Internalization and trafficking of mouse 24p3 protein in L929 cells

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Abstract

It has been shown that mouse 24p3 protein is a dexamethasone-inducible secretory protein which suggests the involvement of an autocrine mechanism in the L929 cell line. The aim of this study was to investigate the possibility of this mechanism in L929 cells and to also demonstrate further processing of this protein after association with L929 cells. We have characterized the internalization of 24p3 protein in L929 cells with fluorescein isothiocyanate- and Alexa555-labeled protein supplement instead of secreted 24p3 protein. As endocytotic inhibitors could reveal the inhibition of protein internalization, we confirmed the existence of receptor-mediated 24p3 protein internalization in L929 cells by carrying out an inhibition test. Plus-chase experiment of L929 cells with biotinylated 24p3 protein demonstrated a release of internalized 24p3 protein into the extracellular region. The protein recycling inhibitor, bafilomycin A1, arrests the transport of 24p3 protein by accumulating in early endosome, but no effect could be observed in protein release from early to late endosome by nocodazole. These results provide the first evidence on recycling of apo-24p3 protein in L929 cells.

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Introduction

Lipocalins were found to be important extracellular carriers of hydrophobic molecules in many species (Bishop *et al.* 1995, Flower 1996, Akerstrom *et al.* 2000). There is increasing evidence to claim that these proteins are involved in a variety of physiological processes (Bratt 2000, Logdberg & Wester 2000, Devireddy *et al.* 2001). There is less knowledge regarding the mechanisms by which lipocalins exert their biological effects. But, it is well accepted that these proteins are able to bind to specific cell receptors (Wojnar *et al.* 2003, Devireddy *et al.* 2005) and internalize into the cells (Elangovan *et al.* 2004, Devireddy *et al.* 2005). Due to limited data concerning the internalization mechanism of lipocalins, the molecular mechanisms are very unclear at the moment. 24p3 protein is an inducible lipocalin of L929 secretory proteins (Garay-Rojas *et al.* 1996). After secretion, 24p3 protein has been suggested to undergo an autocrine mechanism to play a specific role in the cells (Garay-Rojas *et al.* 1996). Until now, this has not been clarified. Based on the concept of autocrine mechanism, it has been hypothesized that 24p3 protein may follow unique pathways of secretion and internalization, allowing it to have the biological function. The knowledge regarding the internalization and trafficking route of 24p3 protein in this cell is limited. Our recent study has given support to this hypothesis describing the effects of 24p3 protein on L929 cell cycle (P-T Li, Y-C Lee, N Elangovan and S-T Chu, unpublished observation). Based on this view, we attempt to clarify the trafficking of 24p3 protein in L929 cells. It has been suggested that acidification could hold coated pits on the cell membrane so that they can no longer pinch off from the plasma membrane to form an intracellular vesicle (Sandvig *et al.* 1986, Cosson *et al.* 1989). Alteration of microfilaments or microtubules with the inhibitors, cytochalasin and vincristine, can result in the prevention of endocytosis (Rikihisa *et al.* 1994, Puja *et al.* 2004), and therefore, we could confirm the protein internalization. It is not clear whether 24p3 protein is recycled from the cells or degraded upon its entry into the cells. Based on that, the budding of endosome carrier vesicle (ECV) from early endosomes has been demonstrated to be inhibited by bafilomycin A1 (Johnson *et al.* 1993). Besides that, nocodazole blocks transport from early to late endosomes at a later stage, resulting in the accumulation of cargo in ECV (Bayer *et al.* 1998). Using these inhibitors, we measured the accumulation status of 24p3 protein in cells and clarified the protein-tracking pathway. To address this question, we used the L929 cell line as a model. From the data, it is evident that 24p3 protein can be internalized and recycled after binding to the cells suggesting the existence of an autocrine mechanism.

Materials and Methods

Chemicals and biochemicals

An enhanced chemiluminescence plus kit (ECL) was purchased from Amersham-Pharmacia Biotech. Alexa Fluor

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555 carboxylic acid and succinimidyl ester were from Molecular probes (A20174), and fluorescein isothiocyanate (FITC) was from Merck (No. 124546). Anti-mouse IgG-horseradish peroxidase conjugate prepared from goats and anti-mouse IgG FITC-conjugated antibody were obtained from Sigma. The 24p3 protein was purified from diethylstilbestrol (DES)-stimulated female mice as has been previously described (Chu *et al.* 1996). All chemicals were of reagent grade quality.

Cell culture

L929 cells were maintained in DMEM:F12 supplemented with 10% fetal calf serum. The cells were seeded into 24-well plates (or coverslips) and left to acclimate for at least 24 h before being treated. To detect protein internalization, cells were incubated with 1·0 μM fluorescein-labeled 24p3 protein. After various durations, the population of cells was observed under a microscope.

Protein labeling

FITC-labeled 24p3 protein (FITC-24p3) was prepared by incubating purified 24p3 protein (5 mg/ml) with fluorescein isothiocyanate (FITC, 5 mg/ml) in sodium bicarbonate buffer (200 mM), pH 9·0, for a period of 20 h at 10 °C. Unconjugated FITC was removed by gel filtration on a PD-10 column (Amersham). Alexa 555-labeled 24p3 protein (2 mg/ml) was prepared by incubating with 2 mg/ml Alexa Fluor 555 carboxylic acid, succinimidyl ester in 100 mM sodium bicarbonate, pH 8·3, for 1 h at room temperature. After reaction, the free Alexa 555 was removed by centrifugation using microcon YM-10 (Amicon, USA). Biotinylated 24p3 protein (1 mg/ml) was prepared by incubating the same molar ratio of Sulfo-NHS-LC-Biotin (Pierce, IL, USA) in 0·1 M $NaHCO_3$, 0·2 M NaCl buffer, pH 9·0, for 2 h at room temperature under darkness. After labeling, free Sulfo-NHS-LC-Biotin was removed by centrifugation using microcon YM-10.

Detection of FITC-24p3 protein interaction with L929 cells

Cells at a concentration of 1×10^5 were cultured on coverslips for 48 h, washed twice with PBS and fixed with methanol, and again washed with PBS to remove the organic solvent. The cells were overlaid separately with 100 μl PBS, 100 μl PBS containing 5 μg FITC-labeled 24p3 protein, and 100 μl PBS containing 5 μg FITC-labeled 24p3 and 50 μg unlabeled 24p3 protein for competitive assay. The cells were incubated in the dark for 1 h at 37 °C, and washed twice with PBS and cold acetone, pre-chilled at −20 °C before use. The cells were again washed twice with PBS and observed under a fluorescence microscope.

Protein extraction from L929 cells

Cells at a concentration of 1×10^5 were cultured in the medium in the presence of 1·0 μM biotinylated 24p3 protein. The surface protein was washed away with glycine buffer and incubated with serum-free medium for various times. The cells were washed with PBS and trypsinized with EDTA/ trypsin for 1 min to resuspend the cells and then washed twice with PBS. The cells were resuspended in hypotonic buffer (10 mM HEPES, 10 mM KCl, and 1·5 mM $MgCl_2$, pH 7·9) containing 1× Protease inhibitor Cocktail (PI Cocktail, Cat No. 1697498, Roche Molecular Biochemicals) and 2% SDS, and then extracted by sonication for 25 min on ice. Then the cell debris was removed by centrifugation at 500 ***g*** for 15 min. The protein extract was subjected to SDS-PAGE and western blot analysis. The culture medium which contained recycling proteins was concentrated by centricon YM-10 and subjected to SDS-PAGE and western blot analysis.

Western blot analysis

The dissolved proteins in the sample buffer were resolved by SDS-PAGE on a 12% acrylamide gel slab. Proteins were transferred on to PVDF membrane by semi-dry electrotransfer (1 mA/cm^2) at room temperature for 2 h. The transferred proteins were detected using HRP-conjugated streptavidin diluted to 1:10 000. The reactive bands were visualized using an ECL kit and exposed on X-ray film.

Flow cytometry and fluorescence microscopy

Flow cytometry data were collected using a COULTER EPICS XL flow cytometer (Beckman-Coulter, FL, USA). The fluorescence was initiated by excitation at a wavelength of 488 nm and measured via a 525 nm filter. The fluorescence intensity of FITC was quantified for 10 000 individual cells.

L929 cells were cultured in the absence or presence of 1 μM fluorescein-labeled 24p3 protein for 30 min, washed twice with PBS, fixed by methanol, and washed again with PBS to remove the organic solvent. For examination of nuclear morphology, cells were stained with 2 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI) to stain DNA and were observed under fluorescence microscope (AH3-RFCA, Olympus, Tokyo, Japan).

Results

FITC-labeled 24p3 protein binding to L929 cells

In the previous report (Garay-Rojas *et al.* 1996), it has been shown that 24p3 protein could be expressed and secreted via dexamethasone induction. This induction was further amplified by an autocrine mechanism. It implied that the 24p3 protein should act on the cells. In order to clarify this

issue, we supplemented 24p3 protein in the medium to confirm the interaction of this secreted 24p3 protein with L929 cells; we labeled 24p3 protein with FITC. To study the binding process, L929 cells ($1{\cdot}2\times10^5$) were grown on coverslips in MEM medium for 24 h and were analyzed under bright field (Fig. 1, left panel) and fluorescence microscopy (Fig. 1, right panel). Efficient binding of the L929 cell with 5 μg FITC-24p3 protein was observed after 1 h incubation, followed by the removal of unbound protein by washing twice with PBS. Then fixing with cold acetone, FITC-24p3 protein was found to bind to the cell membrane (Fig. 1B and E). In the competition assay, L929 cells were incubated for 1 h with 5 μg FITC-24p3 protein with or without 50 μg unlabeled 24p3 protein (Fig. 1C and F). The efficiency of FITC-24p3 protein binding was inhibited by ten-fold with the unlabeled 24p3 protein, whereas in the control, the L929 cells devoid of FITC-24p3 protein (Fig. 1A and D) showed no fluorescence on cells. Thus, in our competition study, we confirm that 24p3 is specifically bound to the cell surface.

Inhibitors of internalization of 24p3 protein

Following 24p3 protein binding to the cells, we intended to identify the further processing of this protein in the cells. We proposed that protein internalization would occur at the

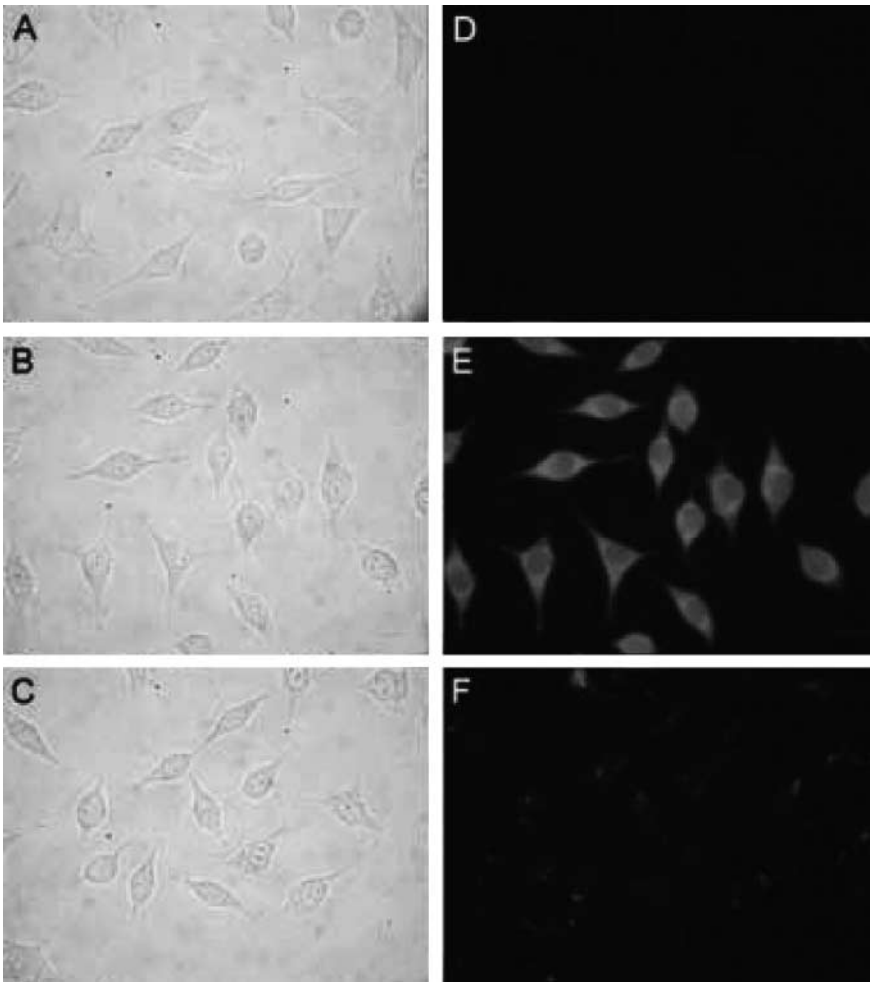

Figure 1 24p3 protein specific binding assay. L929 ($1{\cdot}2\times10^5$) cells were grown on coverslips in MEM medium. After 24 h, cells were washed twice with PBS buffer, then fixed with methanol for 2 min. FITC-labeled 24p3 protein was supplemented and incubated for 1 h at 37 °C under darkness. Before observation under the microscope, the free protein was removed from the cells with cold acetone. (A to C) bright field observations; (D to F) observed under fluorescent microscopy. (A and D) Control cells without 24p3 protein incubation; (B and E) with 1 μM FITC-labeled 24p3 protein; (C and F) with 5 μg FITC-labeled 24p3 protein, and 50 μg unlabeled 24p3 protein. Magnification × 400.

moment of binding. In the endocytic pathway, endocytosed molecules are delivered to early endosomes and then trafficked to the recycled endosomal compartment or to late endosomes and lysosomes for degradation. To investigate whether the internalization of 24p3 protein is via receptor-mediated endocytosis, we tested the association of 24p3 protein with the cell by cytosol acidification. Low cytoplasmic pH inhibits the endocytosis of the cell by blocking the pinch off at the endocytotic vesicles from the cell surface (Cosson *et al.* 1989). Certain weak acids have the ability to rapidly penetrate the cell membrane in its associated form. Hence, the cytosol was acidified by acetic acid; once in the cytosol, the acid dissociates, thereby lowering the cytosolic pH. Significant inhibition of FITC-24p3 protein internalization was observed in the acetic acid-treated L929 cell (Fig. 2B) when compared with the control (Fig. 2A). This treatment inhibits the pinching off of the coated pits with persistent attachment to the plasma membrane, leaving the vesicles on the cell surface. Obviously, the intracellular fluorescent distribution is different from the control cell. The fluorescence image is distributed more like a smear status. Under this condition, less amount of FITC-24p3 protein internalized into the cell and acetic acid inhibited endosomal vesicle formation.

The polymerization of microfilaments is a requirement for protein endocytosis. Cytochalasin B is a depolymerizing agent, which can inhibit actin polymerization (Rikihisa *et al.* 1994). Cells were treated with cytochalasin B, which prevents polymerization of actin, and were analyzed by immunofluorescence microscopy. L929 cells were treated with cytochalasin B (10 μg/ml) for 1 h before inducing internalization with FITC-24p3 (1 μM). Treatment with cytochalasin B inhibited receptor-mediated internalization of the FITC-24p3 significantly in L929 cell lines (Fig. 2C). Cytochalasin B completely inhibited the vesicle movement towards nuclei and the fluorescent vesicle appeared adjacent to the inner side of the plasma membrane of the cells. These results suggest the involvement of actin polymerization in 24p3 protein internalization.

The involvement of the microtubule network in L929 cell internalization of FITC-24p3 protein was examined with the microtubule assembly inhibitor, vincristine. Vincristine is a cell cycle-dependent drug that arrests cell mitosis during metaphase by preventing tubulin polymerization as well as by inducing depolymerization (Puja *et al.* 2004). In our experiment, we used vincristine for inhibiting the cytosolic microtubule polymerization, which might prevent the endosome movement. The fluorescence microscopy showed that vincristine causes cellular morphological retraction and flattening, indicative of microtubule disruption. This vincristine treatment affects the majority of cell internalization, especially in early to late endosomes. In control cells, FITC-24p3 protein internalized into the cells showing a bright fluorescence staining in the middle region, resulting in

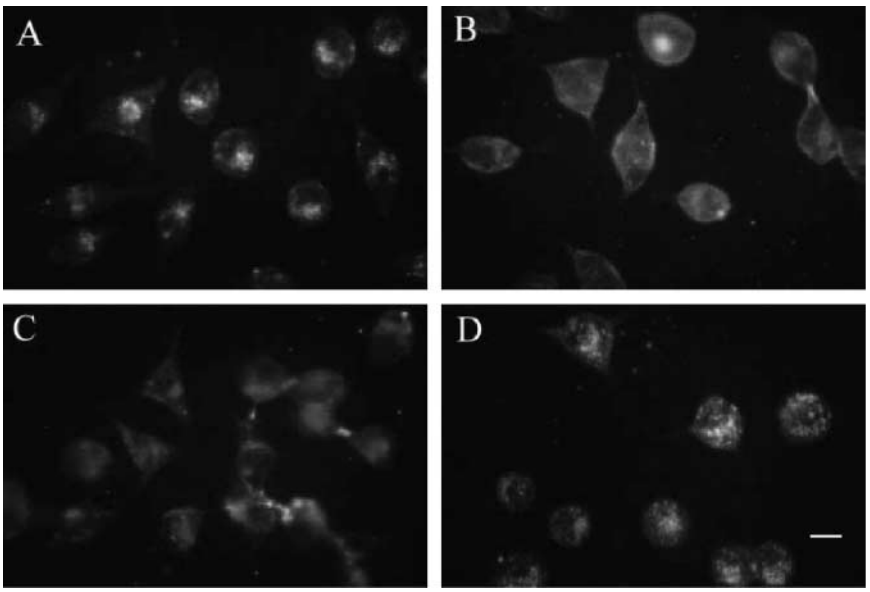

Figure 2 Effect of inhibitors on 24p3 protein internalization. L929 ($1{\cdot}2\times10^5$) cells were grown on coverslips overnight, treated with different inhibitors, and incubated with 1 μM FITC-labeled 24p3 protein for 30 min under CO_2 incubator at 37 °C. After incubation, free proteins were removed by 50 mM glycine and 150 mM NaCl (pH 3·0) buffer for 1 min. They were washed twice with PBS and fixed with methanol:acetone (1:1, v/v) for 5 min. (A) Control without inhibitor treatment; (B) treated with 10 mM acetic acid; (C) treated with 10 μg/ml cytochalasin B; (D) treated with 100 mM vincristine (bar = 10 μm).

accumulation in the endosomes (Fig. 2A). After treatment with vincristine, the cell morphology may be affected due to the dissembling of microtubules, whereas the bright fluorescent vesicles were still present in the cells (Fig. 2D). This observation implied that the microtubular inhibitor has no effect on the accumulation of the protein in the intracellular region and suggests 24p3 protein maintenance occurs only in early endosomes after internalization.

Receptor-mediated endocytosis of 24p3 protein on L929 cells

The transglutaminase inhibitor, monodansyl cadaverine (MDC), which inhibits clustering and internalization of the ligand–receptor complexes into clathrin-coated vesicles (Davies *et al*. 1980), was used as a demonstration compound. For direct evidence of receptor-mediated endocytosis of 24p3 in L929 cells, and also to demonstrate the effect of MDC (transglutaminase enzyme inhibitor essential for endocytosis of receptor), an inhibitor of receptor-mediated endocytosis of 24p3 protein receptor, a fluorescent microscopic experiment was carried out using Alexa555-24p3 protein conjugate as a probe. The binding of Alexa555-24p3 protein conjugate to L929 cell was carried out at 37 °C in a humid chamber. The cells were mounted and observed under a fluorescent microscope (Fig. 3A). The cells were internalized with Alexa555-24p3 protein, which is bright and highly fluorescent. On the other hand, L929 cells were pre-incubated with MDC (50 μM) for 30 min followed by supplementation with Alexa555-24p3 protein (1 μM) and MDC (50 μM) and further incubation for 30 min. The proteins on the cell surface were removed with washing buffer (50 mM glycine, 150 mM NaCl, pH 3·0), which could wash away the 24p3 protein not entering into the coated pits. The data showed a significant decrease or no fluorescence in the cells (Fig. 3B). These results suggest that the 24p3 protein undergoes internalization by receptor-mediated endocytosis in the L929 cell. Either with or without adding MDC, the nuclear stain, DAPI(1 μg/ml)-stained L929 cells served as control (Fig. 3C and D).

Localization of internalized 24p3 protein in L929 cells

Cells were exposed to inhibitors that are known to differentially inhibit the assembly of clathrin-coated pits, actin polymerization and microtubule assembly. We have analyzed the endocytic receptor internalization of 24p3 protein in L929 cells. Figure 4A showed the receptor-mediated endocytosis of L929 cell, along with Alexa555-24p3 protein. Apart from this, in order to illustrate that 24p3 protein undergoes a lysosomal degradation or

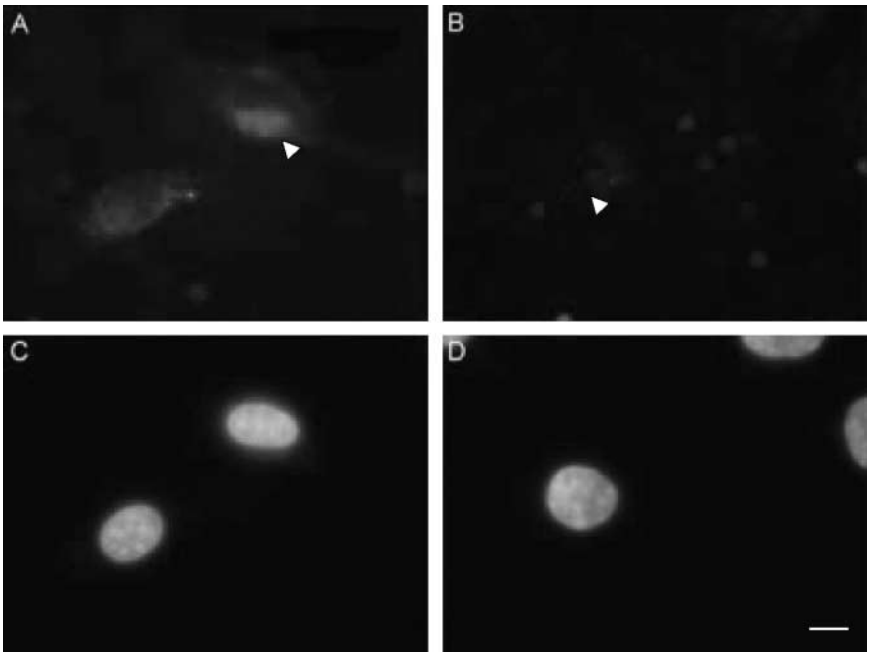

Figure 3 Effect of MDC on 24p3 protein internalization in L929 cells. Cells were grown on coverslips overnight at 37 °C in MEM medium, and were pre-incubated with 50 μM MDC for 30 min before protein internalization assay. Then, cells were incubated with 1 μM Alexa555-24p3 protein and 50 μM MDC for another 30 min. Before observation, the surface proteins were removed with 50 mM glycine and 150 mM NaCl (pH 3·0) buffer for 1 min. They were washed twice with PBS and fixed with methanol:acetone (1:1, v/v) for 5 min (A and B). Arrows indicate the location of Alexa555-labeled 24p3 protein. The cell nuclei were stained with DAPI (1 μg/ml) for 10 min (C and D). (A and C) Control cell without MDC treatment; (B and D) cells treated with MDC (bar = 10 μm).

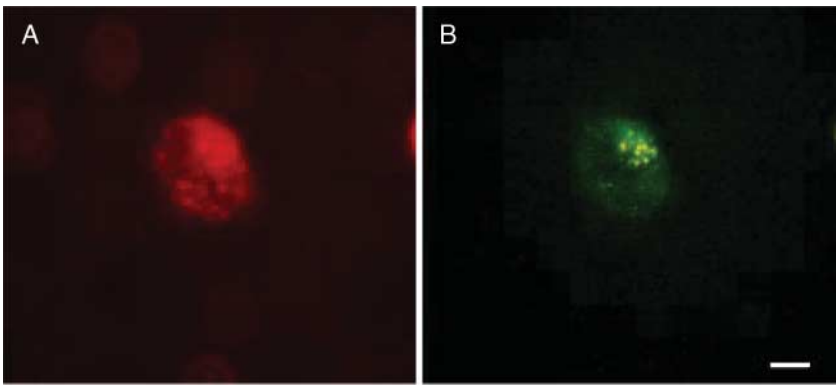

Figure 4 Intracellular location of 24p3 protein in L929 cells. L929 ($1{\cdot}2\times10^5$) cells were grown on coverslips overnight, treated with different inhibitors and incubated with 1 μM Alexa555-labeled 24p3 protein for 30 min under CO_2 incubator at 37 °C. After incubation, free proteins were removed by 50 mM glycine and 150 mM NaCl (pH 3·0) buffer for 1 min. They were washed twice with PBS and fixed with methanol:acetone (1:1, v/v) for 5 min. For blocking the non-specific binding of antibody, the fixed cells were incubated with 2 mg/ml BSA in PBS for 30 min. After washing twice with PBS, the cells were incubated with FITC-labeled Lamp-2 antibody (100-fold dilution) overnight at 4 °C under darkness. Using blue excitation for FITC-labeled Lamp-2 antibody observation and green excitation for Alexa555-labeled 24p3 protein observation, the samples were observed under fluorescent microscopy. (A) Alexa555-24p3 protein; (B) FITC-Lamp-2 antibody (bar=10 μm).

recycling mechanism, we measured the location of 24p3 protein and Lamp-2 protein. Lamp-2 protein is a late lysosome- and lysosome-associated membrane protein, localized on the periphery of these two cellular compartments (Chen *et al.* 1985). We used Lamp-2 protein as a late lysosome and lysosome marker to identify the location of internalized 24p3 protein. The Alexa555-24p3 protein and FITC-labeled anti-Lamp-2 antibody would fluoresce in red and green respectively, under the fluorescent microscope. On merging these two images, the resulting yellowish-orange color indicated the co-localization of 24p3 protein and Lamp-2 protein. In contrast, detection with FITC-labeled Lamp-2 antibody showed less or no co-staining in the late endosome or/and lysosome compartments and only small amounts of Alexa555-24p3 protein co-existed in the late endosome or/and lysosome (Fig. 4B). These results suggest that 24p3 protein is endocytosed in L929 cells via 24p3 protein receptor, whereas only little protein may be forwarded to the late endosome thus indicating involvement of a recycling pathway.

Recycling of 24p3 protein in L929 cells

The ability of 24p3 protein to internalize and recycle from the endocytotic compartment back to the cell surface or extracellular environment has been biochemically analyzed. Cells were incubated in serum-free MEM medium with biotinylated 24p3 protein for 30 min at 37 °C to allow internalization. The endocytosed pool of proteins was thus protected from reduction and remained biotinylated. After protein internalization, the protein on the cell surface was washed with glycine buffer (50 mM glycine, 150 mM NaCl, pH 3·0) and then incubated with the protein-containing cells in MEM medium at 37 °C for various time intervals. The cell proteins were harvested at various time intervals and electrophoresed. The eletrophoresed protein was transferred on to a PVDF membrane through Western blot and detected by HRP-conjugated streptavidin. The band intensity was analyzed by EZ-1D software (Fig. 5B). The internalization of 24p3 protein was detected (Fig. 5A) and showed a time-dependent decrease in intracellular 24p3 protein with a maximum decrease at 3-h incubation and then increasing in the fourth hour (Fig. 5A, upper panel). These chase experiments showed that internalized 24p3 protein could be recycled back to the cell surface or extracellularly. A definite increase was observed in biotinylated 24p3 protein after 1-h chase and was present in almost detectable amounts after 2 and 3 h chase in the media (Fig. 5A, lower panel). Interestingly, a decrease in extracellular protein and an increase in intracellular protein again at the fourth hour, suggest that internalized 24p3 protein can be recycled to the cell surface or to the extracellular region, and also re-enter the cells. We can therefore conclude that extracellular 24p3 protein triggers an endocytosis and recycling from early endosomes.

Bafilomycin A1 and nocodazole on endocytotic transport in L929 cells

In order to investigate the internalization, recycling, and endocytic transport system of 24p3 protein, we used the L929 cell line for flow cytometry analysis. The receptor-mediated internalization of 24p3 protein could be detected in L929 cells. We next investigated the recycling of the 24p3 protein in L929 cells. Internalization of 24p3 protein was studied by treatment with FITC-24p3 protein. During the recovery

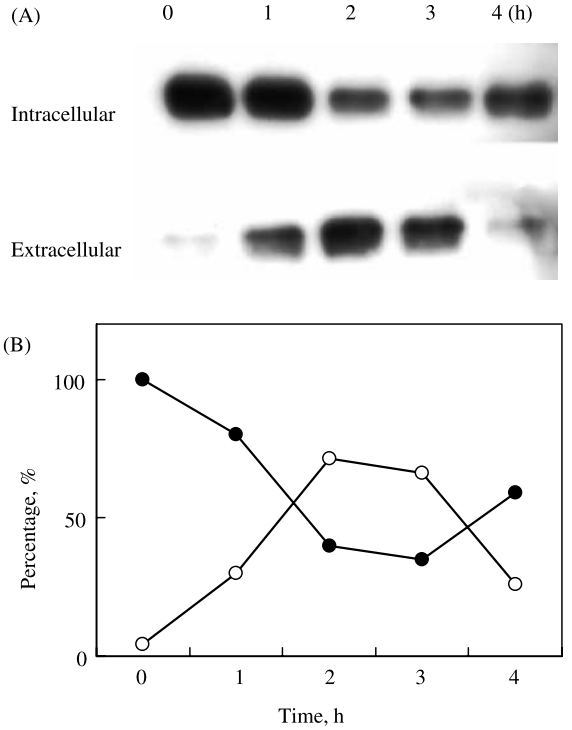

Figure 5 Intracellular processing of 24p3 protein in L929 cells. Cells (5×10^5) were grown on 35 mm dish overnight, and incubated with 1 μM biotinylated 24p3 protein for 30 min at 37 °C. After washing with 50 mM glycine and 150 mM NaCl (pH 3·0) buffer for 1 min to remove the free protein from the cell surface they were incubated under serum-free MEM medium for various durations. Cell protein extracts were loaded on SDS gel electrophoresis for western blotting assay via anti-24p3 protein antibody. (A) Upper panel is cell lysate extractant detected by antibody, lower panel is extracellular proteins detected by antibody; (B) Band intensity was determined by EZ-1D analysis software of (A) and is given as a percentage of control 24p3 protein detection. (●) Indicates the intracellular 24p3 protein; (○) indicated the extracellular 24p3 protein.

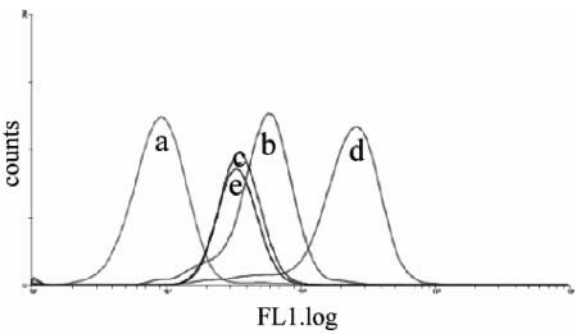

Figure 6 Effect of bafilomycin A1 and nocodazole on endocytotic transport in L929 cells. Cells (5×10^5) were grown on 35 mm dish overnight and incubated with 1 μM biotinylated 24p3 protein for 30 min at 37 °C. After washing with 50 mM glycine and 150 mM NaCl (pH 3·0) buffer for 1 min, to remove the free protein from the cell surface, and then incubated under serum-free MEM medium for 3 h. FITC-labeled 24p3 protein levels were assessed using flow cytometry as described in Materials and Methods. (a) Negative control cells; (b) after FITC-labeled protein incubation for 30 min; (c) incubation in serum-free medium for 3 h after protein internalization; (d) incubation in serum-free medium containing 200 nM bafilomycin A1 for 3 h after protein internalization; (e) incubation in serum-free medium containing 20 μM nocodazole for 3 h after protein internalization.

phase, cells were incubated either in medium alone or in medium amended with bafilomycin (200 nM) or in medium amended with nocodazole (20 μM) for 3 h at 37 °C. Figure 6*a* shows the cells without FITC-24p3 protein incubation and Fig. 6*b* shows the FITC-24p3 protein uptake after incubation for 30 min. In correspondence with the results obtained in the control cells (Fig. 6*a* and *b*), bafilomycin arrested the forward movement of 24p3 protein moving forward or recycled (Fig. 6*d*). The resulting accumulation of protein in endosomal carrier vesicle could be measured. In contrast, depolymerization of microtubules with nocodazole was able to block 24p3 protein movement to late endosome, but the data showed complete recycling of 24p3 protein (Fig. 6*e*). Recycling of 24p3 protein to the plasma membrane was observed after 3-h incubation of L929 cells in the medium (Fig. 6*c*) As a result, we observed that 24p3 protein internalized via an endosomal carrier vesicle in early endosome and might be recycled to the extracellular position or medium, which could not enter in the late endosome.

Discussion

The 24p3 protein has been shown to involve an autocrine mechanism (Garay-Rojas *et al.* 1996), but no evidence has been found in L929 cells. Using fluorescent-labeled protein, we have shown that 24p3 protein can internalize into L929 cells. Previous studies from our laboratory (Elangovan *et al.* 2004) and from other investigators (Devireddy *et al.* 2005) have provided evidence that 24p3 protein internalizes when binding to the cells. Our present study is the first evidence to show that 24p3 protein involves autocrine behavior in L929 cells. In this study, we have shown by acidification and by a cytochalasin B inhibition test that significant amounts of fluorescent-labeled 24p3 protein internalized into the cells. The MDC inhibition assay also revealed the receptor-mediated endocytosis mechanism of 24p3 protein in L929 cells. In support of this, Devireddy *et al.* (2005) has shown 24p3 protein internalization in Hela cells. In addition, our studies have provided details on the pathway involved in the internalization and recycling of 24p3 protein in L929 cells. We propose that suppression of polymerization of microtubules may actually reduce the cell function in transporting internalized molecules. We demonstrated here that vincristine did not affect the accumulation of 24p3 protein in the intracellular compartment. If suppression of microtubule dynamics affects cellular transport, it should be limited to 24p3 protein degradation since the microtubule

network is required for protein trafficking. In fact, no difference in distribution of fluorescent image in vincristine-treated cells was observed when compared with the control cells (Fig. 2). Hence, it is suggested that the protein-containing vesicles do not migrate towards the late endosome. These studies demonstrated clearly that 24p3 protein internalized via receptor-mediated endocytosis and was then secreted (Fig. 5). The 24p3 protein appears in the media within 1 h of instituting the chase, suggesting rapid recycling. At the fourth hour of incubation in MEM, there is a decrease in the amount of 24p3 protein found in the media and also a simultaneous increase inside the cells, thus the re-internalization of 24p3 protein may occur. To investigate the influence of the bafilomycin A and nocodazole on recycling and on the lysosomal pathway, we have applied flow cytometry to determine the endocytotic component labeled with FITC. Recycling receptors selectively accumulate in the tubular extensions of early endosomes, which then bud off to yield a population of transport vesicles that mediate recycling, whereas most ligands are concentrated in the vesicular parts of early endosomes (Geuze *et al.* 1987). The bulk volume containing the ligands is then routed through late endosomes to lysosomes or is recycled. Bafilomycin A1 completely inhibits the delivery of internalized protein to ECV without significantly affecting the internalization step (Claque *et al.* 1994). Moreover, early endosomes become highly tubular in the presence of bafilomycin A1 (D'Arrigo *et al.* 1997). In contrast to bafilomycin A1, nocodazole treatment of the cells results in inhibition of movement of early to late endosome, whereas the ECV will be recycled (Bayer *et al.* 1998, Miaczynska & Zerial 2002).

However, after treatment with the inhibitor bafilomycin, the ECV formation was inhibited at low pH in L929 cells, resulting in accumulation of FITC-24p3 protein in early endosome (Fig. 6*d*). Since the nocodazole blocks the 24p3 protein transport from ECV to late endosome, thereby preventing the recycling process, the protein accumulates at ECV showing high intensity of FITC fluorescence in flow cytometry detection. In contrast, FITC-24p3 protein diminished in the presence of nocodazole suggesting the disruption of microtubule network in L929 cells that would not diminish the level of 24p3 protein, preventing its forward movement to the late endosome. These results emphasized that 24p3 protein is recycled through exocytosis from ECV. Based on the above results, we hypothesize that recycling is a continuous process in which 24p3 protein is internalized and then routed back out of the cell.

Richardson (2005) has mentioned that both apo- and holo-24p3 proteins can be bound and internalized by its receptor, each resulting in different biological functions. At present, it is unclear whether apo- and holo-24p3 proteins are along the same tracking pathway. In conclusion, we have demonstrated that significant amounts of internalized apo-24p3 protein escape the lysosomal pathway and enter the recycling compartment for re-secretion. This finding is important in light of the role of apo-24p3 protein in cellular function and may provide a key understanding of its physiologic relevance in cells. The challenge for further studies is to characterize the holo-protein tracking pathway, and determine whether 24p3 protein plays a dual function in the cells.

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References

Akerstrom B, Flower DR & Salier JP 2000 Lipocalins: unity in diversity. *Biochimica et Biophysica Acta* **1482** 1–8.

Bayer N, Schober D, Prchla E, Murphy RF, Blaas D & Fuchs R 1998 Effect of bafilomycin A1 and nacodazole on endocytotic transport in Hela cells: implications for viral uncoating and infection. *Journal of Virology* **72** 9645–9655.

Bishop RE, Penfold SS, Frost LS, Holtje JV & Weiner JH 1995 Stationary phase expression of a novel *Escherichia coli* outer membrane lipoprotein and its relationship with mammalian apolipoprotein D. Implications for the origin of lipocalins. *Journal of Biological Chemistry* **270** 23097–23103.

Bratt T 2000 Lipocalins and cancer. *Biochimica et Biophysica Acta* **1482** 318–326.

Chen JW, Murphy TL, Willingham MC, Pastan I & August TJ 1985 Identification of two lysosomal membrane glycoproteins. *Journal of Cell Biology* **101** 85–95.

Chu ST, Huang HL, Chen JM & Chen YH 1996 Demonstration of a glycoprotein derived from the 24p3 gene in mouse uterine luminal fluid. *Biochemical Journal* **316** 545–550.

Claque MJ, Urbe S, Aniento F & Gruenberg J 1994 Vacuolar ATPase activity is required for endosomal carrier vesicle formation. *Journal of Biological Chemistry* **269** 21–24.

Cosson P, de Curtis I, Pouyssegur J, Griffiths G & Davoast J 1989 Low cytoplasmic pH inhibits endocytosis and transport from the trans-Golgi network to the cell surface. *Journal of Cell Biology* **108** 377–387.

D'Arrigo A, Bucci C, Ton BH & Stenmark H 1997 Microtubules are involved in bafilomycin A1-induced tabulation and Rab5-dependent vacuolation of early endosome. *European Journal of Cell Biology* **72** 95–103.

Davies PJA, Davies DR, Levitzki A, Maxfield FR, Milhaud P, Willingham MC & Pastan IH 1980 Transglutaminase is essential in receptor mediated endocytosis of alpha2-macroglobulin and polypeptide hormones. *Nature* **283** 162–167.

Devireddy LR, Teodoro JG, Richard FA & Green MR 2001 Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* **293** 829–834.

Devireddy LR, Gazin C, Zhu X & Green MR 2005 A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell* **123** 1293–1305.

Elangovan N, Lee YC, Tzeng WF & Chu ST 2004 Delivery of ferric ion to mouse spermatozoa is mediated by lipocalin internalization. *Biochemical and Biophysical Research Communications* **319** 1096–1104.

Flower DR 1996 The lipocalin protein family: structure and function. *Biochemical Journal* **318** 1–14.

Garay-Rojas E, Harper M, Hraba-Renevey S & Kress M 1996 An apparent autocrine mechanism amplifies the dexamethasone- and retinoic acid-induced expression of mouse lipocalin-encoding gene 24p3. *Gene* **170** 173–180.

Geuze HJ, Slot JW & Schwartz AL 1987 Membranes of sorting organelles display lateral heterogeneity in receptor distribution. *Journal of Cell Biology* **104** 1715–1723.

Johnson LS, Dunn KW, Pytowski B & McGraw TE 1993 Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Molecular Biology of the Cell* **4** 1251–1266.

Logdberg L & Wester L 2000 Immunocalins: a lipocalin subfamily that modulates immune and inflammatory responses. *Biochimica et Biophysica Acta* **1482** 284–297.

Miaczynska M & Zerial M 2002 Mosaic organization of the endocytic pathway. *Experimental Cell Research* **272** 8–14.

Richardson DR 2005 24p3 and its receptor: dawn of a new iron age? *Cell* **123** 1175–1177.

Rikihisa Y, Zhang Y & Park J 1994 Inhibition of infection of macrophages with *Ehrlichia risticii* by cytochalasins, monodansylcadaverine, and taxol. *Infection and Immunity* **62** 5126–5132.

Sandvig K, Tcnnessen TI, Sand O & Olsnes O 1986 Requirement of a transmembrane pH gradient for the entry of diphtheria toxin into cells at low pH. *Journal of Biological Chemistry* **261** 11639–11644.

Sapra P, Moase EH, Ma J & Allen TM 2004 Improved therapeutic responses in a xenograft model of human B lymphoma (Namalwa) for liposomal vincristine *versus* liposomal doxorubicin targeted via anti-CD19 IgG2a or fab fragments. **10** 1100–1111.

Wojnar P, Lechner M & Redl B 2003 Antisense down-regulation of lipocalin-interacting membrane receptor expression inhibits cellular internalization of lipocalin-1 in human NT2 cells. *Journal of Biological Chemistry* **278** 16209–16215.

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