous staining of this membrane-labeled MDCK cell with other fluorescent dyes revealed that the focal intracellular CFP staining at the center of the cell corresponds to the endoplasmic reticulum and the Golgi apparatus. This intracellular localization of membrane-targeted CFP is reasonable because these organelles are included in the standard pathway for membrane proteins to be sorted into the cell membrane. The broad CFP staining at the cell periphery is perhaps the vesicles approaching the cell membrane. CFP was not present in the mitochondria (Fig. 2A). Thus, it is likely that the intracellular presence of membrane-targeted CFP itself would not interfere with cellular function. The MDCK cells expressing membrane-targeted CFP would be useful in principle for the studies of volume changes related to osmotic stress, apoptosis, ischemia-reperfusion injury, and various pharmacological interventions.

In conclusion, we have developed a method for measurement of cell volume using laser scanning confocal microscopy and an MDCK cell line expressing membrane-targeted CFP, which would be useful for further investigation of cellular volume regulation.

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REFERENCES

- 1) MacKnight A., Leaf A., *Physiol. Rev.*, **57**, 510–572 (1977).
- 2) Okada Y., "Cell Volume Regulation. The Molecular Mechanism and

- Volume Sensing Machinery," Elsevier, Amsterdam, 1998.
- 3) Lang F., "Cell Volume Regulation," Karger, Basel, 1998.
 - 4) Zelenina M., Brismar H., *Eur. Biophys. J.*, **29**, 165—171 (2000).
 - 5) Volkl H., Paulmichl M., Lang F., *Ren. Physiol. Biochem.*, **11**, 158—173 (1988).
 - 6) Takata K., Tajika Y., Matsuzaki T., Aoki T., Suzuki T., Abduxukur A., Hagiwara H., *J. Pharmacol. Sci.*, **96**, 255—259 (2004).
 - 7) Simmons N., *Gen. Pharmacol.*, **13**, 287—291 (1982).
 - 8) Namekata I., Yamagishi R., Kato Y., Nakamura R., Tanaka H., Shigenobu K., *Bioimages*, **12**, 61—69 (2004).
 - 9) Kawai H., Suzuki T., Kobayashi T., Sakurai H., Ohata H., Honda K., Momose K., Namekata I., Tanaka H., Shigenobu K., Nakamura R., Hayakawa T., Kawanishi T., *J. Pharmacol. Sci.*, **97**, 361—368 (2005).
 - 10) Iida-Tanaka N., Namekata I., Kaneko M., Tamura M., Kawanishi T., Nakamura R., Shigenobu K., Tanaka H., *J. Pharmacol. Sci.*, **104**, 397—401 (2007).
 - 11) Rothstein A., Mack E., *Am. J. Physiol.*, **262**, C339—C347 (1992).
 - 12) Woll E., Ritter M., Haller T., Volkl H., Lang F., *Nephron*, **74**, 150—157 (1996).
 - 13) Yu W., Sokabe M., *Jpn. J. Physiol.*, **47**, 553—565 (1997).
 - 14) Okada Y., Maeno E., Shimizu T., Dezaki K., Wang J., Morishima S., *J. Physiol.*, **532**, 3—16 (2001).