Article is available online at http://www.webio.hu/por/2003/9/2/0083

ARTICLE

Overexpression of ROCK in Human Breast Cancer Cells: Evidence that ROCK Activity Mediates Intracellular Membrane Traffic of Lysosomes

Yukio NISHIMURA,[1] Kazuyuki ITOH,[2] Kiyoko YOSHIOKA,[2] Kazuo TOKUDA,[1*] Masaru HIMENO[1]

[1]Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan,
[2]Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

Small GTPase Rho and its downstream effectors, ROCK family of Rho-associated serine-threonine kinases, are thought to participate in cell morphology, motility, and tumor progression through regulating the rearrangement of actin cytoskeleton. Here we present evidence that transfection of human breast cancer cells with cDNA encoding a dominant active mutant of ROCK causes dispersal of lysosomal vesicles throughout the cytoplasm without perturbing the machinery of the endocytic pathway. The intracellular distribution of lysosomes and endocytosed transferrin, an early endosomal marker, were further assessed by confocal immunofluorescence microscopy. In the active ROCK transfected cells the lysosomal proteins, cathepsin D, LIMPII, and LAMP1, were found throughout the cytoplasm in dispersed small vesicles, which were not accessible to the endocytosed Texas Red-labeled transferrin. 3D-image analysis of lysosomal distribution in the active ROCK-transfectants revealed abundant punctate signals in the peripheral region of the basal plasma membrane. Cells expressing vector alone did not exhibit these alterations. Wortmannin, a phosphatidylinositol 3-kinase inhibitor, induced LIMPII-positive/transferrin negative large vacuoles in the perinuclear region, and disappearence of the dispersed small vesicular structures. To our knowledge, this is the first evidence that increasing ROCK expression contributes to selective cellular dispersion of lysosomes in invasive breast cancer cells. (Pathology Oncology Research Vol 9, No 2, 83–95, 2003)

Keywords: ROCK; RhoA; Cathepsin; D; LIMPII; lysosomes; endosomes; cytoskeleton

Introduction

It is known that members of the small GTPases family control cell adhesion and motility through reorganization of the actin cytoskeleton and regulation of acto-myosin contractility.[1-6] It has been reported that small GTPase RhoA[7] is expressed at a relatively higher level in metastatic tumors and its expression levels positively correlate with the stage of the tumors,[8] and that the overexpression of active RhoA in the cell facilitates its translocation from the cytosol to the plasma membrane, where its activation results in stimulation of the actin-myosin system followed by cellular invasion both *in vitro* and *in vivo.*[9] One of the target molecules of Rho is the family of Rho-associated serine-threonine protein kinases (ROCK)[10] which also participates in cell-to-substrate adhesions, stress-fiber formation, and stimulation of actomyosin-based cellular contractility, and it has been demonstrated that ROCK, like Rho, is involved in tumor invasion.[11] In invasive tumor cells, an increased expression of lysosomal cathepsins and an altered redistribution of lysosomes toward plasma membrane have been reported for a variety of tumors.[12,13] The secretion from and/or plasma membrane association of cathepsins in tumor cells has been implicated in tumor invasion and metastasis.[14,15] We reported previously that the transfection of mutated small GTPase c-Ha-*ras* oncogene into human breast epithelial cell line MCF-10A, known to increases its invasiveness[16] and metastatic ability,[17] increased a more peripheral distribution of lysosomal pro-

Received: May 26, 2003; *accepted:* June 15, 2003
Correspondence: Yukio NISHIMURA, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan, e-mail: nishimur@bioc.phar.kyushu-u.ac.jp, Tel & Fax: +81-92-642-6617
**Present address:* Fuji Gotemba Laboratories, Chugai Pharmaceutical Co, Ltd, Gotemba, Shizuoka, Japan

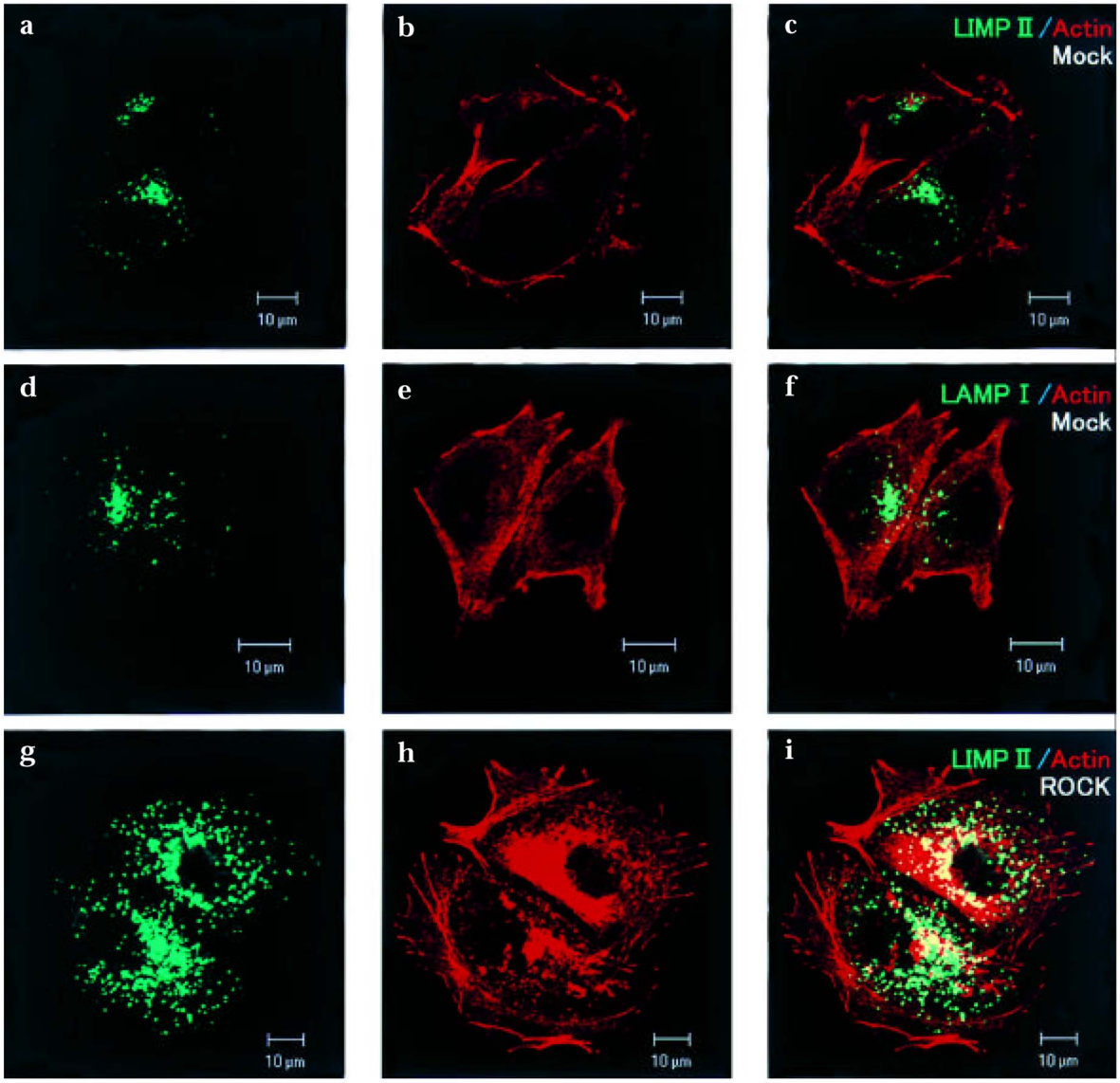
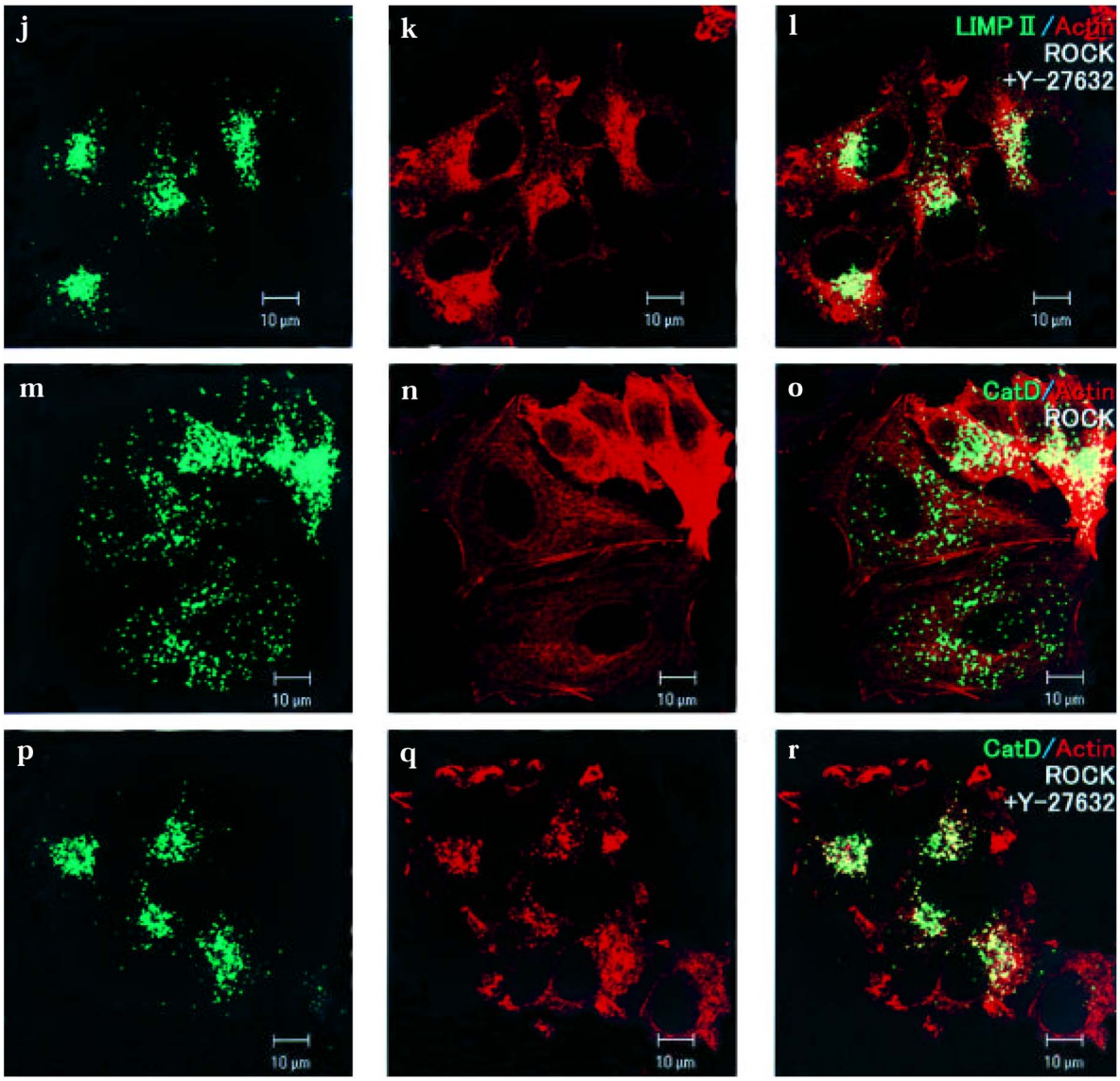


Figure 1. *Immunocytochemical localization of lysosomal proteins and actins in the mock transfectants or the active ROCK transfectants. The mock transfectants (a-f), the active ROCK transfectants (d-r), or the active ROCK transfectants incubated with Y-27632 (j-l, p-r), a selective ROCK inhibitor, for 30 min, were fixed, and were then double-labeled to localize LIMPII, LAMP1 or cathepsin D (green fluorescence) with specific antibodies and actin with Texas red-phalloidin (red fluorescence), respectively, as described in Materials and Methods. The secondary antibodies were Alexa 488-conjugated anti-rabbit IgG or FITC-conjugated anti-mouse IgG. Superimposed images of lysosomal proteins and filamentous actin are shown in c, f, i, l, o, and r. Note that in the mock transfected cells, the lysosomal proteins are mainly localized around the perinuclear region. By contrast, in the active ROCK transfectants, the dispersed cellular localization of lysosomal proteins are observed. Furthermore, the induced actin stress-fiber formation is clearly seen in the active ROCK transfectants. In the presence of Y-27632, the actin stress-fibers in the active ROCK transfectants are considerably reduced, and this inhibitor substantially disrupts the actin stress-fiber network within 30 min (h). Bar, 10 μm*

teins, cathepsin D and LAMP1, compared to the parental cells.[18] Conceivably, these abnormalities of the intracellular localization of lysosomes may reflect an important process for tumor invasion.

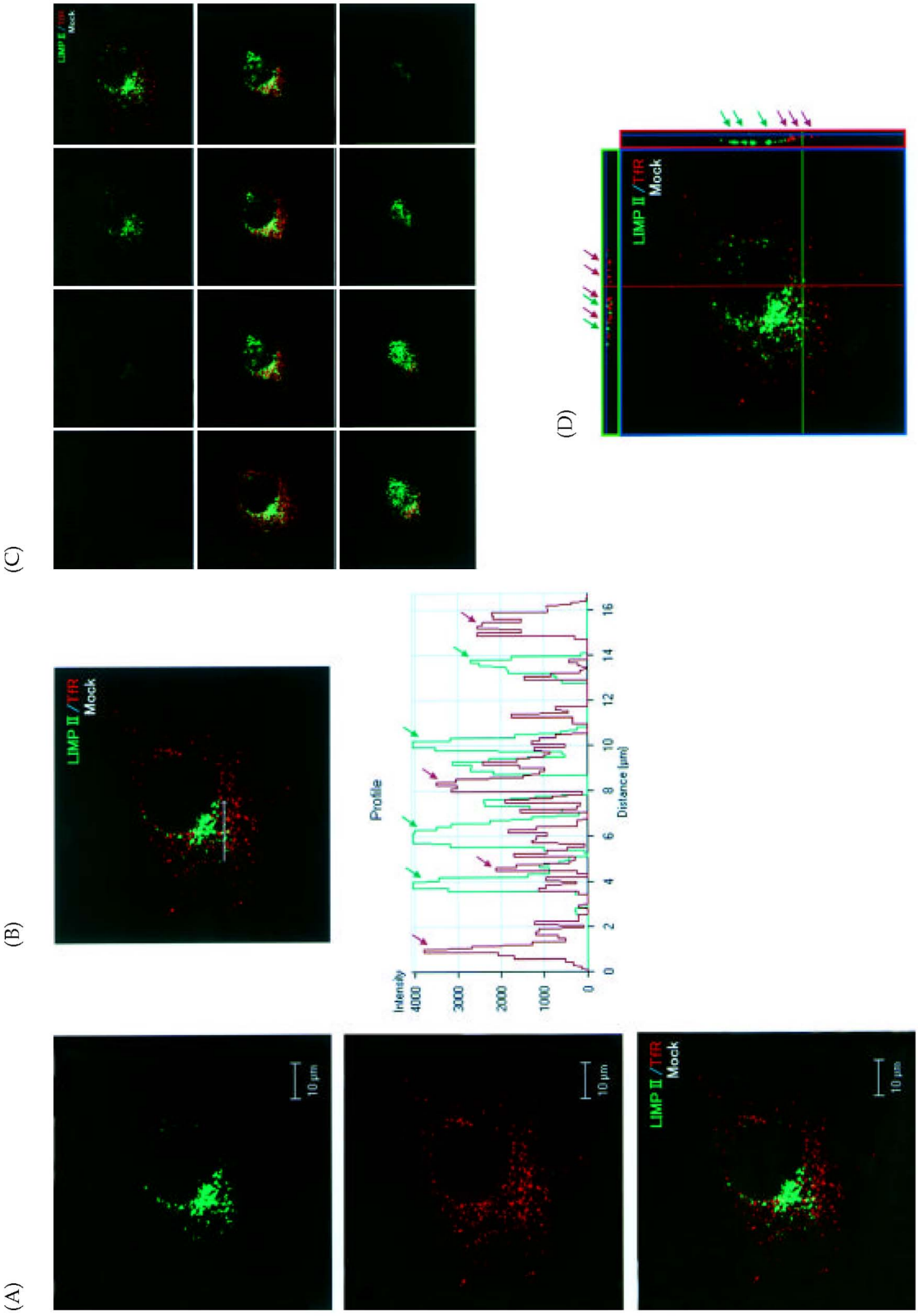
We have recently reported that the dominant active RhoA mutant (VRhoA) or the wild type RhoA (wtRhoA) transfected to MM1 rat hepatoma cell line, causes the centrifugal distribution of lysosomes toward cell periphery.[19] This alteration



in the subcellular translocation of lysosomes was found to be impeded by the presence of Y-27632,[20] a selective inhibitor of Rho-associated protein kinase, p160ROCK which has been demonstrated to be a downstream target of RhoA.[10,21-24] Further analysis revealed that the dispersed lysosomes retain their acidity and they are not co-localized with early endosomes, and are not accessible to the endocytosed anti-transferrin receptor antibody.[25] Thereby, we postulate that the small GTPase RhoA and ROCK signaling selectively regulates the membrane traffic and biogenesis of lysosomes in the endocytic pathway in invasive tumor cells.[25]

In the present study, in order to further investigate the role of ROCK on the intracellular membrane traffic of lysosomes in tumor cells, we analyzed the intracellular localization of lysosomal proteins by confocal immunofluorescence microscopy in the dominant active ROCK transfected MCF-7 human breast cancer cell line. We examined the intracellular distribution of lysosomes by using specific antibodies to lysosomal aspartic proteinase cathepsin D or LIMPII/LGP85, which is distributed within the endocytic organelles at the highest concentration in lysosomes/late endosomes as observed for the other lysosomal glycoproteins, LAMP-1 and LAMP-2.[26-31]

Here we found evidence that the active ROCK mutant transfected into MCF-7 cells causes the centrifugal distribution of lysosomes. We demonstrate that these dispersed lysosomes are not co-localized with early endosomes and are not accessible to the endocytic tracer, Texas Red-transferrin. To


(A)
10 μm
10 μm
LIMP II / TfR
Mock
10 μm
(B)
LIMP II / TfR
Mock
Profile
Intensity
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
Distance (μm)
(C)
(D)
LIMP II / TfR
Mock

date, there is no evidence demonstrating the mechanism by which intracellular movement and trafficking of lysosomes is altered in the active ROCK-transfected cells. We postulate that the small GTPase RhoA and ROCK activity selectively regulates the membrane traffic and biogenesis of lysosomes in the endocytic pathway in invasive cancer cells.

Materials and Methods

Cell cultures and transfection of catalytic domain of human ROCK (Δ4ROCK)

MCF-7 human breast cancer cells (JCRB0134)[32] were obtained from Health Science Research Resources Bank (Osaka, Japan) and cultured in minimum essential medium (MEM) (Invitrogen, San Diego, CA, USA) with non-essential amino acids (Invitrogen), 0.1 mM Na pyruvate (Invitrogen), 10 μg/ml insulin (Invitrogen) and 10% fetal calf serum (FCS, Equitech-bio, TX, USA). cDNA encoding catalytic domain of human ROCK (Δ4ROCK),[11] was subcloned into FLAG-tagged pcDNA3 vector (Invitrogen). Stable cell lines were generated by transfection of constructs or empty vector using Lipofectamine Plus reagent (Invitrogen), and selected in the presence of 800 μg/ml G418 for 4 weeks.

Antibodies

Normal goat serum were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Alexa 488-labeled affinity-purified goat anti-rabbit IgG and FITC-labeled affinity-purified goat anti-mouse IgG were purchased from Molecular Probes (Eugene, OR, USA). A monoclonal anti-human lysosome-associated membrane protein-1 (LAMP-1) H4A3 was purchased from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD, USA), and the Department of Biological Sciences, University of Iowa (Iowa City, IA, USA), under contract NO1-HD-2-3144 from the National Institute of Child Health and Human Development. Texas Red-conjugated phalloidin, and SlowFade antifade reagent were purchased from Molecular Probes (Eugene, OR, USA). Antisera were raised in rabbits (New Zealand white male) against the mature form of rat liver lysosomal cathepsin D[33,34] and the native form of LIMPII/LGP85[29] as described previously, and monospecific cathepsin D IgG or anti-LIMPII IgG was prepared, respectively. An IgG fraction was affinity-purified by protein A Sepharose CL-4B, followed by immunoaffinity chromatography using antigen-Sepharose 4B. Wortmannin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of reagent grade and were obtained from commercial sources.

Immunofluorescence microscopy

Immunofluorescence microscopy has been described previously.[19,25] Cells were grown for 3 days in 6-well plates on glass coverslips in M-MEM-10% FCS. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, permeabilized in PBS containing 0.1% saponin. After washing with PBS, cells were blocked with PBS-2 mg/ml bovine serum albumin. All subsequent antibody and wash solutions contained 0.1% saponin. The mock transfectants or the active ROCK transfectants were then incubated with specific primary antibodies (rabbit anti-cathepsin D IgG, rabbit anti-LIMPII IgG, or mouse anti-LAMP-1 IgG1, mouse anti-transferrin recepor IgG2, respectively), for 2 hours and washed. Then cells were incubated for 1 hour with secondary antibody of Alexa 488-conjugated affinity-purified goat anti-rabbit IgG, or Texas Red-conjugated affinity-purified goat anti-mouse IgG at 20 μg/ml, followed by three washes in PBS containing 0.1 % saponin. In some experiments, the active ROCK transfectants were treated with 15 M of Y-27632, ROCK inhibitor, for 30 min, before cell fixation. To label early endosomes in the wortmannin-treated cells, the active ROCK transfected cells were treated with 100 nM

Figure 2. *Intracellular distribution of lysosomal membrane glycoprotein LIMPII in the mock-transfected MCF-7 cells. The mock transfectants (MCF-7 cells transfected with vector alone) were fixed, and double-labeled to localize LIMPII (green fluorescence) and transferrin receptor (red fluorescence) with specific antibodies, respectively, as described in Materials and methods. The secondary antibodies were Alexa 488-conjugated anti-rabbit IgG or Texas Red-conjugated anti-mouse IgG. Superimposed images of LIMPII and transferrin receptor are shown in c of panel A, B, C, and D. Profile analysis of the intensity of each fluorescence channel, Alexa 488 and Texas Red, along with white horizontal arrow is shown. The green arrows indicate the examples of LIMP-II-positive structures (green fluorescence) but are negative for transferrin receptor-positive structures (red fluorescence). The red arrows indicate structures that are positive for transferrin receptor but are negative for LIMPII. Optical sections acquired along the Z-axis were acquired with increments of 0.3 μm as described in Materials and Methods. Each X-Y optical section (0.3 μm) is shown in Panel C and digits in the upper left corner of each panel indicate the distance of the sections to the cell bottom. Orthographic section (1.04 μm from the cell bottom) of the cell is shown in panel D and each cellular localization of Alexa 488-positive LIMPII and Texas Red-positive transferrin receptor is indicated at the top horizontal window of panel D for X-Z section (green horizontal line) and at the right vertical window of panel D for Y-Z section (red horizontal line), respectively. Note that LIMPII stainings (green fluorescence) are concentrated in small punctate structures around the perinuclear region, and are clearly distinct from those of transferrin receptor. Bar, 10 μm.*

wortmannin for 30 min at 37°C and then incubation was continued for 20 min in the plain culture medium containing Texas Red-conjugated transferrin and wortmannin. The cells were fixed and stained for LIMPII or cathepsin D with primary antibody and secondary antibody conjugated to Alexa 488. In controls, either preimmune serum (rabbit or mouse) was substituted for the primary antibody or no primary antibody was used. Finally, cells were mounted with SlowFade anti-fade reagent and observed on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany), equipped with krypton/argon laser sources. To obtain a 3D-imaging data sets or to generate optical sections of the cells by confocal laser scannning microscopy, 30-40 optical sections of (X-Y section) along the Z-axis were acquired with increments of 0.3 μm using 63×oil immersion objective (NA 1.4), and the fluorescent data sets were analyzed by LSM 510 software. For the simultaneous observation of double-immunolabeled cells by Alexa 488 or Texas Red, both 488- and 568-nm laser lights were used for excitation at the same time, and the single-image of the immunolabeled specimen was observed. In these cases, cross-talk of the emission signals was verified by comparing separately obtained Alexa 488 and Texas Red images in each experiment in advance, especially regarding the observation of double-positive sites. Images were converted to TIFF format, and contrast levels of the images were adjusted using Adobe Photoshop 5.5 software (Adobe Co.) on PowerMac G4 Cube (Apple Computers).

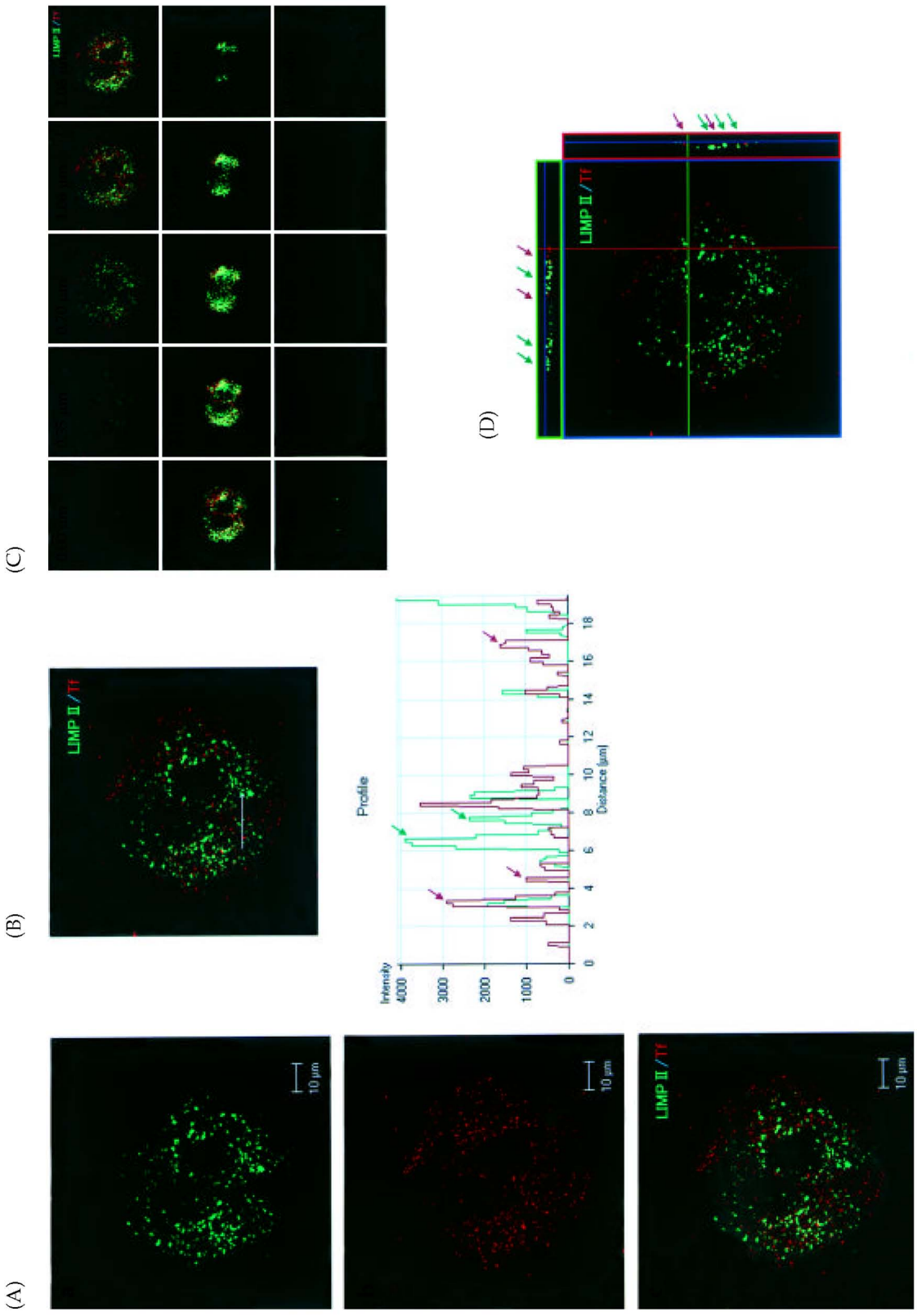
Results

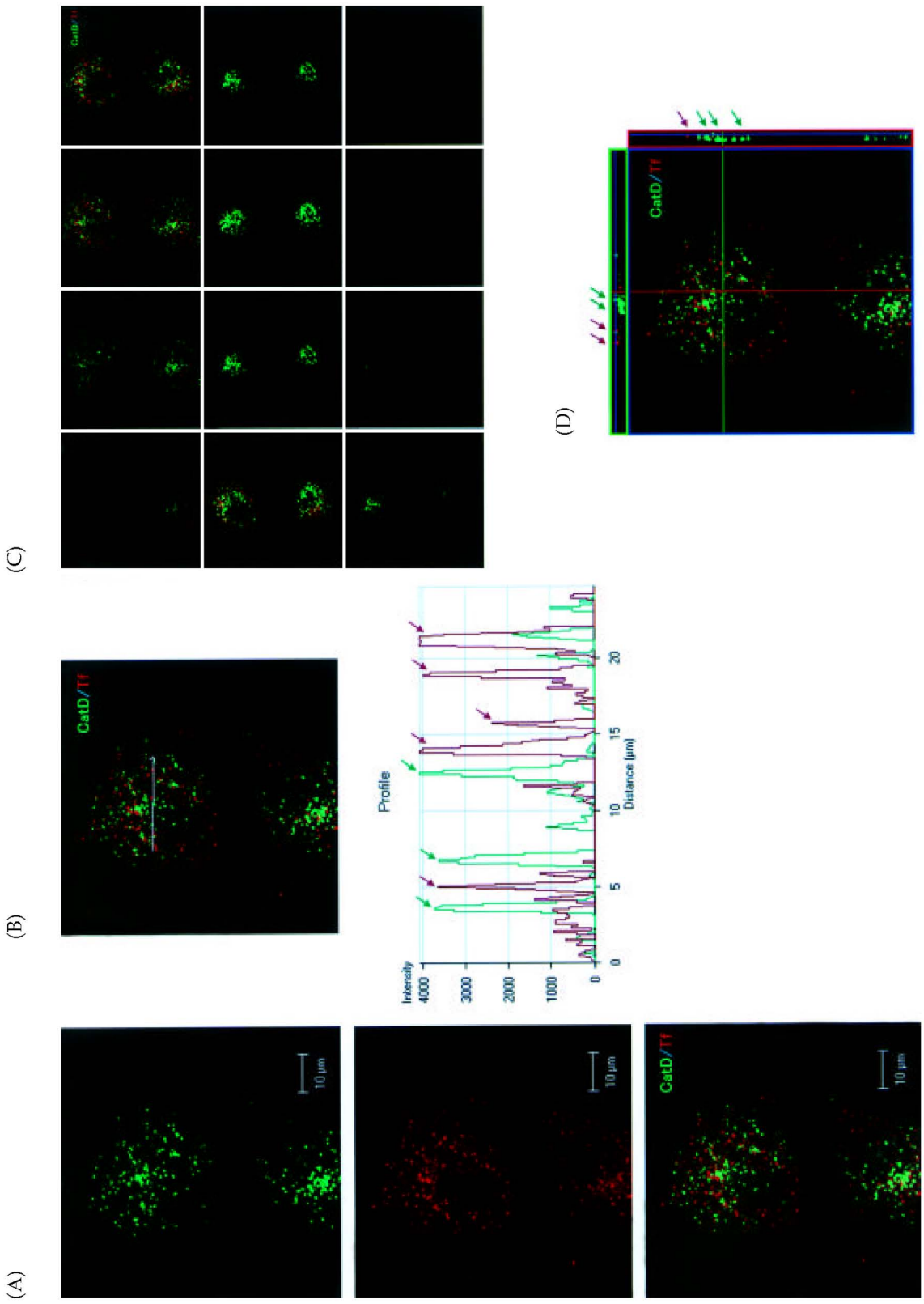
Expression of Δ4ROCK, a constitutively active truncation mutant of p160ROCK, induces strong stress-fiber formation and a radial dispersion of lysosomes in breast cancer cells

It has been reported that transfection of rat hepatoma cells with cDNA encoding a dominant active mutant of ROCK conferred invasive activity, independently of serum and Rho. In contrast, expression of a dominant negative, kinase-defective ROCK mutant substantially attenuated the invasive phenotype, therefore, indicating that ROCK plays an important role in tumor cell invasion.[11] To investigate the role of ROCK activity in the intracellular membrane traffic of lysosomes, we have undertaken immunofluorescence microscopy to determine whether ROCK activity is involved in the regulation of lysosomal biogenesis. ROCK cDNA encoding a truncation mutant of p160ROCK, the constitutively active Δ4ROCK, containing the N-terminal kinase domain and a portion of the coiled-coil region was transfected into MCF-7 human breast cancer cell line, and stable transfectants expressing ROCK were isolated. To determine the intracellular localization of cathepsin D, LIMPII, or LAMPI and actin cytoskeleton, the transfected cells were double-labeled with Texas Red labeled-phalloidin for actin cytoskeleton and stained for lysosomes with cathepsin D, LIMPII or LAMP1 (***Figure 1***). In the mock-transfected cells there is no distinct formation of stress-fibers, and the stainings of LIMPII and LAMPI were present primarily in small punctate structures in the perinuclear region, which is typical pattern of lysosomal vesicles.[18,19,25] In contrast, in the cancer cells expressing fΔ4ROCKcDNA, the cells had elongated polar cell shape with pointed edges and ROCK expression markedly enhanced the formation of stress-fibers, and these stress-fibers terminated at pointed edges. These observations suggested that ROCK is important in regulating actin cytoskeleton reorganization. Furthermore, confocal immunofluorescence microscopy revealed that cathepsin D- and LIMPII-positive small punctate structures were widely redistributed throughout the cytoplasm in transfected cells. The dispersed punctate structures stained for LIMPII were almost co-localized with those for LAMP1 (data not shown). In the presence of Y-27632, a selective inhibitor of p160ROCK, the cellular redistribution of lysosomes was impeded, the inhibitor promoted reclustering of lysosomes toward the perinuclear region. These results are consistent with those reported previously.[19,25]

Next, to determine intracellular distribution of lysosomes, 3D-images of the mock-transfected MCF-7 cells were cap-

Figures 3 and 4. *Endocytosed Texas Red-transferrin downloaded into the early endosomes is not delivered to the dispersed LIMPII- or cathepsin D-positive compartments in the active ROCK transfectants. The active ROCK transfectants were preincubated for 20 min with the Texas Red-transferrin in plain complete medium, and then cells were fixed, and stained with antibodies against LIMPII (green fluorescence in Figure 3) or cathepsin D (green fluorescence in Figure 4). Merged fluorescence signals for LIMPII or cathepsin D and the internalized Texas Red-transferrin are shown in c of panel A, B, C, and D. Orthographic sections (0.7 μm for LIMPII and cathepsin D) are shown by green horizontal line for X-Z section and red vertical line for Y-Z section in panel D, respectively, and cellular localization of Alexa 488-positive LIMPII or cathepsin D and Texas Red-transferrin is indicated at the top horizontal window of panel D for X-Z section and at the right vertical window of panel D for Y-Z section. Details for the figure are described in Figure 2. Note that the internalized Texas Red-transferrin is able to reach the early endosomes seen as small punctate structures in the cell periphery after 20 min incubation, but transferrin-positive structures are not co-localized with LIMPII-positive or cathepsin D-positive structures, suggesting that the dispersed lysosomes do not fuse with early endosomes in the active ROCK transfectants. Bar, 10 μm*


(A)
10 μm
10 μm
LIMP II / Tf
10 μm
(B)
LIMP II / Tf
Profile
Intensity
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
18
Distance (μm)
(C)
(D)
LIMP II / Tf


(A)
10 μm
10 μm
CatD/Tf
10 μm
(B)
CatD/Tf
Profile
Intensity
4000
3000
2000
1000
0
0
5
10
15
20
Distance (μm)
(C)
(D)
CatD/Tf

tured by confocal immunofluorescence microscopy in the paraformaldehyde-fixed cells. 30-40 optical sections along the Z-axis were acquired with increments of 0.3 μm. As shown in *Figure 2,* in the mock-transfectants, LIMPII-positive lysosomal small punctate structures were mainly clustered in the perinuclear region which is approximately in the middle region (1.04 m through 3 μm) of the cell, and the localization of lysosomes was distinct from that of transferrin receptor-reactive early endosomes. A distinct localization of LIMPII-positive small punctate structures from that of transferrin receptor was further confirmed by the profile of the intensity measurement of each fluorescence channel within the cell as shown in *Figure 2B.* Also we observed a similar distribution of cathepsin D-positive structures within the cytoplasm of the mock-transfectants (data not shown).

Endocytosed transferrin is not accessible to the dispersed lysosomes in the active ROCK transfectants

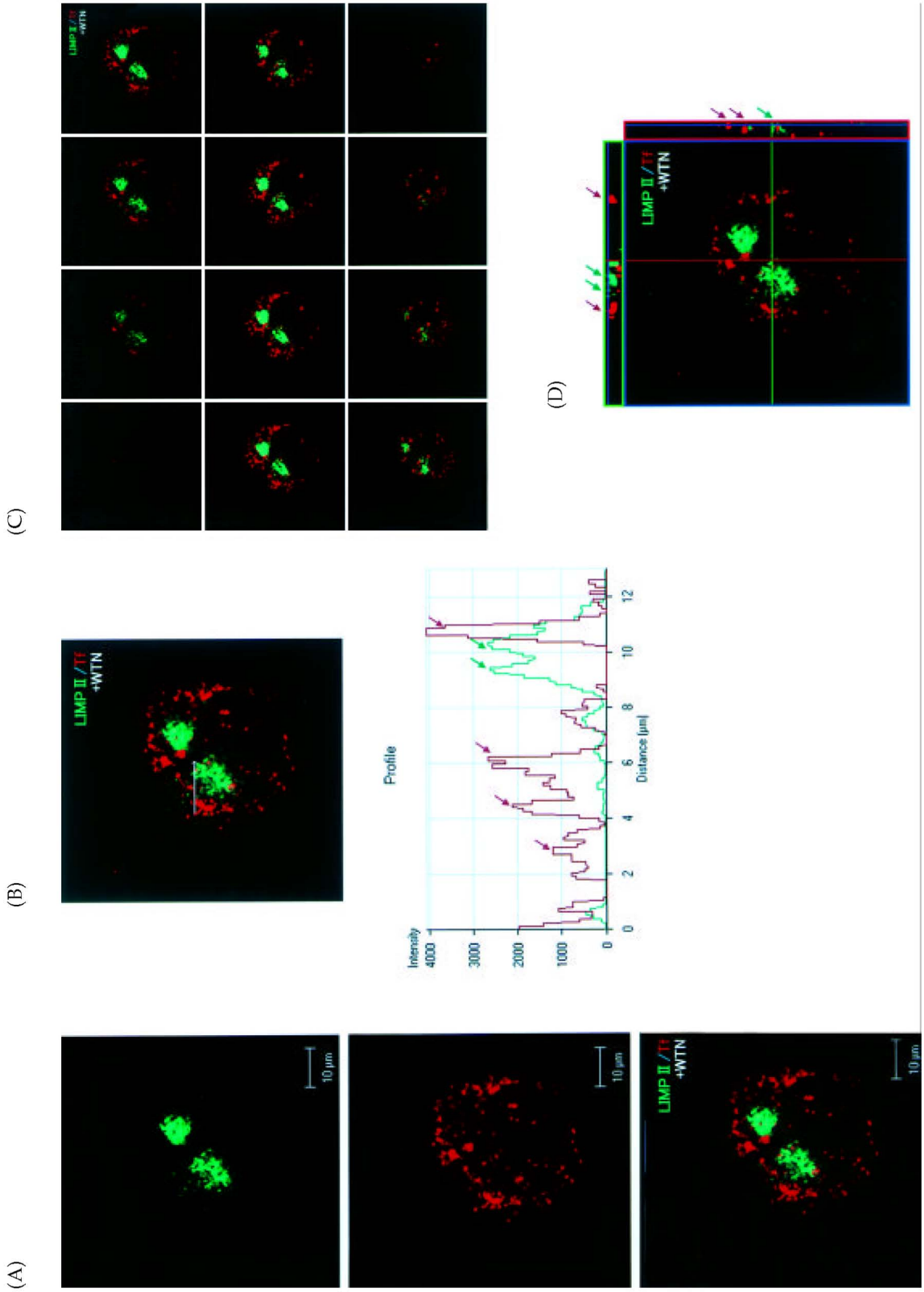
We further examined the cellular distribution of lysosomes stained for LIMPII or cathepsin D in the active ROCK-transfected cells. The results revealed a dispersed punctate distribution of LIMPII-positive vesicular structures throughout much of the cytoplasm. Also, 3D-image analysis of the active ROCK-transfected cells revealed dispersion of lysosomal vesicles toward basal plasma membrane and an abundant and peripheral/subplasmalemmal punctate signals (data not shown). Because the dispersed small vesicles were punctate, round in shape and had a peripheral distribution, we supposed that the dispersed vesicles may be of endocytic origin such as early endosomes, recycling endosomes, late endosomes or lysosomes, the content of which are transported both *in vivo* and *in vitro* in a microtubule-dependent fashion.[35] To examine the identity of LIMPII-immunoreactive dispersed vesicular structures seen throughout the cytoplasm in the active ROCK transfectants, the active ROCK-transfected cells were examined by double labeling immunofluorescence confocal microscopy using antibodies for LIMPII, and the transferrin receptor, or EEA1 (early endosomal antigen).[36] We found that the dispersed LIMPII containing vesicular structures show no co-localization with immunostainings of transferrin receptor or EEA1 in the active ROCK transfectants (data not shown).

In the present study, in order to further substantiate whether ROCK-induced dispersed lysosomes are accessible to endocytic markers, the active ROCK-transfected cells were allowed to internalize Texas Red-labeled transferrin for 20 min and the cells were fixed, and were stained for LIMPII (*Figure 3*) or cathepsin D (*Figure 4*) with primary antibody and secondary antibody conjugated to Alexa 488. Transferrin is bound to its receptor at the cell surface and then internalized to the sorting early endosomes in the peripheral region, and to the recycling endosomes in the perinuclear region, and receptor-ligand complexes are recycle back to the cell surface. As shown in *Figures 3* and *4,* Texas Red-transferrin was efficiently endocytosed and was found in early endosomes in the peripheral region. After 20 min incubation, part of the transferrin was also seen in the recycling endosomes in the perinuclear region, suggesting that active ROCK expression does not have any inhibitory effect on intracellular endocytic trafficking. LIMPII- or cathepsin D-positive lysosomes were not co-localized with early endosomes labeled with the endocytosed Texas Red-transferrin throughout the cytoplasm. The spatial relationship within the cell between the distribution of LIMPII- or cathepsin D-positive lysosomes and Texas red-labeled transferrin in the active ROCK transfectants was further assessed by confocal immunofluorescence microscopy. The optical X-Y sections acquired along the Z-axis showed that lysosomes stained for LIMPII or cathepsin D were found in the bottom part of the cell (0.35-0.7 μm), and the endosomes labeled with Texas Red-transferrin in the peripheral region were also seen in the same area, but the staining of dispersed lysosomes was distinct from those of Texas Red-transferrin-positive early endosomes (*Figures 3 and 4*). These results indicate that lysosomes redistributed toward the cell surface were not communicating with the endocytic pathway and the fusion between the dispersed lysosomes and early endosomes does not take place in the active ROCK-transfected cells.

Large swollen vacuoles induced by wortmannin are distinct from early endosomes in the active ROCK transfectants

We showed recently that wortmannin, an inhibitor of phosphatidylinositol 3-kinase, which is known to mediate membrane trafficking at several distinct steps of the early endocytic pathway,[37-40] caused induction of large swollen vacuoles in the cytoplasm of the active RhoA transfectants which were positive for LIMPII or cathepsin D but were not stained with transferrin receptor antibody, suggesting that large vacuoles were derived from lysosomes/late endosomes.[25] These results further imply that wortmannin selectively enhances enlargement of lysosomes/late endosomes, but does not cause the mixing of early endosomes and lysosomes/late endosomes. To further document whether the expression of active mutant of ROCK causes a dispersion of lysosomal LIMPII upstream to early endocytic compartment, we examined the effect of wortmannin on the membrane traffic of LIMPII- or cathepsin D-positive vesicles in the active ROCK-transfected cells. We found that in the active ROCK-transfected cells wortmannin induced a dramatic change of LIMPII-containing structures, and that

Figure 4. *Legend see on the bottom of page 88.*


(A)
10 μm
10 μm
LIMP II / TF
+WTN
10 μm
(B)
LIMP II / TF
+WTN
Profile
Intensity
4000
3000
2000
1000
0
0
2
4
6
8
10
12
Distance (μm)
(C)
(D)
LIMP II / TF
+WTN

LIMPII-positive swelling of large vacuoles in the vicinity of perinuclear region. In the meantime, the dispersed small punctate LIMPII$^+$ structures disappeared from the cytoplasm (data not shown). Wortmannin perturbed the morphology of transferrin receptor-positive early endosomes too and increased swelling of the vacuoles in the cytoplasm, however, these transferrin receptor-positive swollen early endosomes were not co-localized with those of LIMPII-containing vacuoles, indicating that a mix of LIMPII-positive large swollen vacuoles with transferrin receptor-positive large vacuoles does not take place in the active ROCK transfectants. These results are consistent with our recent data on the active RhoA transfectants,[25] namely that LIMPII-positive lysosomes are selectively dispersed in the cytoplasm of cells expressing active RhoA mutant and are not communicating with the early endocytic compartments.

In the present study, to further substantiate whether the dispersed lysosomes are accessible to the early endosomes, the effect of wortmannin was examined on the redistribution of lysosomes. The active ROCK-transfected cells were treated with 100 nM wortmannin for 30 min and then the cells were fed with Texas Red-transferrin for 20 min and the cells were fixed, and were stained for LIMPII. As shown in ***Figure 5***, wortmannin treatment increased the enlarged swollen vacuoles in the perinuclear region of cytoplasm, while the LIMPII-positive cytoplasmic lysosomes disappeared. Furthermore, all LIMPII staining was localized in these swollen vacuoles. However, these LIMPII-positive swollen vacuoles were devoid of staining by Texas Red-transferrin. Similarly, the cathepsin D-positive small vesicles disappeared from the cytoplasm and were also reclustered toward the perinuclear region (data not shown), whereas it remains to be seen whether the cathepsin D-positive vesicles reclustered to the perinuclear region are fused with the LIMPII-positive swollen vacuoles in the wortmannnin-treated cells. Analysis of the optical slices (X-Y sections) throughout the Z-axis further demonstrated that LIMPII-positive large swollen vacuoles accumulated in the perinuclear region, and that these were not positive for endocytosed transferrin. Taken together, these results confirm that small punctate vesicles immunostained for LIMPII or cathepsin D dispersed throughout the cytoplasm are truly lysosomes, thereby, implying that Rho-ROCK signaling induces the selective dispersion of lysosomes in the cytoplasm but the dispersed lysosomes are not communicating with the early endocytic compartments.

Discussion

To investigate the role of ROCK on intracellular membrane trafficking of lysosomes/endosomes, we investigated by confocal immunofluorescence microscopy the cellular distribution of lysosomal proteins and of endocytic tracer in the active ROCK transfected cells. Our novel findings of the present study are that the transfection of a dominant active ROCK in MCF-7 human breast cancer cell line, leads to a dramatic change in the intracellular localization of cathepsin D-, LIMPII- and LAMP1-positive lysosomes. We showed here the evidence by 3D-image analysis of lysosomal proteins by confocal immunofluorescence microscopy demonstrating that the ROCK activity enhances actin stress-fiber formation along with the pericellular dispersion of lysosomes. We showed that active ROCK induced small punctate lysosomes, redistributed in the vicinity of peripheral region of the cells, which were not co-localized with transferrin receptor, EEA1 or with the internalized Texas Red-transferrin. These results indicate that lysosomes redistributed towards cell surface are not part of the endocytic pathway, and further implicate that the fusion between the dispersed lysosomes and early endosomes does not occur in the active ROCK-transfected cells. In contrast, lysosomes, as judged by LIMPII or cathepsin D stainings were primarily localized in the perinuclear region of the mock-transfected cells.

In agreement with a recent study we have demonstrated that transfection of active RhoA (VRhoA) into MM1 rat hepatoma cells causes a remarkable redistribuion of lysosomal proteins towards cell periphery, and that these aberrant small vesicles are not accessible to the early endocytic tracer.[25] These dispersed vesicles retaining their acidity were also reclustered in the vicinity of perinuclear region when the cells were incubated with Y-27632, a selective ROCK inhibitor.[19,25] By contrast, there is no apparent change found in the fluid-phase endocytosis of dextran from cell surface through early endosomes to lysosomes and in the receptor-mediated endocytic pathway.[25] These observations indicate that RhoA-ROCK signaling regulates preferential intracel-

Figures 5. *Endocytosed Texas Red-transferrin is not delivered to the LIMPII-positive swollen vacuolar compartments in wortmannin-treated active ROCK transfectants. The active ROCK transfectants were incubated for 30 min in the presence of wortmannin and then further incubated for 20 min with Texas Red-transferrin in plain complete medium containing wortmannin. The cells were fixed, and stained with primary antibody against LIMPII (green fluorescence) as described in Materials and Methods. Merged fluorescence signals for LIMPII and the internalized Texas Red-transferrin are shown in c of panel A, B, C, and D. Orthographic sections (0.71 μm for LIMPII from the cell bottom) are shown in panel D. Details for the figure are described in Figure 2. Note that Texas Red-transferrin-positive structures are not co-localized with LIMPII-positive swollen vacuoles, and the endocytosed Texas Red-transferrin is not able to reach the LIMPII-positive swollen vacuoles after 20 min incubation. Bar, 10 μm*

lular membrane trafficking of lysosomes without effect on the early endocytic pathway.

Recent evidence suggest that Rab proteins are key regulators of membrane traffic of the biosynthetic and endocytic pathways,[41-43] and that Rab GTPases recruit tethering and docking factors to establish firm contact between the membranes for fusion, after which SNARE proteins become involved in completing the fusion process.[44] It has been reported that the expression of dominant negative Rab7 mutants selectively affects lysosomes itself in HeLa cells, leading to the redistribution of lysosomes throughout the cytoplasm, but they did not observe the co-localization of lysosomal markers and early endosomal markers such as transferrin receptor or Rab5.[45] By contrast, the expression of the dominant active mutant Rab7-EGFP induced aggregated, perinuclear localization of LAMP-, cathepsin D-positive lysosomes, therefore implying that Rab7 is a possible candidate for regulating lysosomal biogenesis and the maintenance of the perinuclear aggregates of late endocytic structures.[45] On the other hand, in BHK cells, the expression of dominant negative mutant Rrab7 causes an increase in the proportion of CI-MPR and cathepsin D in early endosome compartments.[46] In this context, the localization of cathepsin D, LIMPII, and LAMP-1 in the dominant active mutant ROCK in MCF-7 cells as demonstrated in our study is reminiscent of those observed in the dominant negative Rab7 mutant transfected into HeLa cells.[45] However, in contrast to active ROCK-transfected cells in our study, the dispersed lysosomes showed reduced acidity as revealed by the decrease in LysoTracker Red intensity, and endocytic markers were not able to accumulate in the dispersed lysosomal compartments in the cells expressing the dominant negative Rab7 mutant, thereby, suggesting that the lysosomes redistributed in the cells of expressing dominant negative mutant Rab7 are functionally defective in terms of endocytosis.[45] RhoA and ROCK signaling activity may somehow regulate the inactivation step for Rab7. Further analysis will be required to define how the signaling of Rho-ROCK is involved in the regulation of lysosomal membrane traffic in tumor cells.

Rho activity is known to be directly involved in the cytoskeletal organization in tumor cells.[5] It has recently been demonstrated that the cross talk of the Rho/ROCK signaling sufficient for agonist-induced neurite retraction and cell rounding, which is accompanied by elevated phosphorylation of myosin light chains and the disassembly of the microtubules and intermediate filaments in neuroblastoma cells.[47] Thereby, the selective translocation of lysosomes to the cell periphery may be a common mechanism to cells that are involved in the degradation of the extracellular matrix and the basement membrane, which is a critical step in tumor cell invasion. Indeed, in vitro invasive activity of MM1 cells transfected with dominant active mutant ROCK was found to be significantly induced as reported previously.[11]

It has been shown that intracellular movement of lysosomes to the cell periphery is mediated in kinesin-dependent manner (a plus-end directed motor protein).[48,49] Moreover, Rho protein has previously been demonstrated to interact with kinectin, an anchoring protein of kinesin motor.[50] Recently, it has been reported that RhoG, a family of GTPases, interacts with kinectin which has been shown to bind the conventional kinesin and activate its microtubule-stimulated ATPase activity.[51] In active RhoG-transfected cells, an enhanced movement of lysosomes was evaluated morphologically.[51] The molecular mechanisms by which RhoG uses microtubules to exert its cytoskeletal effects remains unknown, and also the biological significance of the interaction of RhoA with microtubule motor protein is unclear at present. RhoA-ROCK signaling may somehow participate in the regulation of lysosome movement toward cell periphery through interaction with microtubule motor proteins. Alternatively, it is possible that the actin cytoskeleton is involved in the regulation of cellular membrane trafficking of lysosomes. Changes of the actin cytoskeleton often correlated with microtubule rearrangements.[52,53] It remains to be seen how RhoA/ROCK signaling pathway regulates the membrane traffic of lysosomes through rearrangement of cellular actin cytoskeleton and microtubule organization.

References

1. *Paterson HF, Self AJ, Garrett MD et al:* Microinjection of recombinant $p21^{rho}$ induces rapid changes in cell morphology. J Cell Biol 111: 1001-1007, 1990.
2. *Perona R, Esteve P, Jimene, B et al:* Tumorigenic activity of rho genes from Aphysia california. Oncogene 8: 1285-1292, 1993.
3. *Kishi K, Sasaki T, Kuroda S et al:* Regulation of cytoplasmic division of Xenopus Embryo by rhop21 and its inhibitory GDP/GTP exchange protein (rhoGDI). J Cell Biol 120: 1187-1195,1993.
4. *Takaishi K, Kikuchi A, Kuroda S et al:* Involvement of rhop21 and its inhibitory GDP/GTP exchange protein (rhoGDI) in cell motility. Mol Cell Biol 13: 72-79, 1993
5. *Hall, A.* Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 10: 31-54, 1994
6. *Prendergast GC, Khosravi-Far R, Solski PA, et al:* Critical role of Rho in cell transformation by oncogenic Ras. Oncogene 10: 2289-2296, 1995
7. *Fritz G, Just I, Kaina B:* Rho GTPases are over-expressed in human tumors. Int J Cancer 81: 682-687, 1999
8. *Yoshioka K, Nakamori S, Itoh K:* Overexpression of small GTP-binding protein RhoA promotes invasion of tumor cells. Cancer Res 59: 2004-2010, 1999
9. *Yoshioka K, Matsumura F, Akedo H, Itoh K:* Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. J Biol Chem 273: 5146-5154, 1998.
10. *Leung T, Manser E, Tan L, Lim L:* A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to the peripheral membranes. J Biol Chem 270: 29051-29054, 1995.
11. *Itoh K, Yoshioka K, Akedo H et al:* An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nature Medicine 5: 221-225, 1999
12. *Sloane BF, Moin K, Sameni M et al:* Membrane association of cathepsin B can be induced by transfection of human breast epithelial cells with c-Ha-Ras oncogene. J Cell Sci 107: 373-384, 1994

13. *Sameni M, Elliott E, Ziegler G et al:* Cathepsin B and D are localized at the surface of human breast cancer cells. Pathol Oncol Res 1: 43-53, 1995
14. *Taniguchi S, Nishimura Y, Takahashi, T et al:* Augmented excretion of procathepsin L of a fos-transferred highly metastatic cell line. Biochem Biophys Res Commun 168: 520-526, 1990
15. *Kageshita T, Yoshii A, Kimura T et al:* Biochemical and immunohistochemical analysis of cathepsins B, H, L and D in human melanocytic tumours. Arch Dermatol Res 287: 266-272, 1995
16. *Albini A, Graf J, Kitten GT et al:* 17ß-Estradiol regulates and v-Ha-*ras* transfection constitutively enhances MCF7 breast cancer cell interactions with basement membrane. Proc Natl Acad Sci US 83: 8182-8186, 1986
17. *Ichikawa T, Kyprianou N, Isaacs JT:* Genetic instability and the acquisition of metastatic ability by rat mammary cancer cells following v-H-*ras* oncogene transfection. Cancer Res 50: 6349-6357, 1990.
18. *Nishimura Y, Sameni M, Sloane BF:* Malignant transformation alters intracellular trafficking of lysosomal cathepsin D in human breast epithelial cells. Pathol Oncol Res 4: 283-296, 1998
19. *Nishimura Y, Itoh K, Yoshioka,K et al:* Small guanosine triphosphatase Rho/Rho-associated kinase as a novel regulator of intracellular redistribution of lysosomes in invasive tumor cells. Cell Tissue Res 342: 341-351, 2000.
20. *Uehata M, Ishizaki T, Satoh H et al:* A key role for p160ROCK-mediated Ca^{++}sensitization of smooth muscle in hypertension. Nature 389: 990-994, 1997.
21. *Matsui T, Amano M, Yamamoto T et al:* Rho-associated kinase, a novel serine threonine kinase, as a putative target for the small GTP binding protein Rho. EMBO J 15: 2208-2216, 1996
22. *Nakagawa O, Fujisawa K, Ishizaki et al:* ROCK-I and ROCK-II; two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett 392: 189-193, 1996
23. *Amano M, Chihara K, Kimur, Y et al:* Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275: 1308-1311,1997.
24. *Ishizaki T, Naito M, Fujisaw, K et al:* p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesion. FEBS Lett 404: 118-124, 1997
25. *Nishimura Y, Itoh K, Yoshioka K, et al:* A role for small GTPase RhoA in regulating intracellular membrane traffic of lysosomes in invasive rat hepatoma cells. Histochem J 34: 189-213, 2002.
26. *Lippincott-Schwart J, Fambrough DM:* Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. J Cell Biol 102: 1593-1605, 1986.
27. *Griffiths G, Hoflack B, Simon, K et al:* The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell 52: 329-341, 1998
28. *Kornfeld S, Mellman I:* The biogenesis of lysosomes. Ann Rev Cell Biol 5: 483-525, 1989.
29. *Okazaki I, Himeno M, Ezaki J et al:* Purification and characterization of an 85 kDa sialoglycoprotein in rat liver. J Biochem 111: 763-769, 1992.
30. *Sandoval IV, Arredondo JJ, Alcalde J et al:* The residues Leu(Ile)475-Ile(Leu)476, contained in the extended carboxyl cytoplasmic tail, are critical for targeting of the resident lysosomal membrane protein LIMPII to lysosomes. J Biol Chem 269: 6622-6631, 1994
31. *Tabuchi N, Akasaki K, Tsuji H:* Two acidic amino acid residues, Asp(470) and Glu(471), contained in the carboxyl cytoplasmic tail of a major lysosomal membrane protein, LGP85/LIMPII, are important for its accumulation in secondary lysosomes. Biochem Biophys Res Commun 270: 557-563, 2000.
32. *Soule HD, Vazguez J, Long et al.:*, A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51: 1409-1416, 1973.
33. *Nishimura Y, Higaki M, Kato K:* Identification of a precursor form of cathepsin D in microsomal lumen: characterization of enzymatic activation and proteolytic processing in vitro. Biochem Biophys Res Commun 148: 335-343, 1987
34. *Nishimura Y, Kawabata T, Kato K:* Identification of latent procathepsins B and L in microsomal lumen: characterization of enzymatic activation and proteolytic processing in vitro. Arch Biochem Biophys 261: 64-71, 1988
35. *Matteoni R, Kreis TE:* Translocation and clustering of endosomes and lysosomes depends on microtubules. J Cell Biol 105: 1253-1265, 1987
36. *Mu FT, Callaghan JM, Steele-Mortimer O et al:* EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. J Biol Chem 270: 13503-13511, 1995
37. *Stack JH, Emr SD:* Genetic and biochemical studies of protein sorting to the yeast vacuole. Curr Biol 5: 641-646,1993.
38. *Brown WJ Emr SD, Plutner H, Balch WE:* Role for phosphatidylinositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. J Cell Biol 130: 797-805, 1995
39. *Davidson H:* Wortmannin causes mis-targeting of procathepsin D. Evidence for the involvement of a phosphatidylinositol 3-kinase in vesicular transport to lysosomes. J Cell Biol 130: 797-805, 1995
40. *Reaves B, Bright NA, Mullock B, Luzio JP:* The effect of wortmannin on the localization of lysosomal type I integral membrane glycoproteins suggests a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway. J Cell Sci 109: 749-762, 1996
41. *Pfeffer SR:* GTP-binding proteins in intracellular transport. Trends Cell Biol 2: 41-46, 1992
42. *Zerial M, Stenmark H:* Rab GTPases in vesicular transport. Curr Opin Cell Biol 5: 613-620, 1993
43. *Chavrier P, Goud B:* The role of ARF and Rab GTPases in membrane transport. Curr Opin Cell Biol, 11: 466-475, 1999
44. *Pfeffer SR:* Transport-vesicle targeting: tethers before SNAREs. Nature Cell Biol 1: E17-E22, 1999.
45. *Bucci C, Thomsen P, Nicoziani P et al.:* Rab7: A key to lysosome biogenesis. Mol Biol Cell 11: 467-480, 2000
46. *Press B, Feng Y, Hoflack B, Wandinger-Nes A:* Mutant Rab7 causes the accumulation of cathepsin D and cation-independent mannose 6-phosphate receptor in an early endocytic compartment. J Cell Biol 140: 1075-1089, 1998.
47. *Hirose M, Ishizaki T, Watanabe N et al:* Molecular dissection of the Rho-associated kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. J Cell Biol 141: 1625-1636, 1998
48. *Hollenbeck PJ, Swanson JA:* Radial extension of macrophage tubular lysosomes supported by kinesin. Nature 346: 864-866, 1990
49. *Nakata T, Hirokawa N:* Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport. J Cell Biol 131: 1039-1053, 1995
50. *Hotta K, Tanak K, Mino A, et al:* Interaction of the Rho family small G proteins with kinectin, an anchoring protein of kinesin motor. Biochem Biophys Res Commun 225: 69-74, 1996
51. *Vignal E, Blangy A, Martin M, et al:* Kinectin is a key effector of RhoG microtubule-dependent cellular activity. Mol Cell Biol 21: 8022-8034, 2001
52. *Timar J, Tang D, Bazaz R, et al.:* PKC mediates 12-(S)-HETE-induced cytoskeletal rearrangement in B16a melanoma cells. Cell Motil Cytoskel 26: 49-65, 1993
53. *Timar J, Bazaz R, Kimler V et al:* Immunomorphological characterization and effects of 12-(S)-HETE on a dynamic intracellular pool of the $\alpha_{IIb}\beta_3$-integrin in melanoma cells. J Cell Sci 108: 2175-2186, 1995