Defective nuclear localization of p53 protein in a Chinese hamster cell line is associated with the formation of stable cytoplasmic protein multimers in cells with gene amplification

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Materials and methods

Cell lines

The following cell lines were used: RAJI, a Burkitt’s lymphoma cell line with mutant p53 protein (IARC, Lyon, France); B7, a TK- derivative of Chinese hamster cell line V79 (Dr C. Colella, IMD, CNR, Pisa, Italy); MTX A, G, L, M, MTX-resistant Chinese hamster cell lines with DHFR gene amplification, selected from B7 using a multistep protocol (15); T3T3, a transformed murine block to neoplastic growth. It is well established that p53 acts as a cell cycle checkpoint protein and drives DNA damage-dependent cell cycle arrest or apoptosis (1).

Loss of p53 function generally occurs by mutational inactivation of the p53 gene, often associated with nuclear accumulation of the mutated protein. Interaction with viral or cellular oncoproteins can also lead to p53 inactivation (2,3) and in fact represents an important strategy of cancer induction by oncogenic viruses (4). Many p53 functions depend on transactivation of specific sets of genes and therefore require p53 to be transported into the nucleus. A third mechanism of p53 inactivation is thus based on defective nuclear localization of the protein (5,6). Lack of p53 function is associated with, and likely caused by, defective nuclear localization in a variety of tumours (7–9). The causes of this atypical localization of p53 are largely unknown. In theory, lack of nuclear localization can be achieved by cytoplasmic sequestration, defective transport through the nuclear membrane or active nuclear exclusion. The defect can reside in the p53 protein itself, in the architecture of the nucleus or in other components of the nuclear or cytoplasmic environment in which p53 is immersed. It has indeed been suggested that the intracellular microenvironment of a particular tumour, with its specific set of genetic abnormalities, may be important in determining and modulating the subcellular localization and function of p53 (10). More specific explanations have also been proposed: in rat embryo fibroblasts, anchorage of a temperature-sensitive p53 mutant to the cytoplasm at 38°C has been suggested to be mediated by short-lived anchor proteins (11); in a neuroblastoma cell line, cytoplasmic localization of wild-type p53 was found to be due to masking of the C-terminal domain, which prevented exposure of nuclear localization signals (NLSs) (12). Recently it was demonstrated that in such a cell line hyperactive nuclear export contributed to cytoplasmic accumulation (13).

Studying p53 expression in transformed cells, we observed that a methotrexate (MTX)-resistant cell line (MTX M) carrying amplified dihydrofolate reductase (DHFR) genes showed cytoplasmic p53 localization, while the parental cell line (B7) showed p53 nuclear accumulation (14). To answer the question of whether the atypical localization was associated with gene amplification, three more MTX-resistant cell lines selected at different levels of resistance were analysed. Moreover, we characterized the p53 protein of MTX M cells, in order to understand the reasons for its cytoplasmic localization. Our data suggest that the cytoplasmic localization of p53 depends on the presence of gene amplification and is caused by the formation of stable p53 multimers in MTX-resistant cells.

Introduction

The tumour suppressor protein p53 plays a central role in the control of cell proliferation and cell survival and is a major

Abbreviations: DAB, diaminobenzidine; DHFR, dihydrofolate reductase; 6-DMA, 6-dimethylaminopurine; DTT, dithiothreitol; HMWBs, high molecular weight bands; LMB, leptomycin B; N-EME, N-ethylmaleimide; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PMSF, phenylmethylsulphonyl fluoride.

Many p53 functions require p53 transport into the nucleus. Mutant p53 also generally accumulates in the nucleus of transformed or neoplastic cells. However, examples of cytoplasmic accumulation of wild-type or mutant p53 have also been reported. Various explanations have been provided for defective nuclear localization. Here we propose a novel example of cytoplasmic p53 localization which occurs in cells showing gene amplification and appears to be due to the formation of stable p53 multimers. We studied a methotrexate-resistant Chinese hamster cell line (MTX M) carrying amplified dihydrofolate reductase genes and derived from a cell line with p53 nuclear accumulation. MTX M showed cytoplasmic p53 localization and, on immunoblots, several extra bands in the high molecular weight region, besides the expected 53 kDa band. p53 localization and the appearance of high molecular weight bands appeared to be correlated with the degree of DNA amplification. However, amplification of dihydrofolate reductase itself was not involved. Changing the p53 phosphorylation status quantitatively influenced the formation of high molecular weight bands. Cell fusion experiments demonstrated that p53 cytoplasmic localization in MTX M is a dominant phenotype. This result suggests that the defect causing lack of nuclear localization in this cell line does not reside in the nucleus. In the cytoplasm of MTX M and of wild-type/MTX M heterodikaryons p53 gives rise to protein complexes that are unable to re-enter the nucleus. The formation of such protein complexes is dependent on the amplification of an unknown gene product.
fibroblast line; CHEF, a Chinese hamster embryo fibroblast cell line with wild-type p53 (Prof. R. Sager, Dana-Farber Cancer Institute, Boston, MA).

**Immunocytochemistry**

p53 was detected using mouse monoclonal antibodies DO-7 (DAKO), DO-1, Bp53 19.1, Pab 241, Pab 248 and rabbit polyclonal CM-1 (Medac Diagnostica). For p53 immunostaining we used: (i) an immunoperoxidase technique with a biotinylated anti-mouse antibody followed by ABC complex (SPA-Bio Division) and diaminobenzidine (DAB) (Sigma, St Louis, MO) as chromogen—the slides were counterstained with haematoxylin and eosin according to the Papanicolau technique; (ii) an immunofluorescence technique with an anti-mouse FITC-conjugated antibody (Sigma)—the slides were counterstained with propidium iodide or diaminophenylindole (DAPI); (iii) an immunofluorescence technique with a biotinylated anti-mouse antibody followed by avidin-Cy3 (Amersham, Little Chalfont, UK) as chromogen.

**Immunoblotting**

To normalize the loading of gels, two Petri dishes for each experimental point were set up. Cells in one dish were trypsinized and counted, while cells in the second were scraped into phosphate-buffered saline (PBS) and, after centrifugation, the pellet was resuspended in lysis buffer [20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 10 mM EDTA, 2 mM phenylmethylsulphonyl fluoride (PMSF)] at a density of 10^5 cells/100 µl lysis buffer. After incubation on ice for 30 min the lysate was centrifuged again, the supernatant collected and the equivalent of 2 x 10^6 cells/lane fractionated by 7.5% SDS–PAGE, then it was transferred to nitrocellulose using a Trans Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA). For detection, the filters were blocked with 1% low fat milk in TBS-T (20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 0.1% Tween 20) and then probed for 1 h with 1 µg/ml anti-human p53 antibody DO-7. After washing in TBS-T, filters were incubated for 1 h with a 1:2000 dilution of a goat anti-mouse peroxidase-conjugated immunoglobulin. Filters were washed three times again with TBS-T and once with TBS and then detected using the ECL system (Amersham). Blots probed with anti-actin antibodies confirmed the reproducibility of the normalization procedures.

**Dot blot analysis**

DNA was extracted, denatured and spotted onto nitrocellulose filters. About 100 ng of pDHFR11, kindly provided by R.T. Schimke (Stanford University, Stanford, CA), was labelled with dig-11-dUTP by multiprime reaction and hybridized to filters according to standard procedures. Dig-11-dUTP labelled pHPT12, a probe containing the HPRT cDNA kindly provided by T. Caskey (Howard Hughes Medical Institute, Huston, TX), was used as a single copy gene reference. After post-hybridization washes, filters were detected using anti-dig peroxidase-conjugated antibody (Boehringer Mannheim, Mannheim, Germany) and the CSPD chemiluminescent system (Boehringer Mannheim). Autoradiograms were analysed by densitometry. DNA loading of blots was normalized using a filter probed with pDHFR11.

**Immunoprecipitation**

Cells grown on Petri dishes were washed and scraped into ice-cold PBS. After centrifugation, the cell pellet was resuspended in 20 µl of 0.2 M N-ethylmaleimide (NEM) on ice for 30 min and then incubated for 1 h. Ten microlitres of the equivalent of 2 x 10^6 cells/lane fractionated by 7.5% SDS–PAGE, then it was transferred to nitrocellulose using a Trans Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA). An aliquot of 10 µl of freshly prepared 0.2 M N-ethylmaleimide (NEM) was added and the sample incubated on ice for 1 h. Ten microtiter of 50% glycerol containing 0.5 M β-mercaptoethanol (β-ME) with 0.001% bromophenol blue were added and the samples loaded on SDS–PAGE gels.

**Protein alkylation**

Cells grown on Petri dishes were washed and scraped into ice-cold PBS. After centrifugation, the cell pellet was resuspended in 20 µl of 1.5% SDS, 20 mM dithiothreitol (DTT), 2 mM PMSF and heated to 85°C for 10 min. An aliquot of 10 µl of freshly prepared 0.2 M N-ethylmaleimide (NEM) was then added and the sample incubated on ice for 1 h. Ten microtiter of 50% glycerol containing 0.5 M β-mercaptoethanol (β-ME) with 0.001% bromophenol blue were added and the samples loaded on SDS–PAGE gels.

**Treatments affecting protein phosphorylation**

Sub-confluent cultures were treated with 1 mM sodium orthovanadate (Na_3OV_4) or 5 mM 6-dimethylaminopurine (6-DMAP) for 3 h at 37°C in complete medium. Cells were washed and the lysates were prepared as described above.

**Treatments affecting nucleus/cytoplasm protein transport**

Sub-confluent cultures were treated with 2 or 20 nM leptomycin B (LMB) for 3 and 8 h at 37°C in complete medium. Cells were washed, fixed and immunofluorescence was performed as described above. LMB was kindly provided by M. Toshiba (University of Tokyo, Tokyo, Japan).

**Fig. 1.** Nuclear p53 localization in B7 cells (a and c) and cytoplasmic p53 localization in MTX M cells (b and d) detected by immunochemistry using DO-7 monoclonal antibody; (a and b) immunoperoxidase technique; (c and d) immunofluorescence technique. Nuclei in (d) were counterstained with propidium iodide. Bars represent 10 µm.
Cytoplasmic p53 in cells with DNA amplification

Cell fusion
Prior to fusion, MTX M cells were incubated with 0.75 µm latex beads (0.3 ml of a solution of 10 drops of beads in 10 ml medium) and the partner cells with 2 µm latex beads (Polysciences). After 24 h, 2–6×10⁶ cells from both cell lines were washed, trypsinized, mixed and centrifuged to remove the complete medium. An aliquot of 0.25 ml of 50% polyethylene glycol (PEG) 1000, 15% DMSO in serum-free medium was added to the pellet at 37°C with gentle stirring of the cells over 1 min. Serum-free medium (0.75 ml) was then added and incubated for a further 3 min, followed by centrifugation. The cells were seeded onto 3 cm diameter dishes and fixed 4–6 h after fusion.

Microinjection
Twenty-four hours prior to microinjection, cells were trypsinized onto small culture dishes. Microinjection was performed using an Eppendorf microinjection system (Microinjector 5242, Micromanipulator 5170) mounted on an Axiovert 35M microscope (Zeiss) with a heated stage. Injections were intranuclear. Plasmid DNA (full-length wild-type or Δ61 murine p53 constructs; ref. 17) was obtained from Dr B. Vogelstein. It was injected in water at a concentration of 0.25 mg/ml. Following microinjection, fresh medium was added to the cell cultures and they were incubated for 24 h before being fixed.

Results
p53 localizes in the cytoplasm of MTX M cells
The MTX M cell line was isolated from B7, a thymidine kinase-deficient derivative of V79 Chinese hamster cells, as highly resistant to MTX via DHFR amplification (15). The DO-7 mouse anti-p53 monoclonal antibody (human epitope, amino acids 20–25) was used to detect p53 in both the MTX M and B7 cell lines. When an immunoperoxidase technique was used, a brown precipitate was seen in the nucleus of B7 (Figure 1a) and in the cytoplasm of MTX M cells (Figure 1b). p53 appeared to be localized to punctate cytoplasmic structures. To exclude the possibility that a higher level of endogenous cytoplasmic peroxidases in MTX M cells could give rise to the abnormal precipitate localization, we either treated MTX M cells with methanol/H₂O₂ or detected the DO-7 reaction with an anti-mouse FITC-conjugated secondary antibody. Peroxidase inactivation did not abolish the cytoplasmic brown precipitate in MTX M cells (not shown) and secondary FITC-conjugated antibody confirmed the cytoplasmic p53 localization (Figure 1c and d).

MTX M cells show DO-7-reactive high molecular weight bands (HMWBs)
Immunoblot experiments in B7 cells showed two bands in the 53 kDa region, migrating slightly faster than those in RAJI cells, used for reference. In MTX M cells, besides the expected 53 kDa bands, several extra bands were observed in the high molecular weight region (Figure 2a). The presence of these bands was not caused by MTX itself, since cells cultured in drug-free medium for 3–4 days had an identical band pattern (not shown).

Cytoplasmic localization and HMWBs are correlated with each other in the presence of gene amplification
MTX M cells were isolated from B7 cells by a multistep selection procedure using increasing MTX concentrations, starting from 3×10⁻⁹ M, a concentration which allows 50% survival in B7 cells (15). At each step of the selection process a cell line was isolated. In total, nine cell lines were isolated and named MTX A–MTX M (Table I).

To establish whether the cytoplasmic p53 localization shown by MTX M cells was associated with the degree of DHFR gene amplification, three more cell lines (MTX A, MTX G and MTX L) isolated at intermediate stages of the multistep selection and expected to bear increasing DHFR gene copy numbers were examined. Whereas MTX A and MTX G showed nuclear p53 localization, MTX L had both nuclear and cytoplasmic p53 localization (Table I). Moreover, MTX L but not MTX A or MTX G showed on immunoblots a band

Fig. 2. Western blot detection of p53 using DO-7. (a) Parental and MTX M cells; (b) parental cells and cell lines resistant to low (MTX A), intermediate (MTX G) and high (MTX L and MTX M) levels of MTX; (c) parental cells, MTX M cells and MTX M cells grown for several days in the absence of selective medium. Numbers on the left represent molecular weight markers (kDa).
Table I. p53 localization and presence of HMWBs in parental cells and in MTX-resistant cells with different levels of drug resistance

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MTX resistance (M)</th>
<th>p53 localization</th>
<th>HMWBs^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7</td>
<td>&lt;3 × 10^-9</td>
<td>Nucleus</td>
<td>Absent</td>
</tr>
<tr>
<td>MTX A</td>
<td>2 × 10^-7</td>
<td>Nucleus</td>
<td>Absent</td>
</tr>
<tr>
<td>MTX B</td>
<td>4 × 10^-7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MTX C</td>
<td>8 × 10^-7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MTX D</td>
<td>1.6 × 10^-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MTX F</td>
<td>6.4 × 10^-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MTX G</td>
<td>2.5 × 10^-5</td>
<td>Nucleus</td>
<td>Absent</td>
</tr>
<tr>
<td>MTX I</td>
<td>1 × 10^-4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MTX L</td>
<td>4 × 10^-4</td>
<td>Nucleus/cytoplasm</td>
<td>Present</td>
</tr>
<tr>
<td>MTX M</td>
<td>1.6 × 10^-3</td>
<td>Cytoplasm</td>
<td>Present</td>
</tr>
</tbody>
</table>

^aHigh molecular weight bands.
ND, not done.

Fig. 3. Detection of DHFR copy number by dot blot. Genomic DNA from parental and MTX-resistant cells probed with DHFR (upper spots) and HPRT to normalize DNA loading (lower spots). MTX M* represents MTX M cells grown for 140 days without selective medium.

pattern similar to that shown by MTX M (Table I and Figure 2b).

MTX M cells were maintained for 140 days without selective medium. At this time they were still resistant to the maximal (1.6 × 10^-3 M) MTX concentration. Unexpectedly, however, immunoblot analysis showed that the HMWBs had disappeared around day 100 (Figure 2c) and concomitantly p53 accumulated in the nuclei (not shown). These results were confirmed using other antibodies, including DO-1 (human epitope, amino acids 20–25), Bp53 19.1 (human epitope, amino acids 21–23) and PAb 241 (human epitope, amino acids 296–305). The latter two antibodies gave a signal of weaker intensity even at higher antibody concentrations. Cytoplasmic p53 localization and appearance of HMWBs seem therefore to be tightly linked.

To determine DHFR gene copy number, DNA from B7, MTX G, MTX L and MTX M cells, together with MTX M cells grown in the absence of selective medium for 140 days, were probed with murine DHFR cDNA. Dot blot analysis showed a slight increase in gene copy number from parental to highly resistant cells (at least four copies for MTX G, six copies for MTX L and eight copies for MTX M) (Figure 3). The MTX M cell line grown in the absence of selective medium did not show any appreciable decrease in gene copy number. DHFR amplification is therefore not directly involved in both phenotypes (i.e. cytoplasmic p53 localization and presence of HMWBs).

Fig. 4. SDS–PAGE after immunoprecipitation with DO-7. Lanes 1, total lysates; lanes 2, immunoprecipitates. Numbers on the left represent molecular weight markers (kDa).

HMWBs in immunoblots from MTX M cells are due to SDS/DTT-resistant forms of p53 complexes

p53 protein from MTX M and B7 lysates were immunoprecipitated using CM-1 anti-human p53 polyclonal antibody and detected with DO-7. Surprisingly, only the 53 kDa band from MTX M lysates was detected (Figure 4). The same result was obtained when: DO-7 antibody was used to immunoprecipitate and CM-1 to detect immunocomplexes; cellular extracts were boiled prior to immunoprecipitation to mimic SDS gel conditions; immunoprecipitation was carried out overnight (not shown).

The HMWBs could not be resolved by boiling the extracts in the presence of β-ME for extended times. On the other hand, when β-ME was omitted, all p53 signals shifted towards the HMWBs (Figure 5).

Finally, MTX M cell extracts were treated with NEM, which alkylates free sulphhydryl groups preventing oligomer stabilization through disulphide bonds. Such treatment caused the disappearance only of the 110 kDa HMWB on SDS–PAGE (Figure 5).

Modulation of protein phosphorylation status quantitatively influences the formation of HMWBs

It is known that p53 is phosphorylated at multiple sites in vitro and in vivo (18). In order to evaluate the influence of protein phosphorylation status on the formation of HMWBs, treatments that inhibit (6-DMAP) or indirectly increase (Na3VO4) protein phosphorylation were performed. The intensity of the 53 kDa band was not affected by either treatment in B7 cells (not shown) or in MTX M cells. In contrast, in MTX M cells the intensity of the HMWBs increased with 6-DMAP and decreased with Na3VO4 (Figure 6) and these effects were dose-dependent (not shown), suggesting that the formation of p53 complexes is sensitive to the phosphorylation status of proteins and possibly of p53 protein. In immunocytochemistry experiments, 0.5 mM treatment with Na3VO4 resulted in a slight decrease in cytoplasmic p53 localization, while the nucleus became slightly positive; a 1 mM treatment, the dose which gave the major effect on HMWBs, caused rounding of the cells, preventing the precise detection of p53 localization (not shown).
Cytoplasmic p53 in cells with DNA amplification

MTX M cells were treated with 2 and 20 nM LMB for 3 and 8 h. After 8 h treatment with 20 nM LMB the p53 signal, detected by immunofluorescence, became stronger and was confined to the nucleus of Chinese hamster fibroblasts (CHEF) at early passage with wild-type p53, used as control cells. The same treatment did not affect p53 localization in the parental cell line B7, which already accumulated p53 in the nucleus, or in MTX M cells, where p53 remained confined to the cytoplasm (Figure 7). Similar results were obtained after shorter treatments and with lower doses.

**p53 cytoplasmic localization in MTX M is a dominant phenotype**

Cell fusion experiments between B7 and MTX M cells were performed to establish which phenotype was dominant. The two cell lines were labelled by inclusion of beads of different diameter in their cytoplasm. Heterokaryons were easily identifiable as binucleate cells containing both small and large beads. Unfortunately, the use of conventional DAB staining prevented the beads from being seen in cells with cytoplasmic p53 accumulation. This staining procedure quenched the fluorescence of the beads, particularly that of the small ones, which were masked by the brown DAB precipitate. However, using immunofluorescence techniques, small and large green fluorescent beads were easily distinguishable against the red fluorescent background indicating p53 accumulation. Thirty heterokaryons were examined: all but one showed only cytoplasmic p53 localization (Figure 8). The exceptional heterokaryon showed p53 localization in the cytoplasm and in one of the two nuclei.

Plasmids encoding an antigenically distinct wild-type p53 (full-length murine p53) were microinjected into the nuclei of MTX M cells. Using PAb 248, which recognizes murine but not hamster p53, abundant p53 nuclear accumulation was found in 24% of microinjected cells (Figure 9a). No cytoplasmic localization was observed with this antibody. In contrast, only cytoplasmic p53 localization was seen using DO-7, which recognizes hamster but not mouse p53 (not shown). Cells microinjected in the nucleus with a plasmid encoding a Δ61 terminal murine p53, which lacks the two NLSs, failed to show nuclear staining with PAb 248 (Table II), as expected.

Finally, MTX M cells were fused with mouse cells which accumulate p53 in the nucleus and the two antigenically distinct p53 proteins in the heterokaryons were detected. All 100 heterokaryons examined showed only cytoplasmic localization using DO-7 (Figure 9b). Among 80 heterokaryons examined using PAb 248, 58 (72%) showed only one p53-positive nucleus (Figure 9c), while the remaining 28% had accumulation in none (18/80, 22%) or both (4/80, 5%) of the nuclei. No cytoplasmic localization was observed with this antibody.

**Discussion**

In this paper a MTX-resistant cell line displaying an abnormal cytoplasmic p53 localization is described. Immunoblot experiments showed the presence of HMWBs containing the p53 epitope in extracts of MTX-resistant cells. Two antibodies which recognize different epitopes of the protein (DO-7 and PAb 241) gave similar results. Moreover, antibody DO-7 gave the same results in immunocytochemical and immunoblot assays of MTX M cells grown in the absence of MTX for several days. Taken together, these observations allowed us to conclude that the cytoplasmic p53 immunostaining and the
immunoblot HMWBs were not artefacts. Immunocytochemistry and immunoblot experiments on cell lines with increasing MTX resistance, and bearing increasing DHFR gene copy numbers, suggested that the cytoplasmic localization and the appearance of p53-positive HMWBs on immunoblots were phenomena related to each other and to the degree of gene amplification. The gene(s) whose amplification is associated with, and probably responsible for, the cytoplasmic localization of p53 could not be identified. The most obvious candidate, the DHFR gene, is not involved, as shown by an experiment in which MTX M cells were grown extensively in non-selective medium. However, the simultaneous disappearance of both the cytoplasmic localization of p53 and the HMWBs (observed after growing cells for >100 days) confirmed that

<table>
<thead>
<tr>
<th>Plasmid injected</th>
<th>Antibody used</th>
<th>Positive nuclei</th>
<th>Positive cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse wild-type</td>
<td>PAb 248</td>
<td>50</td>
<td>None</td>
</tr>
<tr>
<td>Mouse A61 (amino acids 1–326)</td>
<td>PAb 248</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>Mouse wild-type</td>
<td>DO-7</td>
<td>None</td>
<td>All</td>
</tr>
<tr>
<td>Mouse A61 (amino acids 1–326)</td>
<td>DO-7</td>
<td>None</td>
<td>All</td>
</tr>
</tbody>
</table>

*aThe p53 allele carried by the plasmid is indicated; 104–214 cells were microinjected for each sample.*
the two phenomena are correlated. On the other hand, it was found in the same experiment that neither the level of MTX resistance nor DHFR amplification was decreased. The most likely explanation is that some genes co-amplified with DHFR were decreased in number during extensive culturing and were then responsible for the phenotype of MTX M cells. This hypothesis was confirmed by the fact that in MTX M and MTX L cells the amplified DHFR genes are located in an interspersed amplified region of a marker chromosome, suggesting that the amplicon may be a large one, where rearrangements are quite a frequent event (15).

Several experiments were devised to clarify the nature of the HMWBS. The fact that the relative abundance of the HMWBS increased when the extracts were electrophoresed under non-reducing conditions (without β-ME) suggests that these bands were SDS-resistant forms of the p53 dimer/tetramer. Furthermore, when we alkylated proteins with NEM in the cytoplasm of the heterodikaryons and could no longer leave the nucleus for the cytoplasm. When this occurs in a heterodikaryon, all p53 molecules would become mixed in a common pool of molecules that are all modified to forms that are unable to re-enter the nucleus of either cell line. This suggests that: (i) the phenotype of MTX M cells depends on an abnormal status of p53 molecules in the cytoplasm and not on a nuclear import–export machinery defect; (ii) in parental cells there is a flux of p53 molecules in both directions across the nuclear membrane, with a dynamic equilibrium leading to nuclear accumulation. This equilibrium is upset in MTX M cells.

It was demonstrated that p53 is subjected to both nuclear import and export in vitro (29). Our results, based on another, more physiological experimental approach, suggest that endogenous p53 can also be shuttled between the nucleus and cytoplasm. Recently, Stommel et al. (13) demonstrated that p53 export is mediated by leucine-rich nuclear export signals located in the tetramerization domain. They suggested that in neuroblastoma cells masking of nuclear export and import signals through oligomerization may be a mechanism of regulating cytoplasmic and nuclear localization. In fact, in these cells cytoplasmic localization is due to hyperactive nuclear export. In MTX M cells, however, nuclear/cytoplasmic transport is unaffected (see below), but p53 may be trapped in the cytoplasm by p53 molecular modifications (phosphorylation and HMWBs formation). In support of this interpretation, LMB treatment, which trapped p53 in the nucleus of neuroblastoma cells (13), did not change p53 localization in MTX M cells.

The second approach was based on microinjection experiments. When a murine p53 gene was microinjected into the nucleus of MTX M cells, the mouse p53 protein accumulated in the nucleus, while the Chinese hamster protein was still found in the cytoplasm. This result suggests that the murine p53 protein is not involved in the formation of stable multimers, i.e. heteromultimers of p53 from different species did not form. It was recently demonstrated that mutations during evolution of the tetrameric domain of p53 have led to impaired hetero-oligomerization (30). Although mouse and Chinese hamster p53 differ in only one of the nine amino acids involved in stabilization of oligomers (Arg342 in mouse, Glu342 in Chinese hamster), hetero-oligomerization might be impaired, since it has been previously shown that a single mutation can be substantially destabilizing (30). A second hypothesis is that since the murine p53 is under the control of the strong CMV promoter, its overexpression could prevent the formation of heteromultimers with Chinese hamster p53. In either case, the microinjection experiments clearly showed that protein transport from cytoplasm to nucleus is not defective in MTX M cells.
T3T3 murine cells, which accumulate p53 in the nucleus, showed accumulation of murine p53 in only one nucleus of heterodikaryons, presumably that of murine origin. These data allowed us to discard the second hypothesis.

We have tried to provide some reasonable explanations for the gene amplification-dependent cytoplasmic accumulation of p53 protein. An important question is whether p53 cytoplasmic localization affects its function. Interestingly, UV treatment induced the expression of MDM2 in B7 cells but not in MTX M cells (not shown), suggesting that in the latter p53 was completely inactive. However, a number of questions remain unanswered. Of particular importance would be the identification of the amplified gene product that we consider responsible for p53 multimerization. This is a prerequisite for understanding the mechanism by which p53 multimers are stabilized. Even more important would be to determine whether the phenomenon we have described could be responsible for the inactivation of wild-type p53 in MTX-resistant tumour cells in view of the obvious implications in carcinogenesis. For this purpose human tumour cell lines are under stepwise selection with increasing concentrations of MTX to obtain highly MTX-resistant cell lines (work in progress).

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