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Increased Association of Dynamin II with Myosin II in Ras Transformed NIH3T3 Cells

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Abstract Dynamin has been implicated in the formation of nascent vesicles through both endocytic and secretory pathways. However, dynamin has recently been implicated in altering the cell membrane shape during cell migration associated with cytoskeleton-related proteins. Myosin II has been implicated in maintaining cell morphology and in cellular movement. Therefore, reciprocal immunoprecipitation was carried out to identify the potential relationship between dynamin II and myosin II. The dynamin II expression level was higher when co-expressed with myosin II in Ras transformed NIH3T3 cells than in normal NIH3T3 cells. Confocal microscopy also confirmed the interaction between these two proteins. Interestingly, exposing the NIH3T3 cells to platelet-derived growth factor altered the interaction and localization of these two proteins. The platelet-derived growth factor treatment induced lamellipodia and cell migration, and dynamin II interacted with myosin II. Grb2, a 24 kDa adaptor protein and an essential element of the Ras signaling pathway, was found to be associated with dynamin II and myosin II gene expression in the Ras transformed NIH3T3 cells. These results suggest that dynamin II acts as an intermediate messenger in the Ras signal transduction pathway leading to membrane ruffling and cell migration.

Key words Grb2; dynamin II; myosin II; Ras transformed NIH3T3 cell

Dynamins constitute a superfamily of 100 kDa GTPase that has been implicated in vesicle trafficking. Many studies have suggested that dynamin is essential to endocytic membrane fission, caveolae internalization and protein trafficking in the Golgi apparatus of on several cell types [1–4]. Dynamin I is expressed exclusively in the brain [5], dynamin II is found in all tissues [6,7], and dynamin III is limited to the testis, brain, lungs and heart [8,9]. Although there is considerable evidence showing that dynamin is involved in the skeletal protein functions, most studies reported its relationship with the actin protein. Recently, dynamin II was reported to be involved in the formation of podosome rather than the plasmic membrane [10], actin comet formation [4,11], mediation of cell adhesion and the motility of phagocytic cells [12,13], suggesting that its function is different from that of dynamin I. Hence, dynamin II might be involved in the process of cellular change as a partner of the actin-related molecules.

Ras proteins are believed to contribute to the proliferation, invasion and metastatic properties of transformed cells. It was reported that the overexpression of Ras protein increases metastatic potential in the NIH3T3 cell line [14] and the rate of HaCaT cell migration [15]. This suggests that NIH3T3 cells overexpressing Ras migrate faster than normal NIH3T3 cells. It was previously reported that dynamin II is mainly associated with Grb2 in Ras overexpressing NIH3T3 cells [16], suggesting that dynamin II might be a functional molecule on the Ras signaling pathway. This indicates that dynamin II either mediates different cellular functions or is involved in cell migration and the cellular morphological changes in Ras

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overexpressing NIH3T3 cells.

Myosins are mechanoenzymes that bind to and move along the actin filaments towards the end using the energy released by the hydrolysis of adenosine triphosphate [17]. Myosin II, one of the major components of the cytoskeleton in non-muscle cells, produces the motive force necessary for cell movement and cytokinesis through interaction with the actin filament [18]. Migration requires cell communication with the adjacent cells as well as the extracellular matrix components, and is triggered by chemotactic factors, such as platelet-derived growth factor (PDGF) [19,20]. Previous studies have shown that the pathway triggered by PDGF receptor stimulation leads to actin cytoskeletal reorganization and cell migration [21, 22]. From these reports, it is believed that there might be a link between dynamin II and myosin II in the actin cytoskeleton and cell migration.

In this study, we examined whether or not there is an interaction between dynamin II and myosin II in NIH3T3 cells which might be associated with cell migration. The results showed that dynamin II is expressed with myosin II in NIH3T3 and Ras transformed NIH3T3 [NIH3T3 (Ras)] cells, suggesting that dynamin II might be involved in cell migration with the highly expressed myosin II in NIH3T3(Ras) cells. Confocal microscopy showed that dynamin II was associated with myosin II in cell migration. Immunoprecipitation (IP) with myosin II and Western blot of dynamin II were also carried out to confirm the confocal microscopic results of the PDGF stimulation in NIH3T3 cells.

Materials and Methods

Cell culture

NIH3T3 cell line purchased from American Type Culture Collection) Manassas, USA) and H-Ras transformed NIH3T3 cell line kindly provided by Dr. J. S. GUTKIND (National Institutes of Health, Bethesda, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, USA), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (0.25 μg/ml) and 2-mercaptoethanol (50 μM) at 37 ºC in a 5% CO_2 humidified incubator. The cells were detached by incubation with 0.05% trypsin-EDTA (Gibco BRL) at 37 ºC for 10 min.

Chemical treatment

The NIH3T3 cells were plated on glass cover slips in either a six well plate or 100 mm dish until the culture reached 60% confluence. After 24 h, the culture medium was replaced by fresh Dulbecco's modified Eagle's medium and the cells were cultured for an additional 18 h before being stimulated with PDGF. Then the cells were treated with 30 ng/ml PDGF-BB (Sigma, St. Louis, USA) for 5–30 min at 37 ºC.

Preparation of the cell lysate

The cells were collected by centrifugation and washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). Approximately 2×10^7 cells were suspended in 1 ml of the lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 5 mM Na_3VO_4, 10 μg/ml leupeptin and aprotinin, 5 μg/ml pepstatin and 0.5 mM phenylmethylsulfonylfluoride). The lysates were incubated on ice for 60 min and centrifuged at 12,000 rpm for 10 min. The supernatant was used as the whole cell lysate.

Immunoprecipitation and Western blot analysis

As described above, the cells were washed twice using ice-cold PBS and lysed in an ice-cold lysis buffer. After 60 min incubation on ice, the lysate was cleared by centrifugation at 13,000 rpm for 20 min (Eppendorf, Hamburg, Germany) and the protein concentration was determined using an assay kit (Bio-Rad, Hercules, USA). The protein G (KPL, Gaithersburg, USA) bead slurry was washed three times using PBS, and 500 μl of the lysate was added. The cell lysates with the protein G beads in the lysis buffer were incubated with anti-dynamin II (Hudy-2; Upstate Biotechnology, Lake Placid, USA) or anti-myosin II (hSM-V; Sigma) antibody for 2 h at 4 ºC. After incubation, the protein-bead-antibody complexes were washed with PBS and centrifuged at 10,000 *g* for 5 min. The immunoprecipitates were boiled for 5 min in a reducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, loaded onto SDS polyacrylamide gel (15%) and blotted onto nitrocellulose filters (Amersham Pharmacia Biotech, Amersham, UK). The filters were blocked with 5% skim milk in PBST. The primary antibody was anti-dynamin II, anti-myosin II or anti-Grb2 (Transduction Laboratory, Lexington, USA) antibody. After incubation with the horseradish peroxidase-conjugated secondary antibody (Upstate Biotechnology), the signals were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The intensities of the expressed bands were measured using NIH Scion Image software (version 1.1; Scion, Frederick, USA).

Confocal laser scanning microscopy

Double immunofluorescence staining of the NIH3T3 and NIH3T3(Ras) fibroblasts was performed with the antibodies to dynamin II and myosin II (M7648; Sigma) in order to test the hypothesis that dynamin II and myosin II interact within intact cells. Two secondary antibodies, fluorescein-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG (Biosource, Camarillo, USA), were used to distinguish these two proteins. The NIH3T3 cells were grown on glass cover slips for 48 h until the culture reached approximately 60% confluence. The NIH3T3 cells were stimulated with PDGF by rinsing the cover slips bearing the cells briefly in PBS and fixing the cells by immersing them in 4% paraformaldehyde fixative for 20 min at 37 ºC, followed by permeabilization with 0.1% Triton X-100. The cells were blocked with 1.0% bovine serum album in PBS, and incubated with anti-dynamin II or anti-myosin II diluted in PBS containing 1.0% bovine serum album as the primary antibody for 1 h at 37 ºC. After washing three times using PBS, the cover slips were incubated with the affinity isolated secondary antibody. The cover slips were washed three times with PBS and mounted in fluorescent mounting medium. The cells were observed for their epifluorescence using confocal laser scanning microscopy (TCS400; Leica, Wetzlar, Germany). The acquired images were manipulated with ScanWare 5.0 (Leica) and digitized using Adobe Photoshop software (version 7.0, Adobe Photo Systems, Mountain View, USA).

Statistical analysis

Statistical differences were determined using *t*-test or ANOVA with origin version 7.5 statistical software (Microcal Software, Northampton, USA). $P<0.05$ was considered significant.

Results and Discussion

Increased dynamin II interaction with myosin II in NIH3T3(Ras) cells

NIH3T3(Ras) cells, NIH3T3 cells overexpressing Ras protein, lose the contact inhibition characteristic and show morphological changes [23,24]. Previous studies confirmed that the Ras proteins were overexpressed in NIH3T3(Ras) cells compared with NIH3T3 cells [16]. In addition, many cell processes and spindles were observed in NIH3T3(Ras) cells using scanning electron microscopy (data not shown).

As shown in **Fig. 1**, IP-based screening of fibroblast homogenates was carried out using the antibodies to dynamin and myosin to define the components of the cytoskeleton-related protein that is associated with dynamin.

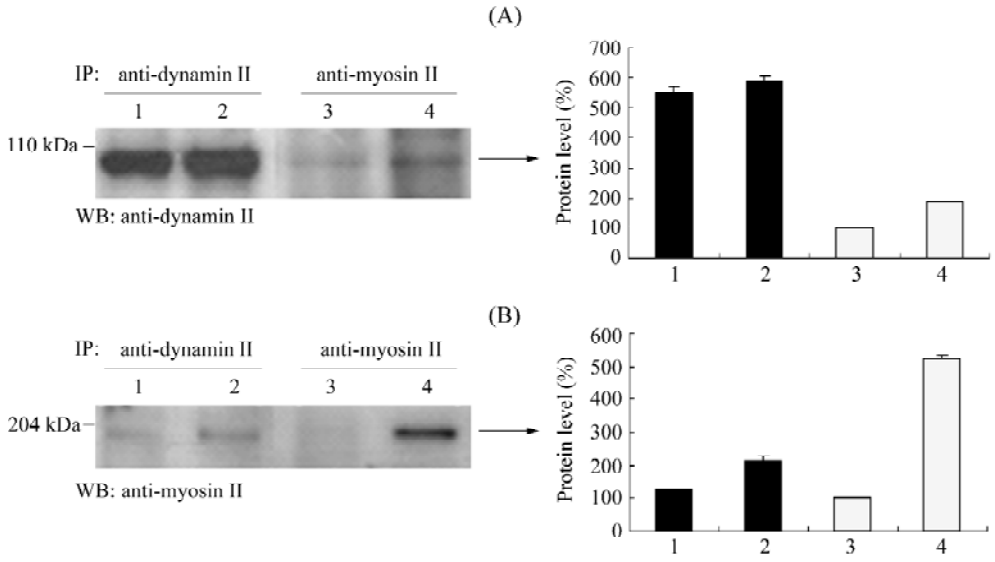

Fig. 1 Immunoprecipitation (IP) and Western blot (WB) analyses showing expression of dynamin II and myosin II in NIH3T3 and Ras transformed NIH3T3(Ras) cells

(A) Expression of dynamin II in NIH3T3 and NIH3T3(Ras) cells. (B) Expression of myosin II. Left, IP and WB results; Right, band analysis results. 1 and 3, the NIH3T3 cell lysates; 2 and 4, the NIH3T3(Ras) cell lysates. Date were mean±SD (n=4).

In particular, the dynamin II and myosin II immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis with corresponding antibody to dynamin II or myosin II. The anti-myosin antibody precipitated with a 100 kDa polypeptide that was recognized by the anti-dynamin antibody. Myosin II was also detected in the dynamin II immunoprecipitate. Interestingly, the expression level of dynamin increased in the presence of myosin in NIH3T3(Ras) cells compared with NIH3T3 cells [**Fig. 1(A)**]. In addition, the myosin expression level was also increased in the presence of dynamin in NIH3T3 cells [**Fig. 1(B)**]. Although several minor bands were observed, the pattern of the total proteins precipitated with either dynamin or myosin, when detected by Coomassie blue staining, was not different from that of the Western blot analysis (data not shown).

Dynamin contains a number of functional domains, including a GTPase domain at the amino-terminal, a pleckstrin homology (PH) domain, a coiled-coil domain and a proline-rich domain [1,25]. Among these, the PH domain is found in many intracellular signaling and cytoskeletal proteins [26,27]. In particular, it has been reported that myosin II is a binding partner to the PH domain in CHO cells [28]. Overall, it is possible that dynamin II and myosin II have a direct interaction through the PH domain of dynamin II. In addition, the higher level of dynamin II co-expression with myosin II might be accompanied with Ras overexpression in NIH3T3(Ras) cells. However, more investigations are required.

These IP-based results were confirmed by examining whether or not dynamin II is co-localized with myosin II in NIH3T3 and NIH3T3(Ras) cells. **Fig. 2** shows the confocal immunofluorescence images of cells detected with the corresponding antibody of dynamin and myosin. In normal NIH3T3 cells, when the localization of dynamin II was examined, a punctuated staining pattern was observed throughout the cell with particularly strong intensity near the nucleus and peripheral region of the cell membrane [**Fig. 2(A)**]; and a similar pattern of myosin was also observed in the NIH3T3 cells [**Fig. 2(B)**]. The intensity of these two co-localized proteins was higher in the NIH3T3(Ras) cells [**Fig. 2(D–F)**] than that in the NIH3T3 cells [**Fig. 2(A–C)**]. The confocal laser scanning microscopy analysis revealed an identical pattern to that

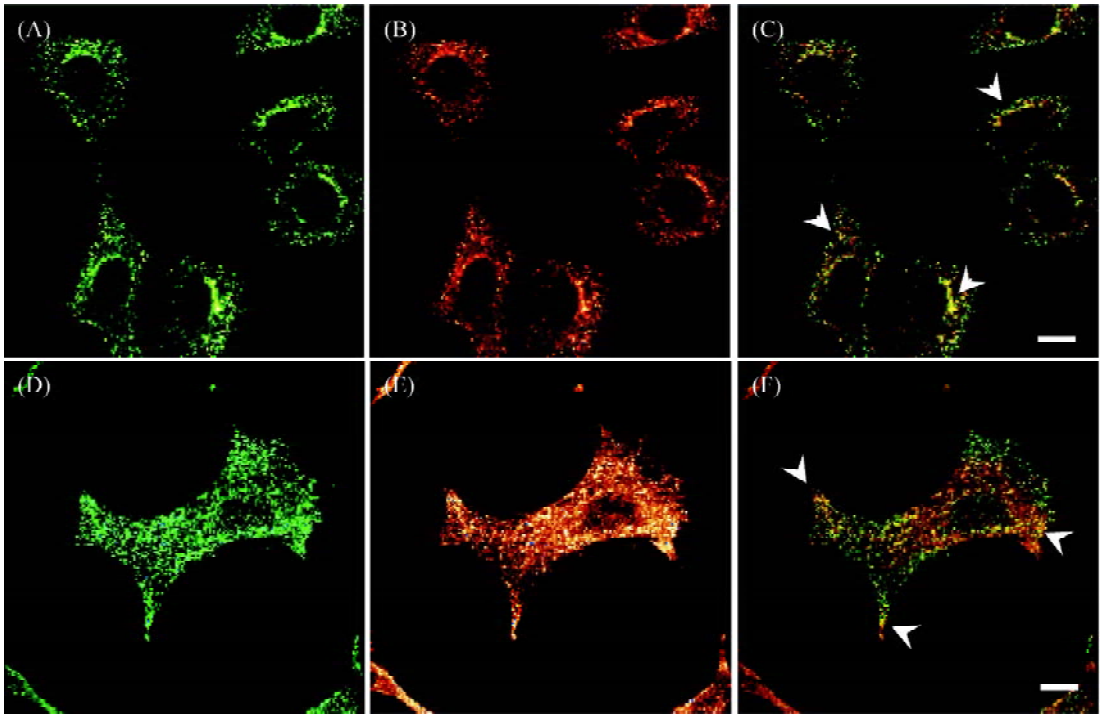

Fig. 2 Confocal images showing the co-localization of dynamin II and myosin II in the NIH3T3 and Ras transformed NIH3T3 [NIH3T3(Ras)] cell lines

The cytoplasmic distribution of dynamin II is similar to that of myosin II. The precise co-localization is shown in the cytoplasm and peripheral region of the cell membrane. The intensity of the co-localized proteins was higher in the NIH3T3(Ras) cells (D–F) than in the NIH3T3 (A–C). Bar=5 μm.

reported elsewhere (data not shown) [18]. **Fig. 2(C,F)** shows the overlapping images highlighting the co-localization of these two proteins. Except for the localization at the periphery of the nucleus, the co-localization observed near the peripheral cell membrane is believed to indicate the region of the leading lamellipodial extension [29]. The co-localization indicated by confocal microscopy is consistent with the biochemical data shown in **Fig. 1**.

Increased interaction of dynamin II with myosin II in PDGF-treated NIH3T3 cells

The NIH3T3 cells were stimulated with PDGF, which is involved in the Ras signaling pathway through the Rac molecule, to determine why increased interaction between these two proteins took place in NIH3T3(Ras) cells [29]. Immunofluorescence staining was carried out using antibodies of dynamin and myosin to determine whether dynamin II is co-localized with myosin in the PDGF-stimulated NIH3T3 cells (**Fig. 3**). In the starved cells, dynamin was localized in the cortical rim along the cell periphery and punctuated spots on the plasma membrane. These cells displayed only modest co-localization between dynamin and myosin in the cortex [**Fig. 3(A)**]. However, after stimulation with PDGF, the NIH3T3 cells assumed a polarized morphology that is characteristic of motile cells [**Fig. 3(B)**] [30,31]. The dramatic change was reflected in the distribution of dynamin and myosin staining, which became more concentrated at the ruffling edge of the cells. This rearrangement of dynamin showed the accumulation of dynamin in the peripheral region of the PDGF-treated cells [4]. IP experiments were carried out on the PDGF-stimulated NIH3T3 cells using the myosin antibody. Dynamin II was detected in Western blot using anti-dynamin II antibody in NIH3T3 cells treated with or without PDGF. The amount of dynamin II that immunoprecipitated with the anti-myosin II antibody increased (**Fig. 4**). An identical amount of myosin II was co-immunoprecipitated under these conditions (data not shown). Interestingly, the dynamin II expression level observed in the anti-myosin immunoprecipitates was increased by approximately 30% (after 30 min) compared with that of the starved NIH3T3 cells (**Fig. 4**).

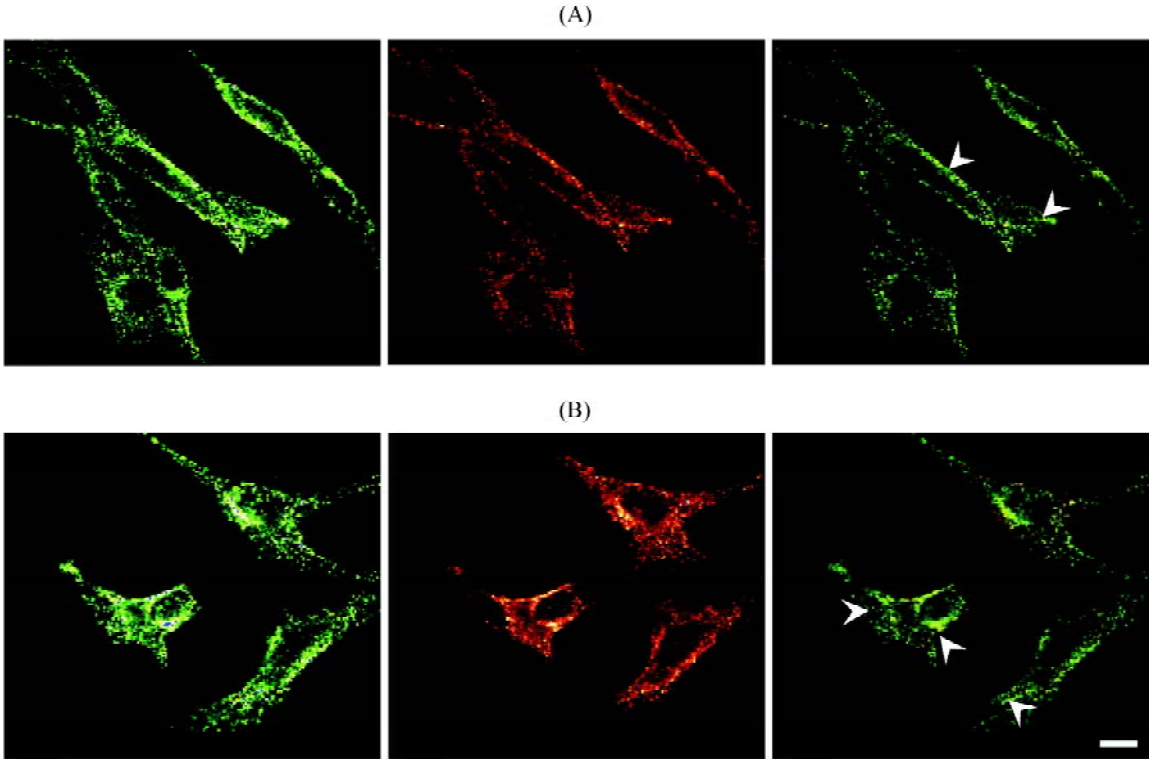

Fig. 3 Localization of dynamin II and myosin II were changed by stimulation with 30 ng/ml platelet-derived growth factor (PDGF)

Dynamin II is associated with myosin II at the cortical membrane ruffles in the PDGF-stimulated NIH3T3 cells. Upper panels, cells without PDGF treatment; lower panels, cells treated with PDGF for 30 min. Bar=5 μm.

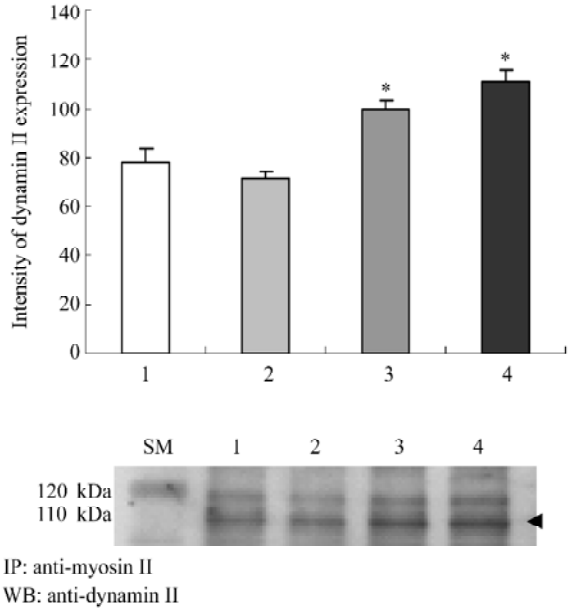

Fig. 4 Dynamin II binding to myosin II in NIH3T3 cells is increased by treatment with 30 ng/ml platelet-derived growth factor (PDGF)

The histogram shows the intensity of the dynamin II expressed on the PDGF-stimulated NIH3T3 cells. Data were represented as mean±standard deviation for four separate cell preparations. **P*<0.05 versus group 2. Western blot (WB) analysis of the anti-myosin immunoprecipitates with the dynamin antibody showed an increasing pattern as a result of PDGF stimulation. 1, cells without PDGF treatment; 2, cells treated with PDGF for 5 min; 3, cells treated with PDGF for 10 min; 4, cells treated with PDGF for 30 min; SM, size marker. IP, immunoprecipitation.

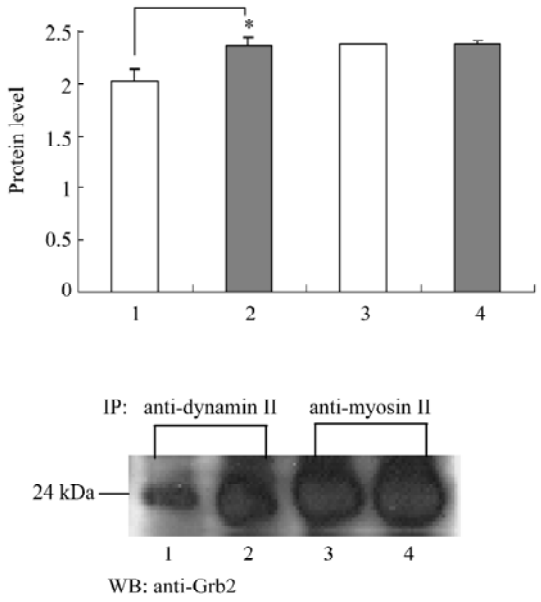

Fig. 5 Western blot (WB) of Grb2, an essential element of the Ras signaling pathway, after immunoprecipitation (IP) of dynamin II and myosin II antibodies in NIH3T3 and Ras transformed NIH3T3 [NIH3T3(Ras)] cell lines

The histogram shows the intensity of Grb2 expression on dynamin II and myosin II. Data were represented as mean±standard deviation of four separate cell preparations. **P*<0.05 versus group 1. 1 and 3, NIH3T3 cells; 2 and 4, NIH3T3(Ras) cells.

Grb2 is essential for multiple cellular functions, but is most well known for its ability to link the epidermal growth factor receptor tyrosine kinase to the activation of Ras and its downstream kinases, extracellular regulated kinase 1 and 2 [32]. The Ras proteins belong to the large Ras superfamily of monomeric GTPase, which contains two other subfamilies, Rho and Rac proteins. The Rho family is involved in relaying signals from the cell surface receptors to the actin cytoskeleton, and the Rab family is involved in regulating the traffic of intracellular transport vesicles [33]. The activation of the PDGF receptor induces Shc expression and forms a complex with increased Grb2 expression [34]. IP was carried out prior to Western blot analysis of Grb2, in order to examine the binding of dynamin to myosin occurring around Grb2 in the signal transduction pathways. These results showed that Grb2 is largely expressed with dynamin II in NIH3T3(Ras) compared with NIH3T3 cells (**Fig. 5**), which is in agreement with previous results [16]. There was no significant difference between NIH3T3 and NIH3T3(Ras) cells when the anti-myosin antibody was used by IP. However, the amount of Grb2 binding to myosin was similar to that of anti-dynamin IP in the NIH3T3(Ras) cells (**Fig. 5**). Overall, these results suggest that dynamin II is associated with myosin II as a signaling molecule involved in cell migration within the Ras-Grb2 signaling pathway.

In summary, the domains of dynamin are known to be important for membrane localization. However, its function in cell migration including actin-related proteins has not been reported. Myosin II is possibly a new binding partner of dynamin II. The *in vivo* binding of dynamin II and myosin II was confirmed in NIH3T3 cells using confocal microscopy. Exposing the NIH3T3 cells to PDGF induced a change in the co-localization of dynamin and myosin from the peripheral region of the nucleus to the ruffled lamellapodial extension, which induced cell migration and actin cytoskeleton formation. A molecular connection of Grb2 was found between dynamin II and myosin II, suggesting that dynamin II might act as an intermediate messenger in the Ras signaling transduction pathway, leading to membrane ruffling and cell migration. However, future studies will be needed to determine the relationship between FAK and PI3K in PDGF signaling in order to

identify the interactions between actin and myosin, and the relationship between dynamin II and myosin inhibitors.

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