**In Vivo Interaction between Dynamitin and MacMARCKS Detected by the Fluorescent Resonance Energy Transfer Method***

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Dynamitin is a subunit of the dynactin complex regulating microtubule-dependent motor functions, and MacMARCKS (Macrophage-enriched myristoylated alanine-rich protein kinase C substrate) is a major protein kinase C substrate regulating integrin activation. The interaction between dynamitin and MacMARCKS has been implicated in integrin-dependent cell spreading. However, the *in vivo* interaction of these two proteins in living cells has not been demonstrated. Spatial and temporal information about the interaction is also lacking. In this study, we used the fluorescent resonance energy transfer method to demonstrate *in vivo* interaction between MacMARCKS and dynamitin with cyan fluorescent protein (CFP)-conjugated dynamitin as the donor fluorophore and yellow fluorescent protein (YFP)-conjugated MacMARCKS as the acceptor fluorophore. The interaction of these two fusion proteins was studied both *in vitro* and *in vivo*, and typical fluorescent resonance energy transfer was observed; the CFP emission peak increased while the YFP emission peak decreased when protein interaction was abolished. Spatial and temporal information was obtained in RAW macrophage cells. In resting macrophage cells, dynamitin-MacMARCKS interaction is concentrated at the cell periphery, although the majority of dynamitin is distributed at the perinuclear region of the cells. When cells were treated with phorbol 12-myristate 13-acetate, both proteins concentrated to perinuclear regions of the cells, and yet the interaction disappeared as the cell spread. Similar events were also observed in 293 cells. Thus, we conclude that dynamitin and MacMARCKS indeed interact in living cells.

The cytoskeleton plays a pivotal role in activating β2 integrin molecules by restraining the integrin molecules on the cell membrane in an inactivated state (1–3). Upon stimulation by PKC1 activation, the cytoskeleton constraint is released and integrin is activated. Both actin and microtubule filaments are involved in constraining integrin molecules. The PKC signal is most likely transduced to cytoskeleton by MacMARCKS protein, a member of the MARCKS (myristoylated alanine-rich protein kinase C substrate) family of PKC substrates (4–6), because mutation or lack of this protein prevents PKC-stimulated molecular mobility of integrin (2), as well as ligand binding by integrin (7–9). In addition, MARCKS protein, the homologue of MacMARCKS, has been shown to be involved in cell spreading (10, 11). MacMARCKS protein contains an N-terminal membrane-targeting domain that is myristoylated at its N-terminal glycine residue. The effector domain, in the center part of the protein, contains the PKC phosphorylation sites, two serine residues at positions 93 and 104.

Interaction between MacMARCKS and dynamitin, a subunit of dynactin, has been observed (12). Dynactin (reviewed in Refs. 13–16), a complex of proteins, is an important regulator of dynein, a microtubule-dependent minus end-directed motor protein (17). The dynactin complex has been suggested as a “molecular bridge” connecting dynein to vesicle membranes (13, 18) and as an enhancement to the processivity of dynein (19). Overexpression of wild type dynamitin (20) or the addition of excess amounts of recombinant full-length dynamitin (21) breaks the dynactin complex, whereas overexpression of the conserved N terminus of dynamitin is not sufficient to break the dynactin complex but is sufficient to disturb the Golgi and endosomes (22). Because dynamitin overexpression dissociates dynein from the mitotic kinetochore (20) and Golgi membrane (23), it has been proposed as a link between dynein and its cargo.

However, interactions between motor proteins and membranes are usually reversible, and one or more regulatory mechanisms may exist. The interaction between dynamitin and MacMARCKS may aid the reversible link, because MacMARCKS is a membrane-bound protein. Both the membrane association of MacMARCKS and MacMARCKS-dynamitin interaction are regulated by PKC-mediated phosphorylation (12, 24). This interaction may provide a link between microtubules and cell spreading (12). Interaction between MacMARCKS and dynamitin has been shown *in vitro* by coimmunoprecipitation from total lysate (12). However, whether these two proteins truly interact in living cells and the spatial and temporal information of such interaction remain unknown. Therefore, we decided to examine whether these two proteins are in proximity in cells, using the fluorescent resonance energy transfer (FRET) method.

FRET is a quantum mechanical phenomenon of radiationless energy transfer from a donor molecule to an acceptor molecule via an induced-dipole interaction. Two conditions must be satisfied for FRET to occur. First, two fluorophores must have the proper spectral overlap, i.e. the emission spectrum of the first fluorophore (donor) must overlap with the excitation spectrum of the second (acceptor) fluorophore. Second, the spatial distances and the relative orientation of the two fluorophores’ transition dipoles must be sufficiently close. The efficiency of...
FRET depends on the inverse sixth power of the distance $R$ between the donor and acceptor. Thus, the distance should not be more than 10 nm. By labeling proteins with fluorophores, FRET can be used to determine the proximity of two proteins. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) greatly enhance the use of FRET. This pair of proteins has been widely used to obtain information both about the existence of FRET and the localization of FRET in live cells, including the interaction of calmodulin and calmodulin-binding peptide (25), calpain activation (26), and oligomerization (clustering) of α-factor receptor (27).

In this paper, we report that when a chimera of MacMARCKS-YFP and a chimera of CFP-dynamitin were expressed in RAW macrophages and in 293 epithelial cells, FRET was observed between these two proteins. When phorbol ester was added to disrupt the interaction of these two proteins (28), FRET disappeared. These data suggest that MacMARCKS and dynamitin indeed interact in the cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—RAW macrophage cells and 293 epithelial cells were purchased from the American Type Culture Collection (Rockville, MD). Recombinant human dynamitin or its mutant cDNA was amplified by polymerase chain reaction (PCR) with the 5′-GGA CTC GGA TCC CCG 9 primer and its 3′-CTG TTC TCC GGA TCC CAA 9 primer, using wild type dynamitin or MacMARCKS-binding domain (MBD) mutant in pGEX plasmid as template (12). The primers included the restriction sites for easy cloning into the pEYFP-N1 upstream of YFP. The term “MacMARCKS-YFP” indicates that YFP is at the C terminus of MacMARCKS. Dynamitin or its mutant cDNA were amplified by polymerase chain reaction with the 5′-GGA CTC GGA TCC CCG 9 primer and its 3′-CTG TTC TCC GGA TCC CAA 9 primer, using wild type dynamitin or MacMARCKS-binding domain deletion (MBD) mutant in pGEX plasmid as template (12). The primers included the restriction sites for easy cloning into pGEX-4T-2 downstream of CFP. The term “CFP-dynamitin” indicates that the CFP is at the N terminus of dynamitin. The sequences of all polymerase chain reaction products were confirmed before use.

The MacMARCKS plasmid (1 μg) and dynamitin plasmid (1 μg) described above were cotransfected into 10⁶ RAW cells or into 5 × 10⁶ 293 cells using the SuperFect liposome method as described in the manufacturer's instructions (Qiagen). The RAW cells were analyzed by FRET and Western blot 20 h after transfection, and the 293 cells were analyzed by FRET and Western blot 16 h after transfection.

**Expression of Fusion Proteins in Escherichia coli**—cDNA encoding MacMARCKS was amplified by polymerase chain reaction with the 5′-GGA CTC GGA TCC CCG 9 primer and its 3′-CTG TTC TCC GGA TCC CAA 9 primer, using wild type dynamitin or MacMARCKS-binding domain deletion (MBD) mutant in pGEX plasmid as template (12). The primers included the restriction sites for easy cloning into pGEX-4T-2 downstream of CFP. The term “CFP-dynamitin” indicates that the CFP is at the N terminus of dynamitin. The sequences of all polymerase chain reaction products were confirmed before use.

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**Expression of Fusion Proteins in Saccharomyces cerevisiae**—cDNA encoding CFP-dynamitin containing a blunt end at its 5′ and SalI sticky end at its 3′ were inserted downstream of GST into pGEX-4T-2 cut with SmaI site at the upstream end and XhoI at the downstream end. cDNA encoding MacMARCKS-YFP containing EcoRI site at its 5′ end and NotI at its 3′ end were inserted into pProEx HTa plasmid downstream of the His tag by using matching restriction sites. After being transformed into the DH5α strain, expression of the fusion proteins was induced by 0.1 mM isopropyl-1-thio-β-p-galactopyranoside at 30 °C for 3 h. The GST-CFP-dynamitin fusion protein was purified using glutathione-Sepharose 4B beads as described (30). The His$_6$ fusion protein of MacMARCKS-YFP was purified using NiNTA-Sepharose as described in the manufacturer's manual (Qiagen).

In Vitro Binding between CFP-dynamitin and MacMARCKS-YFP—The His$_6$ fusion proteins of MacMARCKS-YFP (2 μg each) were incubated with glutathione-Sepharose (50 μl of 50% slurry) conjugated with GST fusion proteins of CFP-dynamitin in 1 ml of phosphate-buffered saline in Eppendorf tubes for 1 h at 4 °C. Meanwhile, glutathione-GST beads were used as negative control. After three washes, Sepharose beads were transferred to fresh tubes and washed again. The GST-dynamitin and the dynamitin-bound MacMARCKS were then subjected to SDS-polyacrylamide gel electrophoresis (9%) and transferred to an Immobilon membrane. MacMARCKS was detected by immunoblotting with the anti-MacMARCKS antibody. The GST-dynamitin fusion proteins and GST on the Sepharose beads were detected by Coomassie blue staining of the same membrane.

**Spectrofluorometric Study of FRET**—The spectrofluorometric study was carried out on an SLM 8000 spectrofluorometer. RAW cells (5 × 10⁶) were transiently transfected with both CFP-dynamitin and MacMARCKS-YFP. After 20 h, the culture was stimulated with or without 100 nM PMA for 15 min. The cells were then scraped off the plate and lysed in 0.5% Triton X-100 for 5 min at room temperature. The emission spectra of CFP-dynamitin and MacMARCKS-YFP in lysate were then measured using an excitation wavelength of 425 nm, which was chosen to maximize the excitation of CFP and minimize the direct excitation of YFP. The spectrofluorometer was set at the ratio mode of 2 nm wavelength intervals, the emission bandpass was 2 nm at 5 integration.

**FRET Measurement and Calculation**—With an ideal pair of fluorophores, the emission spectrum of the donor should overlap largely with the excitation spectrum of the acceptor and absolutely should not overlap with the emission spectrum of the acceptor. At the same time, the CFP/YFP-dynamitin/MBD receptor emission spectrum of the acceptor should absolutely not overlap with the emission wavelength of the donor. However, CFP and YFP showed certain overlapping spectra that needed to be corrected (Fig. 1). We used three sets of filters in our FRET experiments. The CFP filter set was for observing the location and intensity of CFP-dynamitin: excitation filter 440 ± 21 nm, a dichroic beam splitter of 455 nm DRLP, and an emission filter of 480 ± 30 nm. The YFP filter set was for the MacMARCKS-YFP: excitation filter 500 ± 25 nm, a dichroic beam splitter of 525 nm DRLP, and an emission filter of 545 ± 35 nm. The FRET was observed by exciting cells at the CFP wavelength and detecting at the YFP emission wavelength. Thus, the FRET filter set contained the excitation filter at 440 ± 20 nm, a dichroic beam splitter of 455 nm DRLP, and an emission filter of 535 ± 25 nm.

When cells were observed under the FRET filter set, a certain amount of the CFP emission leaked through the FRET filter, and a certain amount of YFP was directly excited by the FRET excitation wavelength. Thus, FRET was observed by exciting cells at the CFP wavelength and detecting at the YFP emission wavelength. Therefore, the FRET filter set contained the excitation filter at 440 ± 20 nm, a dichroic beam splitter of 455 nm DRLP, and an emission filter of 535 ± 25 nm.

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without serum, buffered with 20 mM HEPES at pH 7.2. The bottom of the chamber was an 0.15-mm thick glass that allowed the use of a 100× object lens. We first measured the FRET with the FRET filter, then the CFP with the CFP filter, and finally the YFP with the YFP filter under the exact same exposure time using a CCD camera (Optronic DE750). The FRET was then calculated by

\[ \text{FRET} = \frac{I_{\text{FRET}}}{I_{\text{CFP}}} - 30.84\% \frac{I_{\text{CFP}}}{I_{\text{YFP}}} \]  

(Eq. 5)

To obtain information about the localization of FRET in a cell, the FRET value of each pixel of the total 307,200 pixels was calculated individually using a computer program written in-house and based on the above formula. The calculated FRET value was then plotted pixel by pixel so that a net image of FRET could be presented.

In a sense, FRETN has the same meaning as the equilibrium constant in a chemical reaction.

\[ K_{eq} = \frac{[\text{product}]}{[\text{reactant 1}] [\text{reactant 2}]} \]  

(Eq. 7)

Therefore, the larger the FRETN, the higher the ratio of complex between CFP-dynamitin and MacMARCKS-YFP.

RESULTS

In Vitro Binding between CFP-Dynamitin and MacMARCKS-YFP—Although a previous report has shown that His$_6$-tagged MacMARCKS binds to GST-tagged dynamitin on glutathione-Sepharose beads (12), it was not known whether the fluorescent tag would affect the binding between these two proteins. To verify that attaching fluorescent protein to dynamitin and to MacMARCKS would not affect their binding, we first assayed the binding between purified the GST-CFP-dynamitin fusion protein the and His-MacMARCKS-YFP fusion protein. We used the same method as previously described (12) except that this time a YFP was tagged at the C terminus of MacMARCKS and a CFP was inserted between the N terminus of dynamitin and the C terminus of GST. Fig. 2 shows that the His-MacMARCKS-YFP protein was specifically retained on the GST-CFP-dynamitin beads but not on the GST beads, even though the GST was used in excess amounts. Therefore, tagging fluorescent protein on dynamitin and on MacMARCKS does not inhibit their ability to bind to each other.

Expression of CFP-Dynamitin and MacMARCKS-YFP in RAW and 293 Cells—Once we determined that CFP-dynamitin and MacMARCKS-YFP interact in vitro, we then packaged the cDNA, encoding each of them into the cytomegalovirus-driven expression vectors and transiently expressed them in RAW cells. The expression levels of CFP-dynamitin and MacMARCKS-YFP were determined both by immunoblotting with antibodies against MacMARCKS and dynamitin (Fig. 3) and by surveying with a microscope for cells expressing both blue and green fluorescence. By Western blot, MacMARCKS-YFP in transfected cells migrated at a molecular mass of about 67 kDa, roughly equal to the total of the apparent molecular mass of MacMARCKS (42 kDa) and YFP (26 kDa). In untransfected cells, no corresponding band was observed (Fig. 3). The cotransfected dynamitin was also expressed in these cells; CFP-dynamitin (76 kDa) migrated at the total apparent molecular mass of dynamitin (50 kDa) and CFP (26 kDa). Again, no corresponding band was seen in untransfected cells (Fig. 3). With microscopy, we found that ~20% of the RAW cells expressed both proteins. All transfected cells expressed both fluorescent proteins, and we did not find any cells expressing only one of the transfected constructs. With regard to the ratio of the two proteins in the transfected cells, MacMARCKS-YFP was approximately equal to CFP-dynamitin in RAW cells as determined by their fluorescent intensity quantitated by a CCD camera.

2 The software is free to all researchers upon request.
When we attempted to culture the transfected cells for more than 2 weeks under the G418 selection pressure, all transfected cells eventually died, a result that agreed with a report that overexpression disrupts normal cell division (20).

Characterization of Fluorescent Spectrum of the FRET between MacMARCKS-YFP and CFP-Dynamitin —The FRET assay under a microscope observes only the increase in YFP emission. To be sure that an increased emission at the YFP emission peak indeed results from authentic FRET, one must show a simultaneous decrease in CFP emission and an increase in YFP emission. Therefore, we first examined the fluorescent spectral properties of the MacMARCKS-YFP-CFP-dynamitin complex using a spectrofluorometer. Because portions of these two proteins exist as a complex in lysate from untreated cells but not in cells treated with PMA (12), we therefore studied the MacMARCKS-YFP-CFP-dynamitin complex in RAW cells co-transfected with cDNAs encoding MacMARCKS-YFP and CFP-dynamitin. These cells presumably contain a mixture of un-bound MacMARCKS-YFP, CFP-dynamitin, and bound MacMARCKS-YFP-CFP-dynamitin.

Because the light scattering caused by intact cells in solution interfered with the measurement, 1% Triton X-100 was used to dissolve the cells, and the solution was measured for the emission spectrum excited at 425 nm. The excitation wavelength was selected at the blue side of the CFP excitation wavelength to avoid excessive cross-excitation of YFP (Fig. 1, arrow). Under this condition, we observed an emission peak at 475 nm, which corresponded to the CFP emission, and another peak at 527 nm, which corresponded to YFP emission (Fig. 4). This emission at YFP wavelength should represent a mixed emission of directly excited YFP resulting from a slight overlapping of the YFP excitation spectrum in 425 nm, emission of CFP at the YFP region from the overlapped emission spectrum of CFP, and true FRET. If the interaction between MacMARCKS and dynamitin is blocked, the FRET should disappear, which should translate to a decrease in the YFP emission peak and an increase in the CFP emission peak. Therefore, we treated the cells with 100 nM of PMA for 15 min before adding Triton X-100, because PMA-promoted MacMARCKS phosphorylation decreases the binding between these two proteins (12). Indeed, we observed an ~10% decrease in the YFP emission peak and a corresponding increase in the CFP emission peak. These data indicate that the increase in YFP emission observed using microscopic methods indeed resulted from FRET.

Next we examined the FRET of the transfected cells under the microscope. The FRET and FRETN value of each cell were represented by averaging the 30 areas at the size of 20 × 20 pixels with the highest FRETN value. Then the average FRET and FRETN values of ~30 cells of each sample group were compared with those of the negative control group (cells co-transfected with empty CFP and YFP plasmid). In RAW cells, we found that the FRETN value of CFP-dynamitin and MacMARCKS-YFP-coexpressing cells was 67-fold greater than the control group, which is a statistically significant difference (Table I). To be sure that the positive FRET is not limited only to RAW cells, we also examined 293 cells cotransfected with wild type dynamitin and MacMARCKS. Similar data were obtained in which the FRETN was 159-fold greater than its negative control (Table I).

Because the directly observed image was a mixture of contributions from FRET and from CFP and YFP, we calculated the FRET values pixel by pixel and plotted them using in-house software. This process provided spatial information about the interaction site in live cells. Non-PMA-treated RAW cells maintained their spherical shape, and MacMARCKS-YFP concentrated on the plasma membrane in these cells when compared with the uniform distribution of CFP and YFP mock transfected cells (Fig. 5). Although CFP-dynamitin was primarily distributed in the perinuclear region, the FRET was concentrated at the cell periphery where MacMARCKS was located, thereby suggesting that the portion of dynamitin interacting with MacMARCKS is that located at the periphery (Fig. 5). Because the interaction between MacMARCKS and dynamitin is regulated by PMA-stimulated phosphorylation of MacMARCKS (12), we then tested whether PMA treatment also abolishes the FRET observed in cells coexpressing CFP-dynamitin and MacMARCKS-YFP. After being treated with 100 nM PMA for 15 min, the RAW cells spread out. Most MacMARCKS-YFP was concentrated at the perinuclear region with small residual amounts left on the plasma membrane (Fig. 5). Although CFP-dynamitin was also concentrated at the perinuclear region, no FRET was observed in the perinuclear region or anywhere else in these cells. The FRETN values of these PMA-treated cells were at the same level as the negative con-
trol groups (Table I). This observation suggested that merely having two proteins colocalized in the same subcellular localization was not sufficient to induce FRET. Again, PMA also abolished the FRET between CFP-dynamitin and MacMARCKS-YFP in 293 cells (Table I).

To be sure that the FRET observed had indeed resulted from a true protein-protein interaction and not from a random encounter between two overexpressed proteins in a crowded space, we expressed the MacMARCKS-binding domain deletion mutant of dynamitin (CFP-MBD) instead of wild type dynamitin in RAW cells. Although MacMARCKS was still concentrated at the plasma membrane in RAW cells and the mutant dynamitin still at the perinuclear zone, the mutant dynamitin no longer caused the FRET (Fig. 5), because the mutant dynamitin did not bind to MacMARCKS (12). The FRETN values were extracted from these cells coexpressing the mutant dynamitin and wild type MacMARCKS; the values were close to those of the negative control (Table I). These data provided convincing negative control to support the hypothesis that the FRET between MacMARCKS and wild type dynamitin resulted from actual protein-protein binding. Thus, we conclude that the interaction between MacMARCKS and dynamitin indeed exists in cells and is subjected to the regulation of MacMARCKS phosphorylation.

**DISCUSSION**

In this report, we have show that MacMARCKS and dynamitin occur in proximity in the peripheral membrane in live macrophage cells and on the perinuclear vesicles of live 293 cells. As required by the FRET principle, these two molecules must be within 10 nm of each other for FRET to occur. Considering in vitro binding data and coimmunoprecipitation data supporting their binding (12), we conclude that these two proteins indeed interact in cells. In addition, the in vivo interaction is regulated by PMA-stimulated MacMARCKS phosphorylation, because FRET no longer occurred after cells were treated with PMA. These data correlate with previously reported data that PMA regulates the direct binding of these two proteins in binding assays with both purified proteins and coimmunoprecipitation.

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**TABLE I**

| Cells   | Constructs          | PMA | FRETN      | FRET      | p value   |
|---------|---------------------|-----|------------|-----------|-----------|
| RAW     | MacMARCKS-YFP       | −   | 23.2 ± 19.5| 12.04 ± 6.59| <0.001 (n = 30) |
| RAW     | CFP-Dynamitin       | +   | 0.612 ± 1.60| 2.32 ± 7.82| >0.05 (n = 30) |
| RAW     | MacMARCKS-YFP       | −   | 1.72 ± 3.10| 1.33 ± 2.41| >0.05 (n = 15) |
| RAW     | ED-YFP              | −   | 1.13 ± 1.87| 3.99 ± 6.03| >0.05 (n = 29) |
| RAW     | CFP-Dynamitin       | −   | 0.43 ± 9.20| 2.00 ± 4.25| — (n = 19) |
| 293     | MacMARCKS-YFP       | −   | 45.9 ± 39.0| 10.08 ± 7.67| <0.001 (n = 26) |
| 293     | CFP-Dynamitin       | +   | 1.62 ± 2.56| 2.35 ± 4.12| >0.05 (n = 24) |
| 293     | CFP-MBD             | −   | 1.13 ± 1.87| 3.99 ± 6.03| >0.05 (n = 29) |

**FIG. 5. Localization of FRET in cells.** RAW cells transfected with CFP-dynamitin and MacMARCKS-YFP or with CFP-MBD and MacMARCKS-YFP were treated with or without PMA. Fluorescent images were taken with a CCD camera using CFP, YFP, and FRET filter sets as described under “Experimental Procedures.” The net FRET values were calculated and replotted using software written in-house.
Thus, this report provides the first evidence of in vivo interaction of MacMARCKS and dynamitin in live cells. We can safely say that these two proteins interact with each other in unstimulated macrophages near the cell periphery. During PMA-stimulated cell spreading, the localization of FRET at this site disappears. This observation also agrees with our finding that when the interaction of these two proteins is inhibited, the cells spread (12). Thus, it seems that the interaction is a mechanism of maintaining the cells in a spherical shape. Because intact microtubule is required for the maintenance of cell shape (32), microtubules must be anchored to the plasma membrane or to the microfilaments beneath the plasma membrane. We speculate that dynamitin may be such an anchor, because it is linked to microtubules through a dynactin complex on one end and to the plasma membrane through its interaction with MacMARCKS on the other end. There are two possible ways for this anchor to mediate the involvement of microtubules in cell spreading. One possibility is integrin activation, which is the initial step of cell spreading (33). Our recent work showed that the intact microtubule cytoskeleton is indeed responsible for keeping integrin from being activated. The intact microtubule cytoskeleton could exert its influence by linking to dynamitin through dynactin and dynein. Then interaction of dynamitin with MacMARCKS translates the effect of linkage to dynamitin through dynactin and dynein. Next, this report provides the first evidence of MacMARCKS-Dynamitin Interaction and FRET.

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