ACCELERATED PUBLICATION

The increase in mitochondrial association with actin precedes Bax translocation in apoptosis

Ho Lam TANG*[1,2], Anh-Huy Phan LE†[2] and Hong Lok LUNG*[2]

*Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (SAR), People's Republic of China, and †Department of Molecular and Computational Biology, University of Southern California, Los Angeles, CA 90089, U.S.A.

Accumulating evidence indicates the potential role of actin cytoskeleton in facilitating the mitochondrial recruitment of various pro-apoptotic proteins from the cytosol to initiate apoptosis. In the present paper, we report the observation of the increase in mitochondrial association of actin in early apoptosis. Using cell fractionation and Western blot analysis, we found that mitochondrial accumulation of β-actin occurred before the mitochondrial insertion of Bax and release of cytochrome c in apoptosis. The mitochondrial accumulation of β-actin was observed with various apoptotic stimuli in various cell lines, suggesting that this is a general apoptotic phenomenon in mammalian systems. Using fluorescence microscopy, we have shown that an apoptotic induction triggered the reorganization of the F-actin (filamentous actin) network with an increase in the association with mitochondria, which was observed before mitochondrial fission and nuclear condensation. Perhaps actin could contribute to the initiation of apoptosis by enabling cytosolic pro-apoptotic proteins to be carried to mitochondria by the cytoskeleton-driven trafficking system.

Key words: actin, apoptosis, Bax, cytoskeleton, mitochondrion, translocation.

INTRODUCTION

Apoptosis, a type of programmed cell death, is an evolutionarily conserved biochemical pathway in controlling cell suicide that plays an essential role in regulating normal development and homoeostasis in multicellular organisms [1]. In addition to the metabolic centre, a mitochondrion is an important organelle in regulating apoptosis by receiving upstream pro-apoptotic protein signals from the cytosol, and releasing downstream cell executing factors from mitochondria to initiate apoptotic protease cascades for cell execution [2]. Despite the importance of the mitochondrial insertion of pro-apoptotic proteins in initiating apoptosis, it is still unclear how they translocate from the cytosol to mitochondria.

The cytoskeleton is mainly composed of interconnected networks of actin, tubulin and intermediate filaments, which extend throughout the entire cytoplasm of a cell [3,4]. Apart from structural support, the cytoskeleton functions in cell motility, mitosis and intracellular trafficking using cytoskeletal motors known as dyneins, kinesins and myosins. It is generally believed that dynein and kinesin provide long-distance transport from the periphery to the nucleus or vice versa through microtubules, while myosin provides local transport through the actin filament network [5]. The existence of functional collaboration between the motors and the cytoskeleton networks distributes cargos throughout the entire cell [5].

Accumulating evidence indicates potential linkages between cytoskeletal actin and apoptosis, even though the mechanism is not well understood [6]. The involvement of cytoskeletal actin in apoptosis has been suggested by some morphological studies which show actin cleavage during morphological apoptosis [7]. Studies using chemical inhibitors and genetic mutation to disrupt the cytoskeleton support further the involvement of actin in apoptosis [8,9]. Previous reports on the mitochondrial translocation of the actin-regulatory protein cofilin and the caspase-cleavage fragment of actin during apoptosis [10,11] inspired us to investigate the potential linkage between actin, mitochondria and apoptosis.

MATERIALS AND METHODS

Materials

Anti-β-actin and anti-Bak antibodies were purchased from Cell Signaling Technologies, anti-Bax antibody was supplied by Pharmingen, and anti-β-tubulin antibody was purchased from Calbiochem. All other reagents were obtained from Sigma, unless stated otherwise.

Cell culture

African green monkey renal epithelial COS-7 cells, human cervical carcinoma HeLa cells, and HEK-293T (human embryonic kidney) cell line (from the A.T.C.C.) were cultured as described previously [12]. Cells were seeded on tissue culture plates for 24–30 h until the cell density reached 70 % confluence before being subjected to each experiment.

Apoptotic induction

Apoptosis was induced by 1 μM STS (staurosporine) or 600 μM H_2O_2 in culture medium [Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL)] for the indicated times. For UV-induced apoptosis, old medium was replaced with fresh complete medium 3 h before treatment. The cover of the culture dish was removed, and the cells were exposed to UV irradiation (300 μW/cm^2) for 3 min. Cells after exposure to UV were incubated further at 37 °C for a specific length of time as indicated. Lastly, for serum-withdrawal-induced apoptosis, cells were washed twice with PBS at 37 °C,

Abbreviations used: ER, endoplasmic reticulum; F-actin, filamentous actin; STS, staurosporine.

[1] To whom correspondence should be addressed (email hogan@ust.hk).

[2] All authors contributed equally to this work.

then cultured in DMEM (Dulbecco's modified Eagle's medium) without serum for 5 days.

Subcellular fractionation

Subcellular fractionation was performed as described previously [13]. Briefly, cells were harvested and homogenized by passing the cell mixture through a 22-gauge needle. The homogenate was centrifuged at 1300 ***g*** for 10 min at 4 °C to pellet the nuclei and the unlysed cells. The supernatant was centrifuged further at 17000 ***g*** for 30 min at 4 °C to obtain raw cytosolic and heavy membrane fractions. The soluble cytosolic fraction was the supernatant collected after further centrifugation of the raw cytosolic fraction at 55000 rev./min for 30 min at 4 °C on a Bechman TLA 120.2 rotor. Membrane fractions of ER (endoplasmic reticulum) and mitochondria were purified from heavy membrane fraction by sucrose density-gradient centrifugation: heavy membrane pellet was resuspended in 1 ml of ice-cold MS buffer [210 mM mannitol, 70 mM sucrose, 5 mM Tris/HCl, pH 7.5, 1 mM EDTA and 1 % Complete™ protease inhibitor cocktail (Roche)] and laid on the top of 1.0, 1.2 and 1.5 M sucrose buffer gradient before being centrifuged at 25000 rev./min for 30 min at 4 °C on a Sorvall TST60.4 rotor. Sucrose-density-gradient-purified ER-containing and mitochondria-enriched fractions were collected at 1.0/1.2 M and 1.2/1.5 M interphases respectively, washed with MS buffer, and dissolved in 0.5 % (v/v) Nonidet P40 lysis buffer on ice for 30 min. Approx. 380 μg of mitochondrial fraction protein was recovered in 10^6 healthy or apoptotic COS-7 and HeLa cells, whereas 700 μg was recovered in 10^6 healthy or apoptotic HEK-293T cells.

Western blotting and signal quantification

Samples of 100 μg of protein per lane were separated by SDS/15 % PAGE and transferred on to a Hybond ECL® (enhanced chemiluminescence) membrane (Amersham Biosciences). After blocking, the membrane was incubated overnight at 4 °C with 1:1000 diluted primary antibody, and the signal was detected with the ECL® Western blotting detection system (Amersham Biosciences). For signal quantification, developed X-ray films of the Western blot analysis were scanned, and images were stored in a 24 bit grey-level depth. The signal region was determined by using the Canny edge detection algorithm for image segmentation, as described previously [14]. The strength of signal was extracted by calculating the difference between the average intensity of an identified area and its corresponding background.

Immunocytochemistry

Cells were grown to 70 % confluence on a coverslip. Mitochondria and the nuclei were stained with 50 nM MitoTracker Red CMXRos and 250 ng/ml Hoechst 33342 (Molecular Probes) respectively for 20 min, then the cells were washed twice with PBS and fixed with 3.7 % (w/v) paraformaldehyde for another 20 min. For staining F-actin (filamentous actin), cells were washed and fixed, permeabilized with 0.1 % (v/v) Triton X-100 for 15 min, and stained with Alexa Fluor® 488 Phalloidin (Molecular Probes) at a dilution of 200 μl of PBS/unit for 30 min. Slides were mounted by using the ProLong Antifade Kit (Molecular Probes).

Fluorescence microscopy

COS-7 cells served as the principle model in the present microscopy study owing to their spread morphology and in which the distribution of small organelles within the cells can be easily visualized. Cell images were captured with a monochromatic CoolSNAP FX camera (Roper Scientific) on an Axiovert 200M microscope using a 63× N.A. (numerical aperture) 1.4 Plan-Apochromat objective (Carl Zeiss). Fluorescence signals were analysed by using AxioVision 4 software (Carl Zeiss).

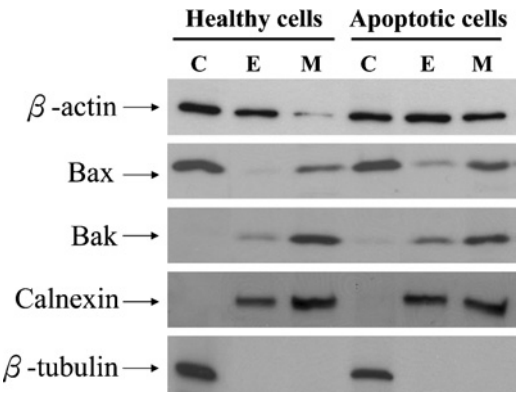

Figure 1 Mitochondrial translocation of β-actin in STS-induced HEK-293T cells

Western blot analysis of β-actin and Bax in cytosolic (C), ER-containing (E) and mitochondria-enriched (M) fractions of HEK-293T cells treated with or without STS for 2 h. The mitochondrial marker Bak indicates that the majority of mitochondria were present in the mitochondrial fraction, while the ER marker calnexin shows that ER was present in both the ER-containing and mitochondrial fractions. β-Tubulin served as a loading control for the cytosolic fraction.

RESULTS AND DISCUSSION

Protein analysis of β-actin in mitochondria after treatment with STS in HEK-293T cells

In order to investigate the potential role of cytoskeletal actin in apoptosis, HEK-293T cells were treated with STS, a general kinase inhibitor that induces caspase-dependent apoptosis [15], for 2 h. Then sucrose density-gradient centrifugation was performed to purify mitochondria from healthy or STS-treated HEK-293T cells for Western blot analysis of β-actin. Figure 1 shows that in healthy HEK-293T cells β-actin was present mainly in cytosolic and ER-containing fractions rather than in the mitochondrial fraction. In the apoptotic cells, β-actin level in the mitochondrial fraction was at least 3-fold higher than in the healthy cells. β-Actin expression remained relatively constant in cytosolic and ER-containing fractions before and after the apoptotic induction. This result contradicts those found by Chua et al. [10], but can be explained by the fact that their subcellular fractionation schema had a lower resolution by having only a two-step sucrose gradient, instead of the three-step gradient used in the present study, which does not allow for proper separation between the ER-containing fraction and the mitochondrial fraction. A pooling of our ER-containing and mitochondrial fractions would have also missed the increase in actin relocalization.

It has been reported that pro-apoptotic proteins such as Bax in the Bcl-2 family translocate from the cytosol to mitochondria in early apoptosis for triggering mitochondrial fission and releasing cell executing factors to the cytosol [2]. In the present study, we also show that Bax translocated to mitochondria after STS induction and, unexpectedly, the increased level of Bax in the mitochondria was moderate when compared with the dramatic increase in β-actin level (Figure 1). These data suggest that the actin level increases in mitochondria in HEK-293T cells after the STS-induced apoptosis and that seems to be an early event during apoptosis.

Increase in β-actin level in mitochondria is an early event in apoptosis

To verify the increase in actin in mitochondria in apoptosis, time-course analyses on the change of β-actin level were performed by

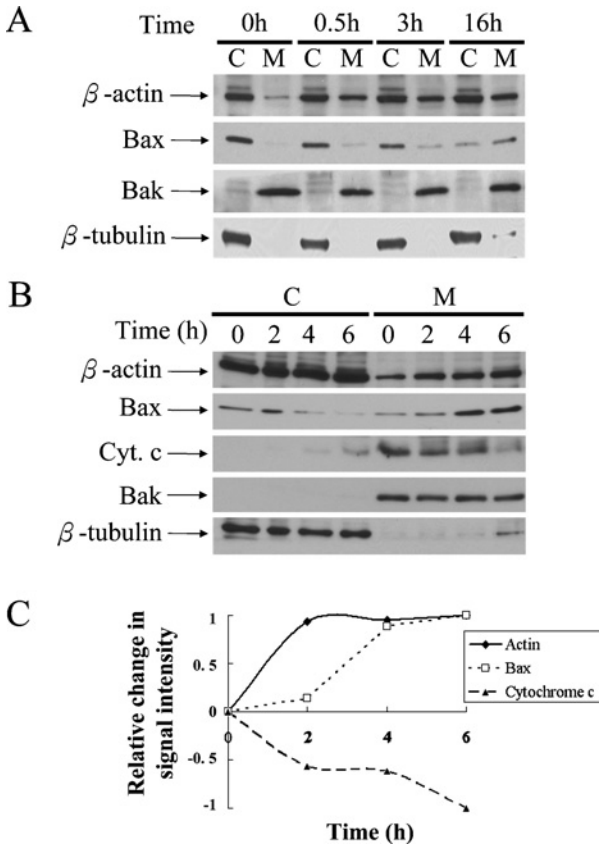

Figure 2 Mitochondrial translocation of β-actin in early apoptosis in STS-treated COS-7 cells and UV-exposed HeLa cells

(**A**) Western blot analysis of β-actin and Bax in cytosolic (C) and mitochondrial (M) fractions of COS-7 cells treated with STS for 0.5–16 h. Bak and β-tubulin served as loading controls for mitochondrial and cytosolic fractions respectively. (**B**) Western blot analysis of β-actin, Bax and cytochrome *c* (Cyt. c) in cytosolic and mitochondrial fractions of HeLa cells from 2 to 6 h after UV induction. (**C**) Quantification of mitochondrial translocation of β-actin and Bax, and cytochrome *c* release in the apoptotic HeLa cells induced with UV. The relative change in signal intensity was calculated as follows: relative change in signal intensity = (signal intensity at a given time − signal intensity at zero time)/(signal intensity at 6 h − signal intensity at zero time).

using two well-characterized models for studying apoptosis: STS-induced apoptosis in COS-7 cells and UV-induced apoptosis in HeLa cells [15,16]. For the first apoptosis model, COS-7 cells were treated with STS for 0.5–16 h, and cell fractionation was performed to enrich mitochondria from healthy and apoptotic COS-7 cells at different time points. The protein levels of β-actin and Bax in mitochondria and the cytosol were detected by Western blot analysis. Figure 2(A) shows that the β-actin level was significantly increased in the mitochondrial fraction after 30 min of the STS treatment, and the β-actin level remained high thereafter. This is in agreement with the increase in β-actin level in mitochondria of HEK-293T cells after treatment with the same apoptosis-inducing agent (Figure 1). Although the increase in mitochondrial accumulation of β-actin was saturated at 30 min after STS treatment, the level of Bax was increased slightly in mitochondria at 30 min and a higher level of Bax was observed in the mitochondrial fraction from 3 to 16 h (Figure 2A). The above data demonstrate that the increase in β-actin in the mitochondrial fraction occurred earlier than the mitochondrial insertion of Bax in STS-induced apoptosis.

A second model of apoptosis was studied by using HeLa cells treated with UV for 2–6 h, and results show a significant increase in β-actin in the mitochondrial fraction at 2 h after UV induction, whereas the Bax level remained relatively unchanged at that time point (Figure 2B). A significant increase in Bax in mitochondria was observed at 4 h followed by the reduction in mitochondrial cytochrome *c* level at 6 h after UV induction. In order to determine the relative rates of increase in β-actin and Bax levels in mitochondria, and the rate of cytochrome *c* release into the cytosol, the signal intensity of protein level was quantified and plotted (Figure 2C). These time-course results in HeLa cells show clearly that the mitochondrial accumulation of β-actin occurred much earlier than the mitochondrial insertion of Bax and release of cytochrome *c*, and this finding agrees with the time-course analysis of β-actin in STS-treated COS-7 cells (Figure 2A). Actin was thought to be involved in late apoptosis as it is cleaved by caspases into C-terminal 15 kDa and N-terminal 31 kDa fragments [7]. Ectopic expression of the 15 kDa actin fragment has been reported to induce cell shrinkage resembling apoptotic cells, and the fragment targets to mitochondria [11]. From the results of the two apoptosis-inducing systems in the present study, it is most likely that the translocation of actin to mitochondria is indeed an early initiation process during apoptosis, and the present study suggests an alternative role of actin in apoptosis.

Reorganization of the actin cytoskeleton in the early stage of apoptosis

To corroborate the mitochondrial translocation of β-actin in the initiation phase of apoptosis, F-actin in healthy and STS-treated COS-7 cells was visualized by fluorescence microscopy. F-actin ran longitudinally across the healthy cells, but 30 min after the apoptotic induction, the longitudinal axis of F-actin decreased significantly (Figure 3A). This was accompanied by the formation of cobweb-like F-actin which radiated from the nucleus to the cytosol (Figures 3B and 3C). After co-staining the healthy or STS-treated cells for their mitochondria, significant portions of the actin filaments were observed to co-localize with mitochondria in COS-7 cells after STS treatment (Figures 3B and 3C). Fluorescent signals from F-actin and mitochondria were quantified and the results show a significant increase in co-localization of F-actin with mitochondria in the apoptotic cells when compared with that in the healthy cells (Figures 3D and 3E). These fluorescence microscopy results strongly support the observation of the increase in β-actin level in mitochondria in the STS-treated COS-7 cells as detected by Western blot analysis (Figure 2A). Besides, typical features of apoptotically dying cells, such as mitochondrial fission, nuclear condensation and cell shrinkage [16,17], were not observed (Figures 3A and 3B) until 1.5 h after STS induction in COS-7 cells (results not shown). This indicates that the increase in mitochondrial association of F-actin is an early event in apoptosis. Furthermore, we have found that, after STS induction, cofilin translocated to the periphery of COS-7 cells and co-localized with F-actin (H. L. Tang, unpublished work). This is in agreement with a recent report by Mannherz et al. [18] in etoposide-induced apoptosis in NRK (normal rat kidney) cells. Cofilin is a key regulator of actin dynamics by creating new actin barbed ends for actin polymerization and is also responsible for the depolymerization of old actin filaments [19]. Reduction in cofilin protein levels with siRNA (small interfering RNA) resulted in the inhibition of both cytochrome *c* release and apoptosis [10]. Thus cofilin might play a role in early apoptosis via the cytoskeletal actin network reorganization.

Mitochondrial translocation of β-actin in response to various apoptotic stimuli

In order to determine the significance of mitochondrial translocation of actin in apoptosis, the effects of various apoptotic

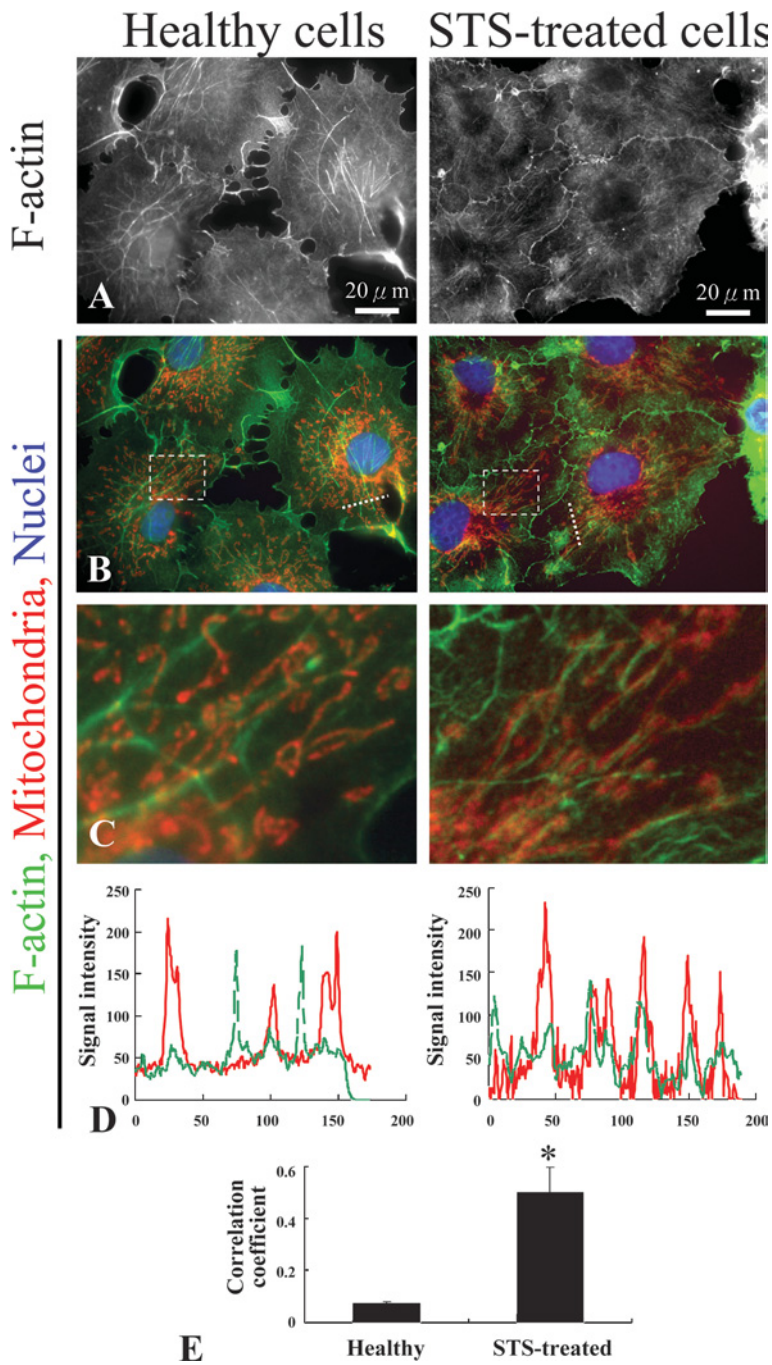

Figure 3 Increase in co-localization of F-actin and mitochondria at an early stage of STS-induced apoptosis in COS-7 cells as determined by fluorescence microscopy

(**A**) Monochromatic F-actin images of healthy and STS-treated (30 min) COS-7 cells. (**B**) F-actin, mitochondria and nuclei were stained with Phalloidin (green), MitoTracker (red) and H-33342 (blue) respectively. (**C**) Enlarged fluorescence images of the corresponding dotted boxes in (**B**). Images presented in (**A**)–(**C**) are representative of three experiments with similar results. (**D**) The fluorescent signal intensity of F-actin (green) and mitochondria (red) was quantified along the dotted line in (**B**). (**E**) Mean ± S.D. correlation coefficient of the fluorescent signals of F-actin and mitochondria in healthy or STS-treated cells in three independent signal-quantification measurements [including the measurement in (**D**) and two extra measurements from two other cells]. The correlation coefficient was calculated as described previously [24]. $*P < 0.01$; Student's t test.

stimuli were studied. Mitochondria were purified from healthy and apoptotic HeLa cells, and β-actin and Bax in the mitochondria were detected by Western blot analysis. Besides treatment with UV, an increase in β-actin level in the mitochondrial fraction was also observed when treated with other apoptotic stimuli such as STS, H_2O_2 and serum withdrawal (Figure 4). Bax was used as a positive control as to indicate the initiation of apoptosis. In response to the various treatments, the mitochondrial β-actin level in the apoptotic cells was approx. 2.5–3-fold higher than in the healthy cells and the increased levels of mitochondrial

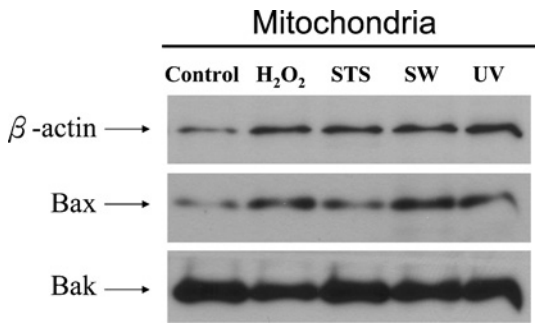

Figure 4 Mitochondrial translocation of β-actin in response to various apoptotic stimuli in HeLa cells

HeLa cells were treated with H_2O_2 for 5 h, STS for 3 h, serum withdrawal (SW) for 5 days or UV for 3 h. The protein levels of β-actin in mitochondrial fraction of the apoptotic cells were detected by Western blot analysis. The β-actin level in the healthy cells served as a control.

β-actin were quite similar. Taken together, these findings indicate that an increase in mitochondrial translocation of actin is a general phenomenon in apoptosis.

The present study provides a new insight into a potential mechanism of actin in signalling apoptosis. It is generally believed that mitochondrial translocation of pro-apoptotic factors depends on diffusion, as it has been shown that the representative pro-apoptotic factor Bax is a soluble protein that is found diffusely throughout the cytosol in healthy cells, but translocates to mitochondria in early apoptosis [16]. However, this general assumption cannot explain how Bax can diffuse to the mitochondria, as this movement is against the concentration gradient. It has been reported that the mitochondrial fission protein Drp-1 translocates from the cytosol to mitochondria [12] and associates with Bax at the mitochondrial fission site during apoptosis [20], whereas the mitochondrial recruitment of Drp-1 is suggested to be facilitated by cytoskeletal actin [21]. Besides, in healthy cells, other pro-apoptotic Bcl-2 family members, such as Bim and Bmf, are associated with the cytoskeletal motors dynein and myosin V respectively [22,23]. During apoptosis, they dissociate from their corresponding motors and insert into mitochondria [22,23]. Indeed, β-actin-deficient cells were reported to be resistant to certain cell-death-induction [9]. It is possible that actin could bridge the gap between mitochondria and the cytoskeleton-driven trafficking system for the recruitment to mitochondria of pro-apoptotic factors associating with the cytoskeleton or its motor proteins. Clearly, further investigation into the molecular mechanisms of this process is required.

We thank Dr Peng Li (Hong Kong University of Science and Technology) for the support of this work; Professor Ming Chui Fung (Chinese University of Hong Kong) for his critical reading and valuable discussion of this manuscript; Chun Wai Ma and Wing Keung Cheung (Hong Kong University of Science and Technology) for Western blot signal quantification; Hoi Yan Law (Carl Zeiss Far East Co. Ltd) and Ka Chun Wu (Hong Kong Observatory) for cell imaging analysis; and Lai Chun Cheung and Ho Man Tang for assistance in preparing this manuscript. This work was supported by the RGC (Research Grants Council) grant from the Hong Kong SAR Government to Dr Peng Li and the Hong Kong University of Science and Technology.

REFERENCES

1 Jacobson, M. D., Weil, M. and Raff, M. C. (1997) Programmed cell death in animal development. Cell **88**, 347–354
2 Wang, X. (2001) The expanding role of mitochondria in apoptosis. Genes Dev. **15**, 2922–2933
3 Hirokawa, N. (1982) Cross-linker system between neurofilaments, microtubules, and membranous organelles in frog axons revealed by the quick-freeze, deep-etching method. J. Cell Biol. **94**, 129–142
4 Svitkina, T. M., Verkhovsky, A. B. and Borisy, G. G. (1996) Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. J. Cell Biol. **135**, 991–1007
5 Mallik, R. and Gross, S. P. (2004) Molecular motors: strategies to get along. Curr. Biol. **14**, R971–R982
6 Gourlay, C. W. and Ayscough, K. R. (2005) The actin cytoskeleton: a key regulator of apoptosis and ageing? Nat. Rev. Mol. Cell Biol. **6**, 583–589
7 Mashima, T., Naito, M., Fujita, N., Noguchi, K. and Tsuruo, T. (1995) Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in Vp-16-induced U937 apoptosis. Biochem. Biophys. Res. Commun. **217**, 1185–1192
8 Posey, S. C. and Bierer, B. E. (1999) Actin stabilization by jasplakinolide enhances apoptosis induced by cytokine deprivation. J. Biol. Chem. **274**, 4259–4265
9 Li, J., Li, Q., Xie, C., Zhou, H., Wang, Y., Zhang, N., Shao, H., Chan, S. C., Peng, X., Lin, S. C. and Han, J. (2004) β-Actin is required for mitochondria clustering and ROS generation in TNF-induced, caspase-independent cell death. J. Cell Sci. **117**, 4673–4680
10 Chua, B. T., Volbracht, C., Tan, K., Li, R., Yu, V. C. and Li, P. (2003) Mitochondrial translocation of cofilin is an early step in apoptosis induction. Nat. Cell Biol. **5**, 1083–1089
11 Utsumi, T., Sakurai, N., Nakano, K. and Ishisaka, R. (2003) C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and targeted to mitochondria. FEBS Lett. **539**, 37–44
12 Frank, S., Gaume, B., Bergmann-Leitner, E. S., Leitner, W. W., Robert, E. G., Catez, F., Smith, C. L. and Youle, R. J. (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell **1**, 515–525
13 Spector, D. L., Goldman, R. D. and Leinwand, L. A. (1997) Cells: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
14 Canny, J. F. (1986) A computational approach to edge detection. IEEE Trans. Pattern Anal. Mach. Intell. **8**, 679–698
15 Bossy-Wetzel, E., Newmeyer, D. D. and Green, D. R. (1998) Mitochondrial cytochrome *c* release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J. **17**, 37–49
16 Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G. and Youle, R. J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. **139**, 1281–1292
17 Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer **26**, 239–257
18 Mannherz, H. G., Gonsior, S. M., Gremm, D., Wu, X., Pope, B. J. and Weeds, A. G. (2005) Activated cofilin colocalises with Arp2/3 complex in apoptotic blebs during programmed cell death. Eur. J. Cell Biol. **84**, 503–515
19 DesMarais, V., Ghosh, M., Eddy, R. and Condeelis, J. (2005) Cofilin takes the lead. J. Cell Sci. **118**, 19–26
20 Karbowski, M., Lee, Y. J., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C. L. and Youle, R. J. (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J. Cell Biol. **159**, 931–938
21 De Vos, K. J., Allan, V. J., Grierson, A. J. and Sheetz, M. P. (2005) Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission. Curr. Biol. **15**, 678–683
22 Puthalakath, H., Huang, D. C. S., O'Reilly, L. A., King, S. M. and Strasser, A. (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell **3**, 287–296
23 Puthalakath, H., Villunger, A., O'Reilly, L. A., Beaumont, J. G., Coultas, L., Cheney, R. E., Huang, D. C. S. and Strasser, A. (2001) Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science **293**, 1829–1832
24 Berenson, L. and Levine, M. (1999) In Basic Business Statistics: Concepts and Applications (7th edn), p. 793, Prentice-Hall, Upper Saddle River

Received 10 February 2006/13 March 2006; accepted 15 March 2006
Published as BJ Immediate Publication 15 March 2006, doi:10.1042/BJ20060241