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ON THE CYTOSOLIC AND PERINUCLEAR MORTALIN: AN INSIGHT BY HEAT SHOCK

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SUMMARY: We have identified, cloned and characterized a 66-kD protein from cytosolic fractions of mouse embryonic fibroblasts and named it mortalin (Wadhwa *et al.*, J. Biol. Chem., **268**, in press, 1993). Immortal fibroblasts were seen to harbor the same or very similar protein, however, localized in the perinuclear locale. The present report is on the differentially localized forms of p66 protein which are biochemically and structurally found to be widely the same. In fact, heat shock treatment could translocate the cytosolic form to the perinuclear position without any detectable biochemical modification. The observed phenomenon adds to the unique identity of mortalin in hsp70 family. Besides, it confers that only the minor differences in the protein probably enroute its differential cellular distribution and the associated function.

Common to all normal diploid cells is their limited division potential in culture which has been widely accepted as a representation of organismic ageing at cellular level (1). Since the first demonstration of this phenomenon in human fibroblasts by Hayflick and Moorhead (2), wide variety of cell assay systems have been employed which suggest the genetic routes of limited replication potential (3-11). More than two hundred changes in biological activity have been shown to occur in cultured normal human fibroblasts as they age *in vitro* (12). Many differentially expressed mRNA and proteins have been isolated from serially passaging cells in culture (reviewed in 13). More recently, human chromosome 1 and 4 (14, 15) and the genes such as *RB*, *c-fos*, *p53* (16-20) have been shown to involve in the control of cell proliferation. However, the precise roots and mechanism of cellular mortality remain hidden so far.

We have employed the cell fusion assay among mortal and spontaneously immortalized clone of mouse embryonic fibroblasts (MEF) to establish the system of natural and conditional ageing which supported the dominant routes of mortality over immortalization (21). The further strategy was to trace the dominant gene expression which is conserved in mortal normal and

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hybrid cells in contrast to its absence in immortal cells. In line with this concept, we traced a 66-kD protein, mortalin, in the cytosolic fractions of normally ageing, MEF and conditionally ageing, hybrids between MEF and MN48-1 cells (22). We have cloned and characterized it to be a novel member of hsp70 family. Further we observed that the protein is differentially localized in immortal cells rather than the absence. The phenomenon of differential distribution of protein is well conserved during immortalization of fibroblasts from three different strains of mouse as well as human fibroblasts (23). The observations apparantly suggested the involvement of protein during cellular mortality and immortalization. Besides, these urged us to delineate the differences between the cytosolic and the perinuclear forms of the protein and to see the heat shock effects. The present report deals with the attempts to differentiate mortalin from mortal and immortal mouse fibroblasts. However, most of the classical biochemical assays have not been informative. On the other hand, cytosolic form of the 66-kD protein in MEF could transiently be dislocated to perinulcear locale by heat shock without any apparent chemical modification indicating that only the minor differences in protein structure could be important for its differential distribution and associated function.

MATERIALS AND METHODS

Cell culture: Mouse embryonic fibroblasts (MEF) were isolated from CD1-ICR, and Balb/c strains of mouse essentially as described in Wadhwa *et al.* (21). Culture conditions, cell hybrids generation between MEF and ouabain- and 6-thioguanine-resistant clone of NIH 3T3 cells (MN48-1) and selection are also detailed there in.

Cell labelling: Cells grown to about 80% confluency in 6-cm dishes were labeled with L-[^{35}S]methionine (50μCi/ml) for 6-8 h in methionine-free Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal calf serum. The cell pellet was lysed in Nonidet P-40 lysis buffer (20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 1% Nonidet P-40) for 30 min on ice and the lysate was obtained by centrifugation at 100,000 x *g* for 20 min at 4 °C. The lysate containing 10^6 cpm of trichloroacetic acid-precipitable counts was used for each reaction.

ATP binding Assay: ATP-agarose (C-8: Sigma) was soaked in phosphate-buffered saline (PBS) overnight at 4 °C followed by 3-4 washings with PBS. L-[^{35}S]Methionine labeled cell lysate (3 X 10^7 cpm) was incubated with ATP-agarose slowly rotating at 4 °C overnight. Bound fraction was sequentially eluted by 0.5 M NaCl, 1 mM GTP and 5 mM ATP in PBS. Each fraction was subjected to immunoprecipitation with excess of anti-p66 antibody (22). Immunocomplexes formed upon incubation with the antibody for 2-3 h on ice were adsorbed to protein A-Sepharose beads and washed thrice with lysis buffer. The complexes bound on the beads were boiled in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris [pH 7.0], 15% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue) or urea lysis buffer [9.5 M urea with 2% w/v Nonidet P-40, 1.6% ampholine (pH 5-8) (Pharmacia), 0.4% ampholine (pH 3-10) and 5% β-mercaptoethanol] and analyzed on one- or two-dimensional 7.5% SDS-polyacrylamide gel. Following electrophoresis, gels were treated with Enlightning solution (Amersham), dried and exposed to an XAR-5 film (Kodak).

V8 protease digestion: Immunoprecipitated p66 protein from L-[^{35}S]methionine labeled cell lysates (10^7 cpm per reaction) was separated on 7.5% polyacrylamide gel. Wet gel was exposed to X-ray film for 8 days. p66 protein was excised from the gel by overlapping with autoradiogramme, and was subjected to one-dimensional peptide mapping with *Staphylococcus aureus* V8 protease (24).

Northern Blot Analysis: Total cellular RNA was extracted and separated on denaturing agarose gel (25). The ultraviolet-cross linked membranes were hybridized with ^{32}P-labeled DNA probes at 65 °C for 12-16 h. Probes used were 2.1-kilobase pair *mot-1* cDNA (22) and 1.7-kilobase pair *PstI* fragment of pA1 as a β-actin specific probe (26).

Heat shock and Immunostaining: For indirect immunofluorescence studies, cells were

plated on poly-D-lysine coated glass coverslips and heat shocked at 42 °C for 1 h in water bath by immersing the coverslips in 60-cm dishes containing prewarmed medium. Cells were fixed with prechilled methanol/acetone (1:1, v/v) mixture for 5 min on ice, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 2% bovine serum albumin (BSA) in PBS for 20 min. They were incubated with anti-p66 antibody (23) (1:600 in PBS with 2% BSA) for 2-3 h at room temperature, washed with PBS with 0.1% Triton X-100 and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (IgG) for 30 min. After 6-8 washing in PBS with 0.1% Triton X-100, cells were overlaid with a cover slip with Fluoromount (Difco) and were examined on an Olympus BH-2 microscope with epifluorescence optics and photographed with Kodak Tri-X pan 400 film.

RESULTS AND DISCUSSION

Cytosolic and perinuclear distribution of p66 protein in mortal and immortal fibroblasts detailed in Wadhwa *et al.* (23) can be seen in Fig. 3. To distinguish the differentially localized p66 protein, the following biochemical assays were performed. MEF and NIH 3T3 cells were labeled with ^{32}Pi for 3-4 h and the cell lysate in Nonidet P-40 buffer (5 X 10^6 cpm) was subjected to immunoprecipitation with anti-p66 antibody. Immunoprecipitated products were separated on 7.5% polyacrylamide gel. ^{32}P-labeled p66 protein was not observed upon autoradiography of the dried gel (data not shown), whereas under the same conditions of immunoprecipitation, p66 protein and its co-immunoprecipitant, actin, were apparent when L-[^{35}S]methionine labeled cell lysates were used indicating that phosphorylation is not involved during cytosolic or perinulcear distribution of p66 protein. *In vitro* autophosphorylation capacity of the protein was checked as described by Csermely and Kahn (27). p66 protein was immunoprecipitated with excess of anti-p66 antibody from the MEF and NIH 3T3 cell lysates containing 200 μg total cellular protein. The immunoprecipitated protein samples were incubated with 10 - 50 μCi [γ-^{32}P]ATP and $MgCl_2$ (10 mM) for 30 min at 30 °C. The reaction was stopped by the addition of EDTA and DTT to 10 and 20 mM respectively. Samples were boiled in SDS sample buffer, separated on SDS-polyacrylamide gel electrophoresis and visualized by autoradiography of the dried gel. No autophosphorylating activity could be detected in either case (data not shown) indicating the lack of kinase type activity in either of the forms of the protein. Further we checked the ATP binding activity of the protein by its ability to bind to ATP agarose. L-[^{35}S]Methionine labeled cell lysates from MEF and NIH 3T3 cells were incubated with ATP-agarose matrix. Bound proteins were eluted by NaCl, GTP and ATP. Each fraction was subjected to immunoprecipitation with excess of anti-p66 antibody. As can be seen in Fig. 1, most of the p66 protein was not bound to ATP-agarose. Its inability to bind ATP indicated the lack of ATPase activity. We have earlier characterized the p66 protein as a member of hsp70 family (22), however, like most of the hsp70 members (28) it may not have ATPase activity.

Upon one-dimensional peptide mapping with *Staphylococcus aureus*V8 protease, exactly similar digestion pattern was obtained from MEF and NIH 3T3 cells (Fig. 2A). The transcript as analyzed by northern analysis with *mot-1* cDNA exhibited the same size from MEF and NIH 3T3 cells (Fig. 2B). The data suggested that the cytosolic and the perinuclear forms do not differ widely or in other words, either the minor structural differences or some other factors are

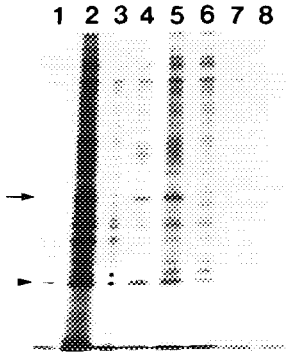


Fig. 1. ATP binding assay for p66 protein. Lanes 2, 3, 4 and 5 show the anti-p66 antibody immunoprecipitants from L-[^{35}S]methionine-labeled MEF cell lysate, from agarose bound fraction, from agarose unbound fraction and ATP-agarose unbound fraction,respectively. ATP-agarose bound proteins were eluted with 0.5 M NaCl, 1 mM GTP and 5 mM ATP in PBS (lane 6, 7 and 8,respectively) and subjected to immunoprecipitation with excess , ten times than the normal (lane 1), of antibody (lane 2-8). p66 protein is indicated by arrow and the coimmunoprecipitant by an arrowhead.

involved for the differential subcellular localizaton of the protein. Cloning and sequence analysis had characterized the protein as a novel member of mouse hsp70 family (22). It shares 68% homology to mouse heat shock related 70; 69% homology to mouse heat shock cognate 70 and 80% homology to yeast mitochondrial heat shock protein/SSC1. It harbors a hsp70 motif, leucine zipper and EF hand motif along with the unique N and C terminals. Heat shock did not induce the synthesis of p66 protein as analyzed by L-[^{35}S]methionine-labeled control and heat shocked cells. However, immunostaining of heat shocked mortal (CD1-ICR MEF, Balb MEF) and immortal (RS4-immortal clone of CD1-ICR MEF, NIH 3T3 and Balb/c 3T3) cells revealed an interesting aspect. Heat shock treatment (42 ^{o}C, 1 h) transiently dislocated the protein from its uniform cytosolic locale to perinuclear position in mortal and from perinuclear to juxtanuclear position in immortal cells (Fig. 3). We have earlier reported that the anti-p66 antibody does not cross react with any of the constitutive or heat shock induced

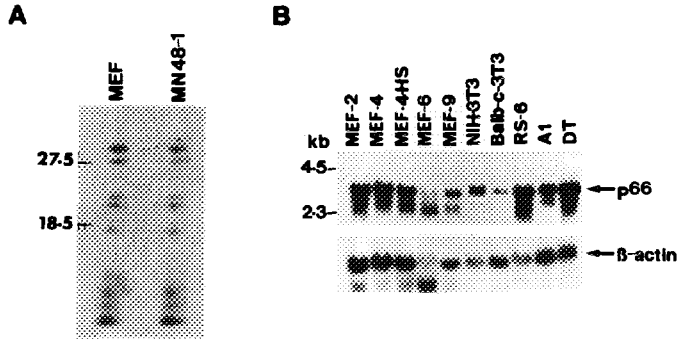
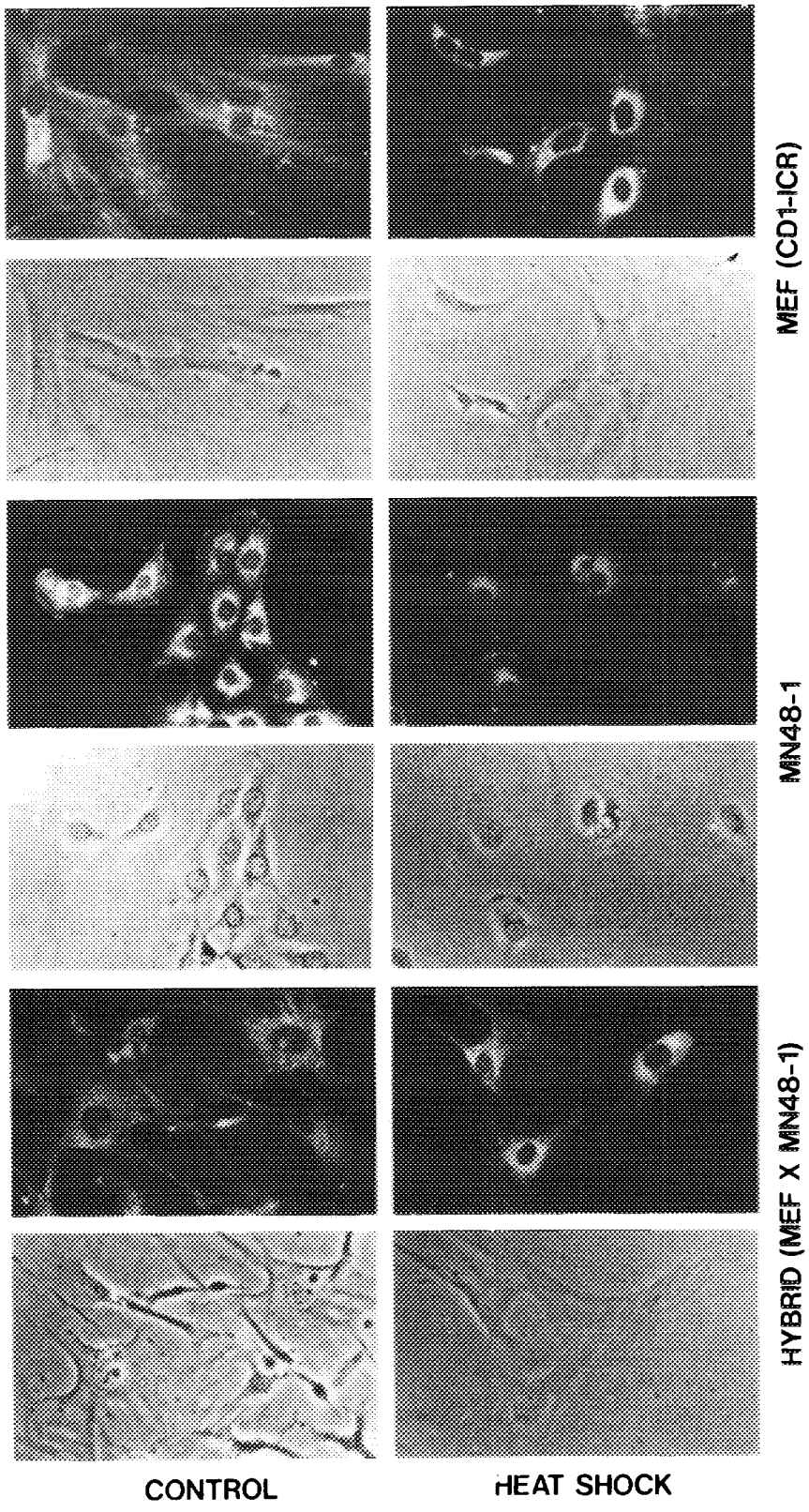


Fig. 2. A. One-dimensional peptide mapping of p66 protein by *Staphylococcus aureus* V8 protease.
B. Northern analysis of p66 transcript in serially passaged mortal MEF; immortal, NIH 3T3, Balb/c 3T3, RS-6, A1 and DT and heat shocked MEF cells.


MEF (CD1-ICR)
MN48-1
HYBRID (MEF X MN48-1)
CONTROL
HEAT SHOCK

proteins. It recognizes only p66 protein on two-dimentional gel analysis (22). Thus, the present observation on heat shock induced translocation of p66 protein are unique and have not been reported for other members of hsp70 family. For comparison, control and heat shocked mortal and immortal fibroblasts were immunostained with anti-hsp70 serum (29). The hsp70 was seen to enter nucleus and concentrate in nucleoli upon heat shock treatment (data not shown), very similar to HeLa cells as demonstrated by Hattori *et al.* (30). The results apparently refer to the special status of p66 protein in hsp70 family. It is also significant to note that unlike hsp70 whose expression has been reported to decrease in cells from aged rats (31), the expression level of p66 protein during serial passaging of MEF (Fig. 2B) remained unaltered. Further, the cytosolic protein from normal fibroblasts and the one from the hybrid fibroblasts undergo similar translocation indicating the similarity of protein from naturally ageing MEF and conditionally ageing (hybrids) fibroblasts whereas the immortal cells exhibit the shift of normal perinuclear form of the protein to the juxtanuclear form. Similar phenomenon was observed in control and heat shocked Balb MEF and Balb/c 3T3 cells. The observations need to be substantiated with immunoelectron microscopical studies for precise subcellular distribution of p66 protein in normal and heat shocked conditions. Further, its association with actin as seen upon the immunoprecipitation assays adds to its complex behavior (23). Immortal cells, and the heat shocked MEF and hybrid cells which exhibit perinuclear immunofluorescence of the protein do show normal distribution of actin fibers (data not shown). The observations suggest that the cellular distribution of p66 protein is not dependent on actin rather it seems to be active to which a particular form of actin may contribute to more or less extent. This statement is derived from the fact that the heat shocked MEF and hybrid cells, and the immortal cells do show the association of p66 protein with actin as seen by immunoprecipitation. The physiological significance of its binding to actin needs to be resolved.

The normal cytosolic and the heat shock induced perinulcear form of mortalin were analyzed on single two-dimensional gel as follows: L-[^{35}S]methionine cell lysate (10^7cpm) from normal MEF cells were mixed with 150 µg of total protein from heat shocked cells. The mixture was then immunoprecipitated with excess of anti-p66 antibody. Immunoprecipitants were adsorbed to protein A-Sepharose, solubilized in urea lysis buffer and analyzed by two-dimensional gel electrophoresis. Gel was stained with silver for visualization of heat shocked p66 protein (perinuclear form) and autoradiographed for the detection of normal cytosolic form. Autoradiogram and the silver stained gel were overlapped to detect differences, if any, in the migration of proteins on two-dimensional gel (Fig. 4). However, the perinulcear and cytosolic forms are not distinguishable indicating that the same protein virtually has the potential for cytosolic and perinuclear distribution. Similar experiments were performed with the combination of the purified protein (200 ng) from control and L-[^{35}S]methionine labeled heat

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Fig. 3. Transient dislocation of p66 protein in mortal (MEF and hybrids) and immortal (MN48-1) cells.
Note the uniform cytosolic distribution of p66 protein in control mortal cells and perinuclear staining in immortal cells. Heat shock dislocates cytosolic form to perinuclear and perinuclear to juxtanuclear location, respectively.

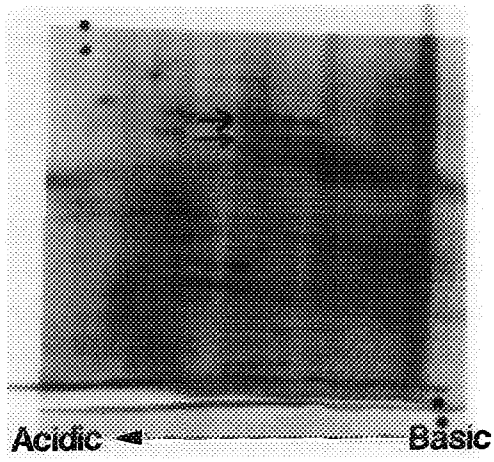

Fig. 4. Two-dimensional analysis of normal (cytosolic) and heat shocked (perinuclear) p66 protein. Silver stained gel shows the heat shocked p66 protein immunoprecipitated from 200 μg of total protein from heat shocked cells (indicated by red arrow) and the autoradiogram shows normal p66 protein immunoprecipitated from 10^7 TCA-precipitable counts from L-[^{35}S]methionine-labeled MEF cell lysate (indicated by black arrow) with excess of anti-p66 antibody. Note the complete overlap of two forms on 2D gel.

shocked MEF cell lysates (10^7 cpm) which supported that the perinuclear form is not chemically modified with respect to the cytosolic form. This data in fact supports the above results which could not distinguish the cytosolic and perinuclear forms from mortal and immortal cells. The conformational changes or the involvement of other factors such as, interaction to other proteins may contribute to the heat shock induced phenomenon. Molecular and fine structural details would be required to unravel the differences or the role of other factors in distribution and the associated function of p66 protein. Nevertheless, the presently demonstrated aspect of mortalin, i.e., perinuclear and cytosolic distribution without apparent biochemical modifications is unique and deserves further investigations.

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