A New Application of Fluorescence Ratio Imaging Technique to *In Situ* Demonstration of the Protein Phosphorylation Rate

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We introduce the methodology of the ratio imaging technique and its new application to *in situ* demonstration of protein phosphorylation. Ezrin/radixin/moesin (ERM) proteins are known to be cross-linkers between plasma membrane and actin filaments (F-actin). Phosphorylation of their COOH-terminal threonine residue was implicated in the regulation of their cross-linking activity. Using a rat mAb 297S specific for COOH-terminally phosphorylated ERM (CPERM) proteins and a mouse mAb CR22 which recognizes total ERM proteins, we detected subcellular distribution of ERM proteins and CPERM proteins in mouse macrophages by indirect immunofluorescence. Total ERM and CPERM proteins were revealed as Texas red- and Alexa 488 (green)-images, respectively, with dual exci-

tation confocal laser microscopy. Obtained Texas red- and Alexa 488-images were saved as TIFF-format files and processed for digital image analysis using a MetaMorph® software. The ratio value in each pixel was automatically calculated by dividing an Alexa 488-image by a corresponding Texas red-image, and built a pseudocolor ratio image. The ratio image showed that the phosphorylation rate of ERM proteins in cytoplasm gradually increased toward the roots of filopodia, and was highest in filopodia. Thus, ratio imaging seems to be advantageous to visualize where ERM proteins are phosphorylated in the cells, not being affected by the total amount of ERM proteins or cytoplasmic volume of the cells.

Key words: Digital image analysis, Fluorescence ratio imaging, ERM proteins, phosphorylation, Macrophages

I. Introduction

In the fields of cell biology and cytochemistry, the fluorescence ratiometry or ratio imaging technique has often been employed for analyzing intracellular Ca^{2+} concentration and pH in living cells [8, 11, 12, 17, 23]. Recently, we have devised an application of the ratio imaging to the analysis of the functional relationship between F-actin and actin-binding proteins [2]. Conventional immunofluorescence microscopy demonstrated that most actin-binding proteins were colocalized with F-actin, and generally enriched in F-actin-rich regions in the cells. However, it was unclear how actin-binding proteins were

differently distributed among distinct F-actin-rich structures, since the amount of actin-binding proteins were much affected by the amount of F-actin. Therefore, the ratio imaging technique was applied to the demonstration of the actin-binding protein concentration relative to F-actin. The ratio images of actin-binding protein/F-actin more efficiently told us where the actin-binding protein would contribute to cell functions than conventional fluorescence images did, since ratio imaging can avoid the effects of other parameters such as the cytoplasmic volume and the F-actin amount [2]. We have considered that the ratio imaging may be useful to analyze not only the relationship between F-actin and actin-binding proteins, but also the relationships between the other two functionally related molecules or between two different forms of the same molecule, such as phosphorylated and dephosphorylated forms.

It is well known that protein phosphorylation and dephosphorylation are one of the most important reactions to regulate protein function and mediate signaling

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in the cell. For example, ezrin/radixin/moesin (ERM) proteins are known to be cross-linkers between plasma membrane and F-actin, and localized predominantly at special regions where F-actin are densely associated with plasma membranes such as filopodia, microvilli and cell-cell/cell-matrix adhesion sites [1, 5, 7, 20, 24, 25]. It was biochemically shown that the phosphorylation of their COOH-terminal threonine residue of ERM proteins was important for regulation of their cross-linking activity, and COOH-terminally phosphorylated ERM (CPERM) proteins might represent the active form of ERM proteins [14, 16]. Recently, Hayashi *et al.* (1999) developed a novel fixation protocol using trichloroacetic acid (TCA) to preserve immunoreactivity of CPERM proteins; and revealed that CPERM proteins were exclusively associated with plasma membrane, whereas total ERM proteins were distributed in both the cytoplasm and plasma membranes by immunofluorescence [10]. In this study, we have further attempted to visualize the phosphorylation rate of ERM proteins in mouse macrophages by fluorescence ratio imaging.

II. Materials and Methods

Reagents

Monoclonal antibodies (mAb), mouse anti-total ERM proteins (clone CR22) and rat anti-CPERM proteins (clone 297S), were kindly provided by Drs. Shigenobu Yonemura and Shoichiro Tsukita, Kyoto University. Specificity and characterization of these mAb were previously reported [10, 14, 20, 26]. Other chemicals were from Sigma Chemical (St. Louis, MO) or Wako Pure Chemical (Osaka, Japan), unless otherwise indicated.

Cells

Mouse bone marrow-derived macrophages were obtained from femurs of C3H HeJ mice and cultured in a BMM medium supplemented with 20% FCS and 30% L-cell conditioned media, a source of macrophage colony-stimulating factor (MCSF) as previously described [3, 22]. After 6 or 7 days of culture, macrophages were replaced on 12 mm circular coverslips in 24-well dishes, and incubated in the culture medium lacking MCSF overnight before use.

Cytochemical staining

Macrophages cultured on circular coverslips were

fixed with 10% trichloroacetic acid (TCA) freshly dissolved in distilled water for 15 min at 4°C [10]. Then, the cells were rinsed in PBS containing 30 mM glycine (G-PBS) four times for 5 min each. These cells were permeabilized with 0.2% Triton X-100 in G-PBS, and pretreated with blocking solution consisting of 10% normal goat serum and 1% bovine serum albumin in G-PBS. They were then incubated with primary antibodies 1 : 1 mixture of mAb CR22 and mAb 297S supernatants for 90 min at room temperature. After rinsing with G-PBS, they were incubated with secondary antibodies, Alexa 488-labeled goat anti-rat IgG (Molecular Probe), and Texas red-labeled goat anti-mouse IgG (Amersham), for 90 min. These secondary antibodies showed non-cross reactivity with the other species IgG.

Confocal microscopy and digital image analysis

Coverslips were mounted on glass slides using Anti-fade (Molecular Probes Inc., Eugene, OR) and observed with a confocal laser scanning microscope (CLSM, Olympus GB200) using $\times 100$ PlanApo lens (NA1.4). Alexa 488- and Texas red-images were obtained by dual excitation at 488 and 568 nm, respectively. Laser intensity (high voltage), aperture size, gain and black level (off set) were appropriately determined so that each signal was enough to be visualized, but few pixels in the interest area were saturated. It is important to adjust both Alexa 488- and Texas red-signals appropriately, and keep the optimal conditions constant through the experiment. We also confirmed that Alexa 488- and Texas red-images were aligned exactly in the same focal plane; Alexa 488- and Texas red-signals were detected only in the 488 nm (green) channel and the 568 nm (red) channel, respectively; and there were few signals in the other channel. For direct digital image analysis, obtained Alexa 488- and Texas red-images were separately saved as 8 bit TIFF-format files. Alternatively, Alexa 488- and Texas red-merged images were preprocessed to separate green and red channels using Adobe Photoshop®, then processed for image analysis. For digital image processing, we mainly employed MetaMorph® Imaging System Version 3.5 (Universal Imaging Co., West Chester, PA) that run on the Windows 95. The ratio imaging was carried out automatically by the ratio command in the MetaMorph Process menu, and presented as spectrum pseudocolor images. For obtaining ratio images on the PowerMac, we tried to use the NIH Image

Fig. 1. Confocal immunofluorescence images and their image analysis of total ERM proteins and COOH-terminally phosphorylated ERM (CPERM) proteins in mouse bone marrow-derived macrophages. The confocal image was taken at an optical slice including the basal adherent portion of macrophages. (A) Total ERM proteins were immunostained using mAb CR22 and Texas red-labeled secondary antibody. (B) CPERM proteins were immunolabeled with mAb 297S and Alexa 488-labeled secondary antibody. (C) A merged image of total ERM proteins and CPERM proteins images was made by superimposing with MetaMorph. (D) The ratio image of CPERM proteins/total ERM proteins was obtained by the MetaMorph image analysis. The image was revealed as a spectrum pseudocolor image corresponding to the ratio values from 0 to 1.3. (E) Line scan analysis on the merged image (C) showing fluorescence intensities of Alexa 488 (green: CPERM proteins) and Texas red (red: total ERM proteins) signals at the position of a dotted line in C. (F) Line scan analysis on the ratio image (D) showing the ratio values of Alexa 488/Texas red at the same line position as in E. Scale bar = 10 μ m.

Fig. 1

1.62, a free software which can be downloaded from <http://rsb.info.nih.gov/nih-image/> or <ftp://codon.nih.gov/pub/nih-image/>. In this case, using the Image Math command in the process menu of the NIH Image, the Alexa 488 image was divided by the Texas red image, then multiplied by a certain coefficient so that an interesting area of the ratio image would be appropriately contrasted.

III. Results

Immunofluorescence using anti-ERM proteins showed that total ERM proteins were very enriched in filopodia of macrophages; and other cytoplasmic regions were also moderately labeled for ERM proteins (Fig. 1A). Similar to total ERM proteins, CPERM proteins were predominantly observed in filopodia. However, cytoplasmic labeling with anti-CPERM proteins appeared to be weaker than that with anti-ERM proteins (Fig. 1B). In the dual color merged image (Fig. 1C), filopodia which were labeled with both anti-ERM proteins (red) and anti-CPERM proteins (green) became bright yellow suggesting that ERM proteins concentrated in filopodia were extensively phosphorylated on their COOH-terminal threonine residue. Other cytoplasmic regions were shown to be dark orange or red. Hence, it was apparent that CPERM proteins were much more in filopodia than in other cytoplasm. However, in the conventional merged image, it was unclear how much ERM proteins were phosphorylated in the cytoplasm, because the amount of ERM proteins was generally small.

The ratio image of CPERM proteins/ERM proteins successively showed that the rate of phosphorylation of ERM proteins was highest in filopodia. In a filopodium, the phosphorylation rate of ERM proteins seemed to be constant from the tip to the root (Fig. 1D), although the conventional images showed that signals for both total ERM proteins and CPERM proteins became weaker at their thinner tip (Fig. 1A–C, E). Furthermore, the ratio image revealed that the rate of phosphorylation gradually increased toward the roots of filopodia. (Fig. 1D). These ratio images were obtained using MetaMorph software, but essentially the same images could be obtained by NIH Image.

The line scan analysis by MetaMorph showed the fluorescent intensity of each signal in specified regions where we drew a line on the merged image (Fig. 1E). Similarly, the line scan analysis on the ratio image indicated ratio values of CPERM proteins/ERM proteins (Fig. 1F) as a graph. Thus, the quantitative analysis of image data and their statistic comparison among the different regions or cell structures would be possible.

More detailed findings on the relationship between CPERM proteins and ERM proteins, which is associated with the cell functions of macrophages, will be described in a separate paper.

IV. Discussion

With the flourishing development of image analysis software and hardware, digital image analysis has been increasingly used as a quantitative tool in morphological and cytochemical studies [13]. In this study, we newly applied the ratio imaging technique to visualizing phosphorylation rate of ERM proteins in macrophages. The conventional confocal images of anti-ERM proteins showed that ERM proteins were predominantly localized in filopodia of macrophages, and some were present in cytoplasm. Similar to anti-ERM proteins, the signal of anti-CPERM protein was prominent in filopodia, however, few signals were seen in the cytoplasm. These findings are consistent with a previous report [15]. It was known that ERM proteins bind to F-actin at their COOH-termini [1], and their binding was regulated by COOH-terminal threonine phosphorylation by protein kinase such as Rho-kinase and protein kinase C- θ [14, 18, 19]. Therefore, it was presumable that phosphorylated ERM proteins would function in filopodia where F-actin was cross-linked to plasma membrane via ERM proteins [14–16]. On the other hand, in most cytoplasmic ERM proteins, their NH₂- and COOH-terminal halves are associated intra- and/or intermolecularly. This head-to-tail association suppresses their binding ability of NH₂- and COOH-termini to plasma membrane and F-actin, respectively [4, 6, 9, 24, 25]. Such head-to-tail association of inactive ERM proteins is broken into active forms by phosphorylation of COOH-terminal threonine residue [14]. Although phosphorylated ERM protein were biochemically shown to be translocated from cytoplasm to apical cell protrusions in NIH-3T3 cells [21], it was still unclear whether ERM proteins were phosphorylated in the cytoplasm before recruiting to the plasma membrane, or phosphorylated just beneath the plasma membrane after recruiting there. The raw immunofluorescence images gave us the impression that phosphorylated ERM proteins localized only in filopodia but not in cytoplasm. However, this seems to be a misinterpretation of the images owing to a smaller amount of ERM proteins in the cytoplasm. In the ratio images of CPERM proteins/total ERM proteins, the rate of CPERM proteins relative to total ERM proteins gradually increased in the cytoplasm toward the roots of filopodia. This suggests that at least some ERM proteins are phosphorylated in the cytoplasm, and then recruited into filopodia. It is unlikely that all ERM proteins are phosphorylated just beneath the plasma membrane in filopodia.

It is also worth noting that the rate of ERM protein phosphorylation was the highest and constant from the root to the tip in a filopodium although signals of both total ERM proteins and CPERM proteins decreased toward the tip because the tip was thinner. Thus, the raw fluorescence images seem to be misleading sometimes because the intensities of fluorescence signals are considerably affected by the total amount of molecules, and the

volume of cell structures. The ratio imaging can avoid such misinterpretations on the raw fluorescence images, and can reveal a visually accurate relationship between both signals.

Furthermore, as shown in Fig. 1E and F, we can quantitatively demonstrate the fluorescence intensity of each signal and its ratio values at specified regions by line scan analysis in MetaMorph programs. It should be noticed that the ratio value of fluorescence intensities does not represent the actual rate of protein phosphorylation. Nevertheless, the ratio values would be worthy of statistical analysis to compare phosphorylation rates among the different regions or cell structures associated with diverse cell functions. We believe that this ratio imaging technique can be widely applied to various cytochemical samples and should allow novel insights into the properties of the cells.

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