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Extramitochondrial Localization of Mortalin/mthsp70/PBP74/GRP75

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Subcellular fractionation and immunofluorescence microscopy were used to identify the specific sites of intracellular residence of mortalin, also called a mitochondrial homologue of the hsp70 family, in immortal human cell lines previously assigned to four distinct complementation groups (A–D) for indefinite cell division. In addition to the mitochondria it was seen in the endoplasmic reticulum (ER) fractions of all the cell lines analyzed. Interestingly, three of the group A cells lines (EJ, GM639, and HT1080), in addition to the mitochondria and ER, exhibited cytosolically (extra-organelle) localized pool of mortalin. These findings demonstrate that mortalin is not present exclusively in mitochondria. Its residence in different organelles may be the basis of differential distribution observed previously in different human cell lines.

Key Words: **cell immortalization; mortalin; intracellular localization.**

Mortalin is a member of hsp 70 family of proteins. It was first cloned from a comparative protein screen between normal and immortal mouse cells. Whereas normal murine cells have a pancytosolic immunostaining pattern, that excluded nucleus, immortal cells showed a perinuclear concentration of the protein (1, 2). Normal human cells also show a pancytosolic immunostaining of mortalin and immortal human cells exhibited one of the four non-pancytosolic staining patterns which interestingly were unique to the members of each of the four complementation groups for immortality (3). It has been established that the limited proliferative potential of normal cells in culture, cellular senescence, is dominant over cell immortality (4, 5) and that there are a limited number of genetic events or biochemical pathways that result in immortalization (6–11). Involvement of mortalin in one or more of these biochemical pathways that regulate proliferative potential of cells, has been suggested by various data. Cell hybrids from two cell lines that complement to yield mortal cells were seen to have pancytosolic mortalin staining pattern (Pereira-Smith, unpublished data). The pattern of mortalin immunostaining in immortal human cells was reversed to the pancytosolic type, typical of normal cells, upon induction of the senescence phenotype by the introduction of normal chromosomes, chromosome fragments or genes or by treatment with 5-Bromodeoxyuridine (12–14).

In case of mouse, two distinct transcripts, mot-1 (mouse cDNA encoding pancytosolic protein) and mot-2 (mouse cDNA encoding perinuclear protein) have been cloned. These are shown to be different only by two amino acid residues in the carboxy-terminus of the protein but have contrasting biological activities. Mot-1 induced senescence of NIH 3T3 cells whereas mot-2 induced malignant transformation of the latter (15, 16). Recently, it has been shown that the malignant phenotype of these cells is mediated, at least in part, by inactivation of wild type p53 that is shown to interact with mortalin (17). Interestingly, coimmunolocalization of mortalin and p53 was found in immortal, but not in normal, mouse and human cells (17).

Only one kind of mortalin transcript has so far been cloned from human cells by independent groups; this is also called PBP74 (an antigen presenting protein), GRP75 (glucose regulated protein), mthsp70 (mitochondrial hsp70) (18–21). Therefore there is no evidence for differences in the primary structure of the molecule in immortal human cells to account for the different staining patterns. Studies involving immunostaining and microscopical analyses have assigned the protein to various sites in cell (2, 18, 22, 23). Some studies have assigned it exclusively to mitochondria (21, 24). Our interest was to investigate whether the differential staining patterns observed for different hu-

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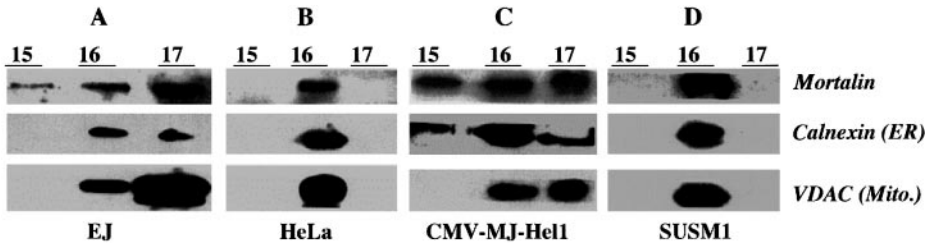

FIG. 1. Western blot analysis of Percoll gradient fractions. Representative cells assigned to four complementation groups (A-EJ, B-HeLa, C-CMV-MJ-Hel1, and D-SUSM1) were subjected to fractionation by Percoll gradient as described under Material and Methods. Fractions were analysed for the presence of mortalin, ER marker-calnexin, and mitochondrial marker-VDAC. Mortalin sedimented in fraction 16 in all the cells (panels A–D), and also fractions 15 and 17 in EJ and CMV-MJ-Hel1 (panels A and C). Fractions 16 and 17 were positive for the mitochondrial as well as ER markers (panels A–D) and fraction 15 was positive only for the ER marker (panel C) indicating that in addition to the mitochondria, mortalin is present in the ER.

man cell lines represented altered mitochondrial distribution or localization of the molecule at different subcellular sites. Therefore we used biochemical fractionation and immunomicroscopy to determine the subcellular residence of mortalin/PBP74/GRP75 in immortal human cells assigned to the different complementation groups.

MATERIALS AND METHODS

Cell fractionation by Percoll gradient. Cells were grown as monolayers to 70–90% confluency in 100 mm-diameter dishes as described (9) and were subjected to Dounce homogenization in 1 ml of HSE Buffer (10 mM HEPES, pH 7.4, 0.25 M sucrose, 5 mM EDTA). The post-nuclear supernatant (3000 × g) was diluted to a final concentration of 35% Percoll. It was layered on a 1 ml cushion of 2M sucrose on which 1.3 ml fractions of 30%, 27.5% 25%, 22.5%, 20%, and 17.5% Percoll (Pharmacia) in HS buffer (HSE buffer without EDTA) were applied. Centrifugation was performed at 36,000 rpm for 2 h in a Beckman SW41 rotor at 4°C. Following centrifugation, the gradient was fractionated from the top of the tube, at a setting of 20 drops/fraction, with the aid of a Densi-Flow II apparatus (Buchler Instruments) and Bio-Rad Model 2110 Fraction Collector. An aliquot from each was subjected to Western blot analysis with anti-mortalin, anti-calnexin or anti-VDAC antibody.

Cell fractionation by sucrose/deuterium oxide gradient. The post-nuclear supernatant as described above was applied to the top of a shallow discontinuous sucrose gradient in buffered deuterium oxide as previously described (25), without prior isolation of vesicles. Both gradients were fractionated from the top of the tube with a Buchler Densi-Flow apparatus. Fractions were collected and were subjected to Western analyses as described below.

Western blotting. Protein fractions were separated on SDS-polyacrylamide gels and were electrophoretically transferred to nitrocellulose membrane by semi-dry transfer using Tris-Glycine buffer for 20 mins. The blots were incubated with one of three anti-mortalin antibodies: a polyclonal rabbit antibody to the entire protein (1) that specifically recognized mortalin and did not cross react with other hsp70 family members, a goat polyclonal antibody to the C-teminus of the protein (Santa Cruz Biotechnology) and a mouse monoclonal ascites (Stress Gen Biotechnologies). The results presented in the figures are with the Stress Gen antibody. The anti-calnexin antibody was obtained from Stress Gen Biotechnologies and the anti-VDAC antibody was a kind gift from Dr. W. Craigen, Baylor College of Medicine. The proteins were visualized by incubation with HRP-conjugated secondary antibodies.

Confocal microscopy. Cells were plated on glass coverslips in 35 mm tissue culture dishes. Approximately 48 h later, to 75–80% confluent cultures 3 μM Mito-track was added for 10 min. The cells were then washed with phosphate buffered saline, fixed with 3% paraformaldehyde and submitted to mortalin staining,using the polyclonal antibody to the entire mortalin protein, as described previously (1, 3). Microscopy was performed using an Applied Precision DeltaVision microscope (Issaquah, WA) fitted with an Olympus IX70 microscope. Images were acquired via wide field sectioning employing fluorescent light. The stacked images, usually 20–25 sections, were subjected to point spread function analysis for better image quality, on Silicon Graphics software (SGI, Mountain View, CA). Magnification of all cells was maintained at 600×. Mortalin was seen as red, Mito-track as green and areas of overlap as yellow.

Immunoelectron microscopy. Fibrosarcoma (N-ras+) (HT1080) cells belonging to Complementation Group A of immortalization grown to about 70% confluency were fixed with 4% paraformaldehyde and 0.5% glutarldehyde in 0.1 M PBS (pH 7.2) at 4°C for 2 h. These cells were embedded in 10% gelatin in 0.1 M PBS followed by immersion in 20% polyvinylpyrrolidone and 1.84 M sucrose in 0.1 M PBS for 2 h and were frozen in liquid nitrogen. Cryosectioning was done using a Reichert FC4E (Reichert-Nissei) at −100°C. Sections were blocked with 2% BSA for 30 min, and then incubated with the anti-mortalin antibody (1:100 dilution in 1% BSA/50 mM TBS, pH 7.5) for 2 h. After washing with 1% BSA/TBS, sections were incubated with a goat anti-rabbit IgG antibody (1:40 dilution in 0.1% BSA/TBS) conjugated with 10-nm colloidal gold particles (British Biocell International Ltd.) for 1 h. After washing with 0.1% BSA/TBS, sections were fixed with 1% glutarldehyde in TBS for 10 min, and washed with distilled water. Sections were stained with 3% uranyl acetate and embedded with 3% polyvinylalcohol and 0.3% uranyl acetate. After preparation, the sections were viewed and photographed with a Hitachi H-7000 transmission electron microscope at 100 kV.

RESULTS AND DISCUSSION

A physical separation of sub-cellular organelles by partial floatation in shallow, discontinuous Percoll gradients was first attempted. Western blotting with anti-mortalin antibody showed that it separates in fractions 15–17 in some cells (EJ and CMV-MJ-Hel1) (Fig. 1, panels A and C) and only fraction 16 in others (HeLa and SUSM1) (Fig. 1, panels B and D). We used antibodies against VDAC (voltage dependent anion channels) (26) and calnexin (27) to detect the separation of the mitochondria and ER, respectively. As seen in Fig. 1, no clear separation of mitochondria and ER was obtained for most of the cells by this protocol. Both 16 and 17 fractions were positive for mitochondrial and ER markers and therefore did not provide any conclusive hints to the residence of mortalin in these cells.

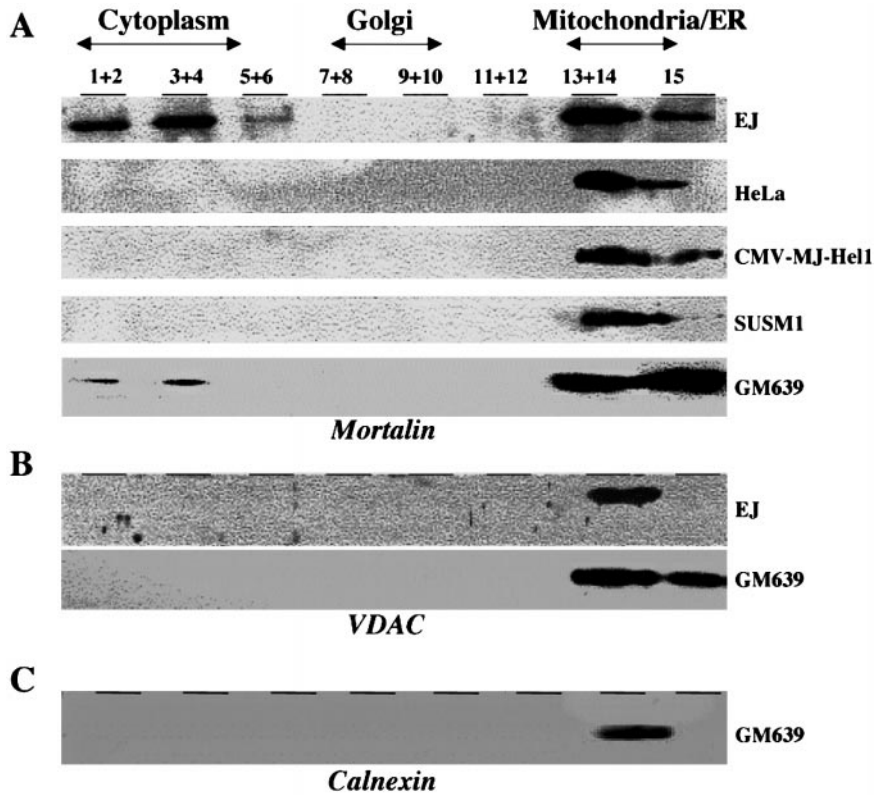

FIG. 2. Western blot analysis of sucrose/deuterium oxide gradient fractions. Fractions obtained from indicated cells by a shallow discontinuous sucrose gradient in buffered deuterium oxide were subjected to Western blotting stained with anti-mortalin or anti-VDAC antibody. Mortalin was detected in fractions 13 to 15 in all the cell lines. These fractions were positive for the mitochondrial and ER markers (panels B and C). EJ and GM639 cells showed sedimentation of mortalin in fractions as early as 1 to 4 (panel A). These fractions were negative for the mitochondrial (panel B) as well as ER markers (panel C). Fraction 15 of EJ cells (mortalin positive and VDAC negative) revealed ER resident pool of mortalin and fractions 1–4 represented nonmitochondrial and nonER pool of mortalin.

However, fraction 15 of EJ cells was mortalin positive but devoid of calnexin as well as VDAC staining. Signal for calnexin and VDAC was 2–4 fold brighter than for mortalin in fractions 16 and 17 (Fig. 1, panel A), therefore, it is unlikely that the absence of calnexin and VDAC reactive bands in fraction 15 was due to less loading. This was indicative of an existence of a pool of mortalin that does not associate with the mitochondrial or ER residence. Similarly, on the basis of calnexin positivity, mortalin positive fraction 15 of CMV-MJ-Hel1 could be called as the ER fraction (Fig. 1, panel C) and indicated its localization in ER. This experiment, although far from being conclusive for precise assignment of mortalin to specific cellular sites, provided hints that mortalin exists in more than just one site. Besides its mitochondrial site, there is evidence for its ER localization in CMV-MJ-Hel1 cells and also non-mitochondrial, non-ER site(s) in EJ cells. Such different intracellular pools of mortalin may also be the basis of differential immunostaining patterns of the protein previously observed for different complementation groups.

We next subjected the cells to another fractionation protocol involving density equilibrium centrifugation of vesicles in shallow, discontinuous sucrose/deuterium oxide gradients. Consistent with its residence in either mitochondria or ER microsomes, mortalin was detected at the bottom of the gradient (fractions 13–15) following centrifugation in all the four cell lines (EJ, HeLa, CMV-Mj-Hel1 and SUSM1; Fig. 2A). However, in EJ cells some of the total mortalin population remained at the site of application on top of the gradient (Fig. 2A). To determine whether this was unique to EJ cells or typical of group A cell lines we analyzed another cell line, GM639 that has been assigned to the group A by genetic studies (9) and by pattern of mortalin immunostaining (juxtanuclear cap) (3). Similar to EJ, GM639 cells also showed mortalin in the early fractions that represent organelle free cytosolic fraction (Fig. 2A). Because of its limited detection in cell lines assigned to complementation group A only, it appeared unlikely that the cytosolic mortalin could reflect leakage from the mitochondria or ER during cell homogenization. Furthermore, we confirmed that this phenomenon represented cytosolic residence of mortalin rather than the fragility of mitochondria or ER by the absence of VDAC (Fig. 2B) and calnexin (Fig. 2C) in these fractions. These data indicated that intracellular

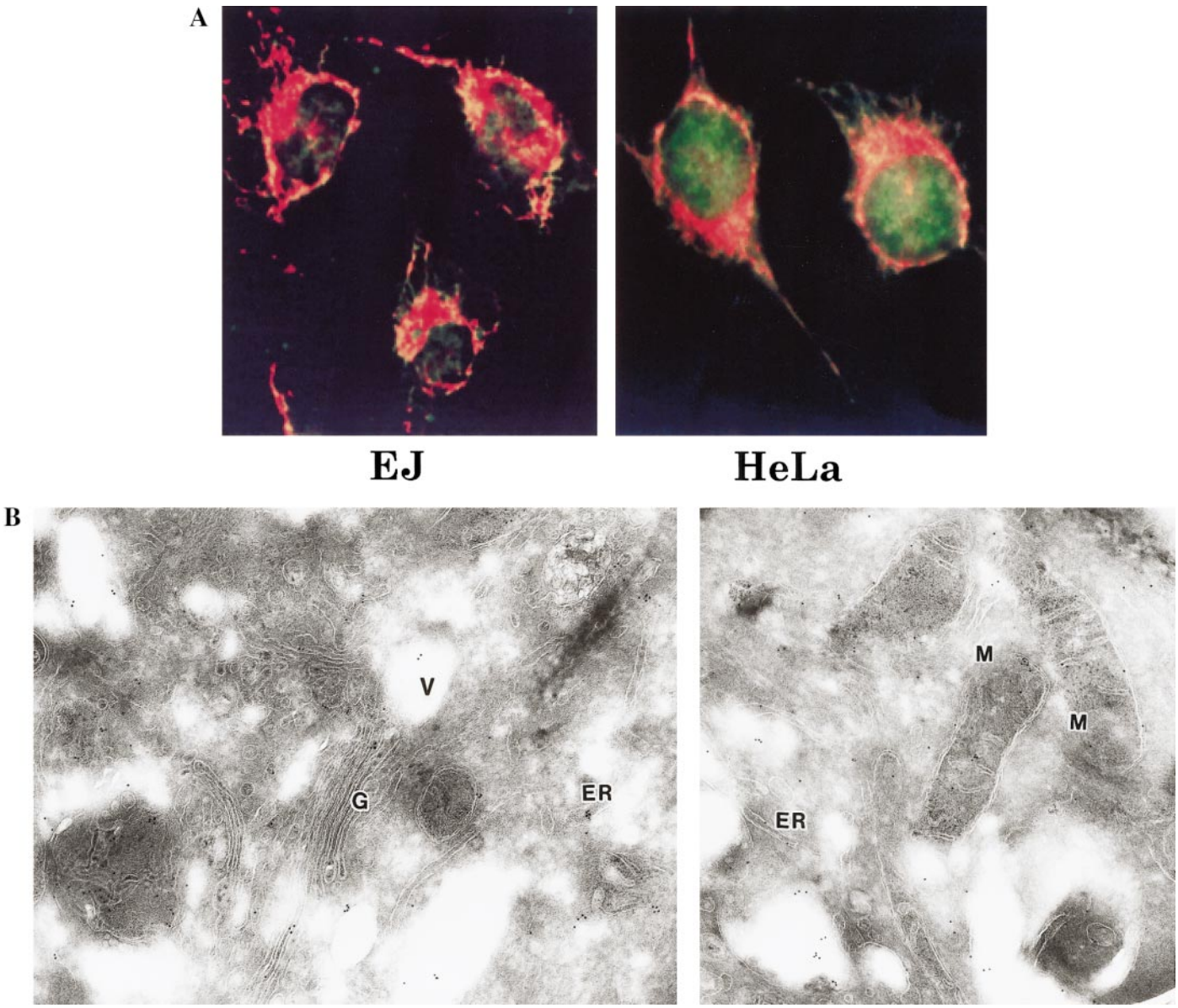

FIG. 3. Microscopical localization of mortalin. (A) Confocal microscopical analysis of mortalin and mitochondrial marker Mito-track. Mortalin was visualized by immunostaining with anti-mortalin antibody and secondary staining with TRITC (red)-conjugated antibodies. Mitotrack staining was seen as green fluorescence. Areas of overlap were seen as yellow. As indicated by the yellow color, mortalin and mitochondrial staining was seen to overlap in EJ cells and HeLa cells. In addition, in both the cell lines there was a significant amount of red staining accounting for nonmitochondrial pool of mortalin. (B) Immunoelectron microscopical analysis of mortalin in HT1080 cells. Mortalin specific staining was detected in mitochondria (M), endoplasmic reticulum (ER), cytoplasmic vesicles (V), and cytosol. Magnification: left panel, ×42,500 and right panel, ×37,500.

niche of mortalin is not restricted to mitochondria and that the cytosolic localization of mortalin may be unique to immortal cell lines assigned to this group.

As shown in Figs. 1 and 2, clear fractionation of the mitochondria and ER was difficult for most of the cell lines used. Therefore double immunostaining for mortalin with the mitochondrial marker, Mito-track and the ER marker DioC6 was performed. In EJ and HeLa cells most of the mitochondrial stain (green) overlapped with mortalin (red) and was seen as yellow color in overlay images. However there was still significant amount of mortalin staining (red) that did not colocalize with mitochondria (Fig. 3A). Similar results were obtained with ER marker, DioC6 (data not shown). Taken together with the biochemical data, it was apparent that mortalin resides in more than just one site in cell. Some other studies have assigned this protein to mitochondria, ER, golgi, cytoplasmic vesicles, cell surface, plasma membrane (20, 21, 23, 24, 28) in different cells but have not been well supported by cell

fractionation assays. Our data demonstrate for the first time that in human cells mortalin localization includes mitochondria, ER and cytosol and this may form the basis of differential distribution of the protein in different cell lines assigned to different complementation groups of immortalization. The fact that we used three independent antibodies in our studies eliminates the possibility that the results are due to the cross-reaction of the anti-mortalin antibody with other proteins. Additionally, we did isolate stable clones of the various cell lines expressing an HA-tagged mortalin protein and obtained the same confocal microscopy results using an anti-HA antibody (data not shown).

As described above, two cell lines (EJ and GM639) assigned to complementation group A exhibited three sites of mortalin residence. We also performed immuno-electronmicroscopy in another member (HT1080) of complementation group A and obtained evidence to its localization in the mitochondria, ER and cytosol/cytoplasmic vesicles (Fig. 3B).

Our studies have identified mitochondria and ER as two major sites of mortalin localization in all cell lines. The relative amount of protein in the mitochondria and ER in different cells may form the basis of differential immunostaining patterns. Besides, its cytosolic residence that has been identified in three of the complementation group A-members may contribute to this phenomenon. How mortalin is targeted to ER rather than mitochondria is not understood at present. However, localization of mortalin in ER is substantiated by another approach in which we have isolated mortalin interacting proteins. This has led to the identification of gp96, an ER protein, suggesting that these two proteins may interact in ER (unpublished observations). In addition, mortalin has been found to interact with interleukin receptor type-I (29) and fibroblast growth factor-1 (30). Such studies support extra-mitochondrial localizations of mortalin. Interestingly, some other proteins that were assumed to be mitochondrial, have recently been detected in non mitochondrial locations including ER, golgi, peroxisomes and secretory vesicles [*reviewed in* 31).

Our finding that a pool of mortalin is localized in the cytosolic fraction of complementation group A members raises the question of whether this is the final intracellular location for this population of molecules or merely represents a cytosolic intermediate en-route to ER. A recent report describes the distribution of fumerase protein between mitochondria and cytosol in yeast (32). Their model suggested that the targeting and distribution occur cotranslationaly and involves folding and retrograde movement of the processed protein back through the translocation pore. Another possibility is that the differential pool of mitochondrial mortalin and consequently its location in other subcellular residences is due to the differential mitochondrial membrane potential of the various immortal cell lines. It has been shown that normal and transformed cells have substantial qualitative differences in their mitochondrial membrane potential (33, 34) leading to the differential uptake and retention of cationic fluorochrome Rh-123 (35). Although, Rh-123 uptake among various cell lines has been shown to differ qualitatively and quantitatively (36), studies have not been performed on a sufficiently large number of human transformed cells assigned to different complementation groups to make this study conclusive. However, in support of this is the report demonstrating that senescence induced by p53 is accompanied by reduced mitochondrial membrane potential (37). Other possible explanations include altered chaperone activity of mortalin or altered interactions with other proteins, such as those associated with the cytoskeleton. Both of these can potentially affect cell division. For example, the stress response of cells from different species has been found to relate to their *in vitro* population doubling potential (T. Kirkwood, personal communication) and changes in actin cytoskeleton have been observed during adenovirus infection and malignant transformation. A recent report of a kinesin knockout mouse described a change in mitochondrial pattern from cytoplasmic to perinuclear (38). Thus, in addition to differential trafficking of mortalin, changes in structural proteins such as kinesin, tubulin and others may, as a result of the immortalization process, alter the intracellular location of mortalin. Further studies on the mechanism of altered trafficking of mortalin between cell lines in the different complementation groups, as well as the involvement of structural protein, warrants further investigation. The results should provide insights into the molecular differences and thereby the mechanisms involved in immortalization of human cells by one of the four pathways.

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