Vol. 197, No. 1, 1993
November 30, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 202-206

Spontaneous immortalization of mouse fibroblasts involves structural changes in senescence inducing protein, mortalin

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Received September 20, 1993

SUMMARY: Mortalin, a novel member of mouse heat shock protein 70 (hsp70) family, is seen to distinguish the cellular mortal and immortal phenotypes by virtue of its cytosolic and perinuclear distribution, respectively. We report here that the cytosolic and perinuclear forms of mortalin from CD1-ICR mouse embryonic fibroblasts and NIH 3T3 cells, respectively, differ by two amino acids, can be distinguished on two-dimensional SDS-polyacrylamide gel. The perinuclear mortalins from RS-4 and Balb/c 3T3 cells harbor the same two amino acids as that of NIH 3T3 cells. However, these when analyzed with C-MEF mortalin did not exhibit the mobility shift equivalent to C-MEF and NIH 3T3 mortalins. The data indicate that the perinuclear mortalin from different immortal fibroblasts are not identical and implicate the possibility of additional structural changes in mortalin during immortalization. Such differences may also contribute to the differential *in vitro* growth characteristics of these immortal cells.

The phenomenon of cellular senescence, the progressive loss of replicative potential of cells in culture, has been extensively used as a model of cellular aging *in vitro*. The genetic routes of cellular senescence have increasingly been invoked especially based upon the cell fusion experiments which have brought in the concept of dominant nature of anti-proliferative genes over the ones that release cells from restrictions on their division potential (immortalization). However, the information on such anti-proliferative genes is limited to less than 15 whereas the positive stimulators of cell division (proto-oncogenes) have been extensively studied. Strategies so far used to isolate genes with anti-proliferative potentials have yielded the identification of tumor suppressor genes [1-3]; anti-proliferative genes such as *BTG1* [4], *prohibitin* [5]

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0006-291X/93 $4.00

and SAG [6]; the genes coding for extracellular growth inhibitory proteins [7, 8] and the growth arrest specific genes [9]. In addition, the over expression of some genes during normal cellular senescence as well as premature aging syndromes have been identified [10]. Mortalin, initially identified as a marker for cellular mortality by cell fusion strategy in mouse fibroblasts [11, 12] is manifested to have differential cellular distribution in mortal and immortal mouse and human fibroblasts [13]. We have recently reported that the cytosolic form ($p66^{mot\text{-}1}$) from CD1-ICR mouse fibroblasts (C-MEF) differs from the perinuclear form ($p66^{mot\text{-}2}$) of NIH 3T3 cells by only two amino acids i.e.,valine to methionine at 618 (V618M) and arginine to glycine at 624 (R624G) amino acid residues, respectively. Whereas the former induced cellular senescence in NIH 3T3 cells, the latter did not impart any effect on cellular phenotype [14]. Presently, upon isoelectric focusing and subsequent size separation of $p66^{mot\text{-}1}$ and $p66^{mot\text{-}2}$ on single SDS-polyacrylamide gel, we found that the former exhibits an additional slow migrating spot as compared to the latter. Perinuclear mortalin from the immortal cell lines such as RS-4 (spontaneously immortalized clone from C-MEF) and Balb/c 3T3, however, did not exhibit equivalent results though these have been seen to harbor the same two amino acids as that of NIH 3T3 cells by reverse transcriptase polymerase chain reaction and sequence analysis. The data present an easy detection of defined structural changes in case of NIH 3T3 and C-MEF cells and also reflect that the perinuclear forms of mortalin from different immortal cells may not be identical and implicit the possibility that more changes in mortalin locus may occur during immortalization.

MATERIALS AND METHODS

Cell culture: Primary fibroblasts from CD1-ICR mouse and the spontaneously immortalized cells NIH 3T3, RS-4 (spontaneously immortalized clone from CD1-ICR mouse embryonic fibroblasts) and Balb/c 3T3 were grown on Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics (100 U of penicillin per ml and 50 mg of streptomycin per ml) [12].

Immunoprecipitation and two-dimensional gel electrophoresis: Cells were grown in 60-mm dishes and harvested at 90% confluency. Cell pellets after washing with phosphate-buffered saline were stored at -20 °C till further use. Alternatively, cells were metabolically labeled in the presence of L-[^{35}S]methionine (50 μCi/ml) for 6-8 h in methionine-free DMEM supplemented with 5% dialyzed fetal calf serum and harvested as above. The cell pellets were 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 obtained after centrifugation at 100, 000 x *g* for 20 min at 4 °C were used for immunoprecipitation and two-dimensional analysis of mortalins with anti-mortalin antibody (polyclonal-identifies single band on western analysis)[12]. Cell lysate containing 150 μg of total protein from primary fibroblasts as estimated by the method of Bradford [15] using bovine serum albumin as standard and 5 X 10^6 cpm trichloroacetic acid-precipitable counts from indicated immortal cells were mixed and immunoprecipitated with anti-mortalin antibody (2 μl) on ice for 90 min. Immunocomplexes were adsorbed onto the protein A-Sepharose beads were solubilized in 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 were separated on the pH gradient of 8 to 5 followed by size separation on 7.5% SDS-polyacrylamide gel. The gel was stained with silver for visualization of cytosolically distributed mortalin from primary fibroblasts (cold

lysate) and autoradiographed for visualization of perinuclearly distributed mortalin from immortal cell (metabolically labeled lysates). The gels were overlapped to see the separation of mortalins from indicated cells on single SDS-polyacrylamide gel.

Reverse transcriptase polymerase chain reaction and sequencing: Total cellular RNA (1 μg) from C-MEF, RS-4, NIH 3T3 and Balb/c 3T3 cells were reverse-transcribed with Superscript (Bethesda Research Laboratories) using oligo (dT) primers. The reaction was subjected to 30 cycles of polymerase chain reaction consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min by using primers corresponding to bases 1731-1750 and 2127-2150 [see Ref. 12]. The amplified product was purified (Gene Clean II kit, Bio 101 Inc.) and sequenced by dideoxy chain termination method using primer corresponding to bases 1841-1857 [see Ref. 12] (Sequenase 2.0 kit, US Biochemicals).

RESULTS AND DISCUSSION

Mortalin, a 66-kDa protein, was initially identified as a mortality marker by virtue of its presence in cytosolic fractions of naturally aging (mouse embryonic fibroblasts) as well as conditionally aging (hybrids between primary and immortal fibroblasts) cells in contrast to the absence in cytosolic fractions of immortal cells [11]. We have characterized it as a novel member of mouse heat shock protein 70 (hsp70) family by cloning and sequence analysis [12]. Immunoprecipitation and immunoscreening analysis indicated that immortal cells harbor the similar protein in perinuclear locale and the phenomenon of differential distribution in mortal and immortal cells was seen to be conserved not only in different strains of mouse but also in human fibroblasts [13]. The biochemical assays to distinguish the cytosolic and perinuclear forms were not informative. Thus the cDNA clone for perinuclear mortalin from NIH 3T3 cells (*mot-2*) was isolated and sequenced [14]. The cloning and sequence analysis of *mot-1* and *mot-2* cDNA from C-MEF and NIH 3T3 libraries indicated changes at two base pair positions in the open reading frame with two corresponding amino acid changes (valine to methionine at 618 and arginine to glycine at 624 amino acid residues) near the carboxyl terminus. The northern analysis has delineated the presence of *mot-1* and *mot-2* transcripts in C-MEF and NIH 3T3 cells respectively, and substantiated the cloning and sequencing results [14].

The present synchronous two-dimensional analysis of the cytosolic and the perinuclear forms of mortalin suggested that the cytosolic mortalin ($p66^{mot\text{-}1}$) from C-MEF can be distinguished from the perinuclear mortalin ($p66^{mot\text{-}2}$) from NIH 3T3 cells, wherein the former separates as three very nearby spots aligning at the region of pI 5.9 and the latter lacks the slow migrating (more basic) spot (Fig. 1A). Since we have detected only *mot-1* transcript in C-MEF cells by northern analysis [14], the two spots common to NIH 3T3 cells do not represent structurally very distinct form of protein. Rather, the absence of slow migrating (more basic) spot in NIH 3T3 sample may either reflect the change from arginine to glycine as described above or some undetectable minor modification of the protein. Furthermore, we have also reported that the transfection of *mot-1* cDNA induces cellular mortality in NIH 3T3 cells whereas the over expression of *mot-2* does not impart any detectable effect in these cells. Thus the two defined changes are associated with the biological function of the

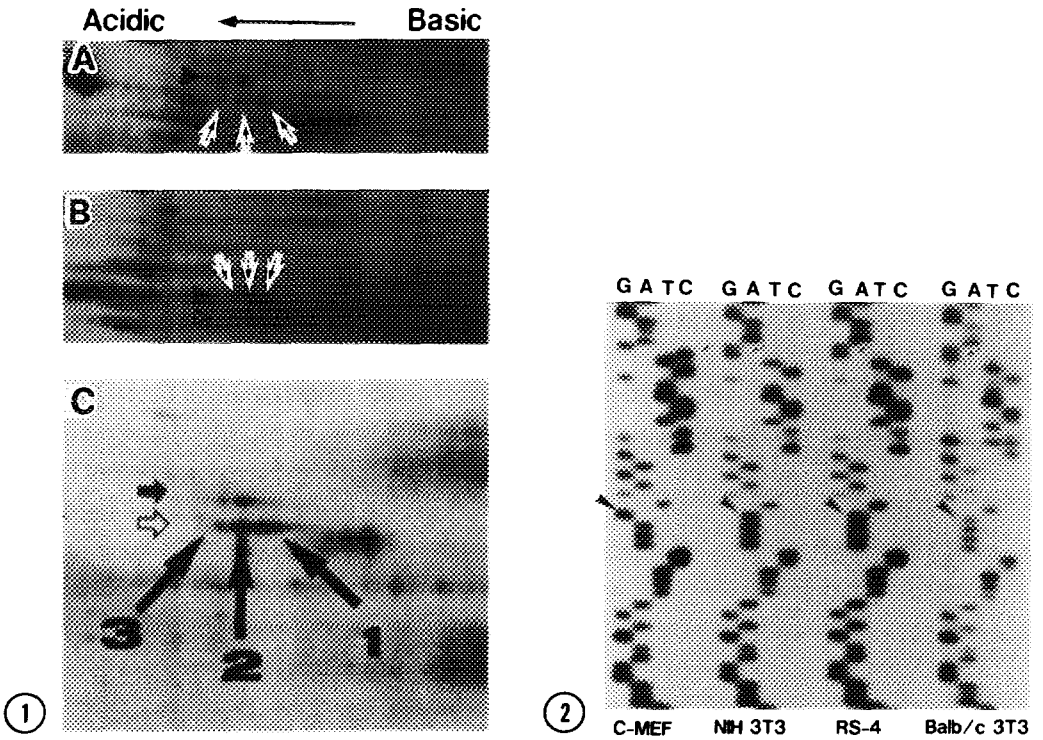

Fig. 1. The separation of cytosolic and perinuclear mortalins on two-dimensional polyacrylamide gel. The silver stained gel and autoradiogram were superimposed to match the signals. In the figure, autoradiogram is shifted up to show both together. Cytosolic mortalin (p66$^{mot\text{-}1}$) is seen as silver stained spots (indicated by open triangles in A & B and open arrow in C, purified mortalin from C-MEF). Perinuclear mortalin is visualized by autoradiography of the same gel as shown by closed triangle in A (NIH 3T3) & B (RS-4) and closed arrow in C (NIH 3T3).

Fig. 2. Reverse transcriptase polymerase chain reaction and sequence analysis showing the changes from G to A (indicated by arrowhead) and from C to G (indicated by arrow) in case of NIH 3T3, RS-4 and Balb/c 3T3.

protein which seems to be mediated by differential cellular distributions. Balb/c 3T3 and RS-4 cells are seen to harbor perinuclearly distributed mortalin [13]. Both of these were determined to exhibit *mot-2* like structure, i.e., methionine at 618 and glycine at 624 amino acid residues, as analyzed by localized reverse transcriptase polymerase chain reaction and sequencing (Fig. 2). The data substantiated that the two reported changes are important and are sufficiently involved for the lack of cytosolically distributed mortalin and the escape from its senescence inducing function as described [14]. However, the complete mortalin structure remained unknown in case of Balb/c 3T3 and RS-4 cells. Having known the differential mobility of mortalins from C-MEF and NIH 3T3 cells (Fig. 1A), we tried the combinations such as C-MEF & Balb/c 3T3, and C-MEF & RS-4 cells. Surprisingly, the equivalent mobility shift was not observed (Fig. 1B) under the similar experimental conditions when C-MEF and NIH 3T3 mortalins could reproducibly be differentiated. Thus the protein from RS-4 and Balb/c 3T3 cells though perinuclearly distributed may not be identical to that of NIH 3T3 cells.

It is significant to note that i) if the above described differential separation of $p66^{mot-1}$ and $p66^{mot-2}$ is due to some minor chemical modification, such modification is not indispensable to escape from senescence inducing function of cytosolic mortalin, $p66^{mot-1}$ and ii) the heat shock induced translocation of mortalin from cytosolic to perinuclear localization was also not distinguished on two-dimensional gel which indicated that minor structural changes rather than the chemical modification are involved in the differential distribution of mortalin [16]. Furthermore, the purified protein from C-MEF cells when mixed with L-[^{35}S]methionine labeled immunoprecipitated protein from NIH 3T3 cells and analyzed similarly, the differential separation of $p66^{mot-1}$ and $p66^{mot-2}$ was confirmed (Fig. 1C). The data implicit the possibility of additional changes at mortalin locus during immortalization of RS-4 and Balb/c 3T3 cells. The two above described changes are seen to be sufficient to escape from senescence inducing function of cytosolic mortalin ($p66^{mot-1}$) whereas the perinuclear mortalin ($p66^{mot-2}$) is seen to be biologically inactive [14]. The biological significance of the additional implicated changes remains unknown. The study thus evokes the need to characterize and define the biological function of other implicated changes which might also contribute to the differential *in vitro* growth characteristics of these immortal cells. Characterization of such changes and elucidation of their biological function by molecular cloning and functional assays are underway.

Acknowledgment. This work was supported by Science and Technology Agency of Japan.

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