

Apoptosis or growth arrest: Modulation of tumor suppressor p53's specificity by bacterial redox protein azurin

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The tumor suppressor protein p53 is known to induce either apoptosis or growth arrest depending on cellular background. We have previously reported that a bacterial redox protein azurin induces apoptosis in J774 cell line-derived macrophages whereas a site-directed mutant M44KM64E azurin shows very little cytotoxicity and fails to induce apoptosis in J774 cells. We now report that purified M44KM64E mutant azurin protein can enter both J774 cells as well as the human breast cancer MCF-7 cells. Entry of M44KM64E mutant azurin in J774 cells causes strong inhibition of cell-cycle progression at the G₁ to S phase and a higher level of transcription of the p21 gene. Corresponding to high p21 levels, the levels of cyclins and cyclin-dependent kinases were greatly lowered in M44KM64E mutant azurin-treated J774 cells. Interestingly, M44KM64E mutant azurin protein failed to elicit inhibition of cell-cycle progression in MCF-7 cells, presumably because of mutation at the retinoblastoma tumor suppressor protein that allows functional E2F formation in MCF-7 cells even in the presence of high intracellular p21 level. Thus, the WT azurin induces apoptosis but little inhibition of cell-cycle progression whereas the M44KM64E mutant azurin is deficient in the induction of apoptosis but mediates strong inhibition of cell-cycle progression, demonstrating the role of a single bacterial protein and its hydrophobic patch in modulating two important functions of p53.

The tumor suppressor protein p53 is a major player in an intricate network that regulates cell growth, genomic stability, and cell death (1, 2). p53 is predominantly a nuclear protein that acts as a sequence-specific transcriptional regulator for many genes, including *bax*, which encodes the apoptogenic protein Bax involved in apoptosis (3), and the gene *p21*, which encodes the protein p21/Waf1/Cip1 involved in the inhibition of cell-cycle progression and growth arrest (4, 5). The process by which p53 mediates either apoptosis or growth arrest has been extensively studied but is still poorly understood. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays has shown that p53-responsive gene expression pattern is highly variable, depending on the p53 protein levels in the cell, the nature of the DNA damage or type of stress used to induce p53, the cell type or cell line used, or the nature of p53 protein modification or complex formation (6). It is known, however, that certain viral or mammalian proteins can direct p53 transcriptional specificity toward *p21/Waf1/Cip1* gene so that introduction or hyperproduction of such proteins in a cell will lead to p53-mediated modulation of p21 level leading to cellular growth or growth arrest. For example, *in vitro* transient-transfection assay with NIH 3T3 or Hep G₂ cells with the hepatitis C virus NS5A protein has demonstrated that NS5A physically associates with p53 and mediates transcriptional repression of *p21/Waf1/Cip1*, thereby promoting cell growth (7). Similarly, BRCA1, which is known to interact physically with p53, has been shown to stabilize the normally labile p53 in WT p53-expressing cells and selectively coactivated the transcriptional specificity of p53 toward genes that direct DNA repair and cell-cycle arrest but not

toward those that direct apoptosis (8). The ability of hepatitis C virus NS5A protein to specifically modulate p53-dependent repression of growth arrest has been inferred to be part of the machinery that directs hepatitis C virus-mediated pathogenesis in target cells (7).

We previously reported that a copper-containing bacterial redox protein azurin, normally involved in electron transfer during denitrification by *Pseudomonas aeruginosa*, enters mammalian cells such as J774 macrophage-like cells (9) and cancer cells such as human melanoma UISO-Mel-2 cells (10). J774 cells are murine reticulum cell sarcoma whose ascites form has the macrophage properties of adherence, morphology, receptors for Ig, and antibody-dependent lysis of target cells (11). On entry into such cells, the WT azurin formed a complex with the tumor suppressor protein p53, stabilized it to raise its intracellular level, generated enhanced levels of reactive oxygen species, and induced apoptosis (9, 10). Azurin has a hydrophobic patch exposed to the surface that is believed to be involved in protein-protein interaction with its *in vitro* electron transfer partners such as cytochrome *c*₅₅₁ (12–14). When two methionine residues in this hydrophobic patch were replaced by two polar amino acids (lysine and glutamic acid) to reduce the hydrophobicity of the patch, the resultant M44KM64E mutant azurin could still enter the J774 cells, as determined by immunoblotting of the subcellular fractions of the cells incubated for various times with the mutant azurin protein, but demonstrated very little cytotoxicity and induction of apoptosis in J774 cells (15). Goto *et al.* (15) further showed that although the WT azurin formed oligomeric complexes with a GST-p53 fusion derivative but not with GST alone as determined by glycerol gradient centrifugation, the M44KM64E mutant azurin was deficient in forming such oligomeric complexes and showed weak complex formation. Using GST pull-down assays with WT azurin and full-length or various truncated derivatives of p53 containing the N-terminal, the middle, or the C-terminal parts of p53, Punj *et al.* (16) have demonstrated that the N-terminal to the middle part of p53 is involved in the binding of azurin, the C-terminal having only a weak binding affinity.

The simultaneous loss of hydrophobicity in the hydrophobic patch of azurin and the loss of the ability to induce apoptosis in J774 cells raised an interesting question: is the hydrophobicity critical for p53-mediated azurin cytotoxicity or does the mutant azurin protein simply change the transcriptional specificity of p53 from apoptosis to other p53-mediated characteristics such as inhibition of cell-cycle progression, resulting in growth arrest? In this paper, we demonstrate that the mutant azurin forms a different type of complex with p53 than the WT azurin, resulting in the switching of p53 specificity from the induction of apoptosis

Abbreviation: CDK, cyclin-dependent kinase.

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to growth arrest. This is thus an interesting example where the same protein, either WT or slightly altered in its hydrophobic region, changes the specificity of p53. Further studies with single or multiple mutational alterations in this region may provide interesting insights as to how azurin promotes either apoptotic response or growth arrest response or perhaps even a combined response in modulating the transcriptional specificity of p53.

Materials and Methods

Bacterial Culture and Isolation of Azurin. *Escherichia coli* JM109 was used as a host strain for hyperproduction of WT and mutant azurins. Culture conditions and protein purification steps were as described before (9, 10).

Cell Culture. The J774 cells were cultured in RPMI medium 1640 containing 2 mM L-glutamine, 10 mM HEPES, and supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. Human breast cancer MCF-7 and MDD2 cells were cultured in MEM with Eagle's salt containing 2 mM L-glutamine, 0.1 mM MEM essential amino acids and supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Cytotoxicity Assay. MTT [3-(4,5 dimethylthiazol-2-yl)-2,5 tetrazolium bromide] assay was used for the measurement of the cytotoxicity of WT and mutant azurin as described previously (9, 10, 15).

Cell-Cycle Analysis. J774 or breast cancer MCF-7 and MDD2 cells were incubated with 1.0 mg/ml WT and mutant azurin for 24 h at 37°C. Cells were then washed twice with PBS and fixed with 70% ethanol at -20°C. Fixed cells were washed twice with PBS and stained by 50 μ g/ml propidium iodide in PBS containing 20 μ g/ml RNase A. For determination of DNA content at various phases in the cells, flow cytometry (Becton Dickinson) was used. At least 10,000 cells were collected in each experiment.

Entry of Azurin into Cells. WT and mutant azurin proteins were conjugated with the fluorescent chemical Alexa Fluor 568 (Molecular Probes) and incubated with J774 or MCF-7 cells for 1 h. Entry of fluorescent chemically labeled azurin into the cells was observed by confocal microscopy (model LC510, Carl Zeiss), as described previously (10).

Immunoblotting. J774 cells were cultured with the WT or mutant azurin (1.0 mg/ml) for 0, 6, 18, and 24 h. Whole cell lysate was prepared as described by Asher *et al.* (17). Monoclonal antibodies raised against p21, p53, Bax (Santa Cruz Biotechnology), cyclin-dependent kinases (CDKs), and cyclins (BioSource International, Camarillo, CA) were used for immunoblotting. Blots were also probed for actin by using monoclonal anti-actin antibody (Sigma) as an internal control. Protein bands were visualized by using ECL reagents (Amersham Pharmacia) as described (9, 10).

Real-Time PCR. For quantitation of p21 transcript, we used the real-time PCR method. J774 cells were either untreated or treated with 1.0 mg/ml WT or M44KM64E mutant azurin for 0, 6, 18, and 24 h. After such treatment, cells were washed with PBS and lysed with QIAshredder (Qiagen, Valencia, CA). Total RNA was isolated by using RNeasy kit as recommended by the manufacturer (Qiagen). The purity of the isolated RNA samples were checked by denatured agarose gel electrophoresis. Primers and probes were designed by using the PRIMER EXPRESS software (Applied Biosystems). The primer set and probe for p21 were as follows: the forward primer, 5'-GCTGTCTTGCACCTCTGGT-GTCT-3'; the reverse primer, 5'-TTTTTCGGCCCTGAGAT-

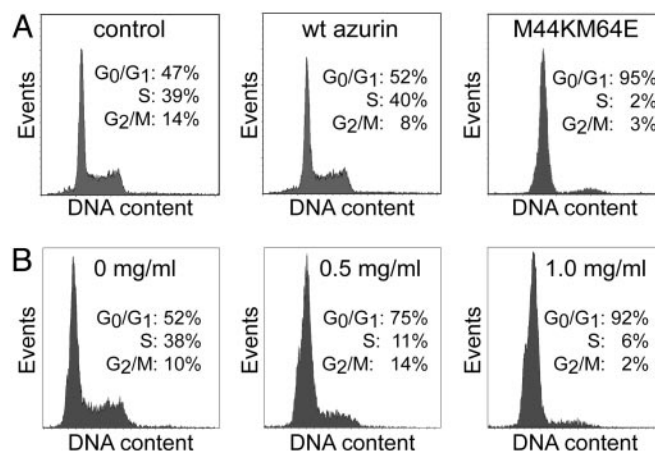


Fig. 1. (A) Flow cytometric analysis of modulation of cell-cycle progression in J774 cells. The procedure has been described in *Materials and Methods*. The percentages of DNA content at various phases are shown. (B) The concentration of M44KM64E mutant azurin that shows maximal effect on the inhibition of cell-cycle progression.

GTTC-3'; and the probe, 5'-AGCGGCCTGAAGATTCCCCG-3'. The gene for GAPDH was used as an internal control. Primers and probe for GAPDH (TaqMan Rodent GAPDH control reagents) were obtained from Applied Biosystems. RT-PCR was performed by using QuantiTect probe RT-PCR kit (Qiagen) according to the manufacturer's instructions. PCR products were continuously measured with ABI PRISM 7000 (Applied Biosystems). The relative amount of p21 transcript was normalized to the amount of GAPDH transcript by using the $2^{-\Delta\Delta CT}$ method (18, 19).

Results

WT and M44KM64E Mutant Azurin Have Different Effects on J774 Cells.

We previously described the isolation of the double mutant M44KM64E azurin where the two methionine residues at positions 44 and 64 in the hydrophobic patch of azurin were replaced by two polar amino acids. We demonstrated that this mutant azurin had very low cytotoxicity in both J774 and UISO-Mel-2 melanoma cellular backgrounds (9, 10, 15), suggesting that the two hydrophobic methionine residues were important in the interaction of azurin with p53 for its stabilization and for induction of apoptosis (15). Replacement of the two Met residues by Lys and Glu results in a lower hydrophobicity of the protein, and such replacements also alter slightly the mobility of the M44KM64E mutant azurin during SDS/PAGE (15). As reported previously, a cytotoxicity assay confirmed that M44KM64E mutant azurin was much less efficient in its cytotoxic action than WT azurin (15).

To examine whether the loss of cytotoxicity is due to lack of entry of the M44KM64E mutant azurin inside J774 cells or whether the lack of cytotoxicity is due to a switch to p53-mediated inhibition of cell-cycle progression, we measured DNA levels at various phases of cell cycle. J774 cells treated with PBS for 24 h demonstrated the gap G₀/G₁ phase level at 47%, S (DNA synthesis) phase level at 39%, and the second gap/mitosis (G₂/M) level at 14% (Fig. 1A, control). Treatment of the J774 cells with 1.0 mg/ml WT azurin had a very similar effect as the control (Fig. 1A, WT azurin). In contrast, incubation of J774 cells in presence of 1.0 mg/ml M44KM64E mutant azurin showed the cell cycle arrested at G₀/G₁ phase (95%) with very little DNA synthesis occurring (2% S phase), suggesting that unlike the WT, M44KM64E mutant azurin significantly inhibits cell-cycle progression at the G₁ to S phase. An experiment with varying M44KM64E concentrations showed that the mutant

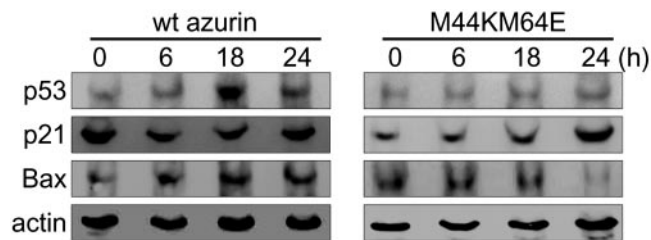


Fig. 2. Determination of levels of p53, p21, and Bax protein by Western blotting. The level of actin was also checked by using monoclonal anti-actin antibody as internal control. Equal amounts of cell extract proteins (30 μ g) were run on SDS/PAGE before immunoblotting (9, 10).

azurin exerted significant inhibition of cell-cycle progression at a concentration of ≈ 1.0 mg/ml during a 24-h treatment period (Fig. 1B).

We previously reported that WT azurin enters J774 cells, forms a complex with p53, and stabilizes the normally labile p53, thus raising its intracellular level (9). The two genes that regulate cell growth and death and that are activated by p53 because they have p53-responsive promoter elements are *p21* and *bax* (20). Consequently, we measured the intracellular levels of p53, p21, and Bax in J774 cells treated with 1.0 mg/ml WT or M44KM64E mutant azurin for 0, 6, 18, and 24 h. The methods of cell extract preparation and immunoblotting have previously been described (9, 10). In presence of WT azurin, the levels of p53 and Bax increased during 18–24 h of treatment (Fig. 2, WT azurin). The levels of the p53-nonresponsive actin remained the same whereas the levels of p21 decreased somewhat during the periods of incubation. In contrast, in M44KM64E azurin-treated J774 cells, the levels of p53 or Bax did not change, but the levels of p21 increased significantly during the 18- to 24-h period of incubation (Fig. 2, M44KM64E). The levels of the internal control, actin, remained fairly constant.

Human Breast Cancer MCF-7 Cells Are Resistant to M44KM64E Azurin Action. We recently reported (21) that WT azurin is cytotoxic to p53-positive human breast cancer MCF-7 cells, inducing apoptosis through stabilization of p53 in a manner reminiscent of WT azurin effect on J774 cells. It was, therefore, of interest to us to examine whether the M44KM64E mutant azurin would inhibit cell-cycle progression in such cells. Treatment of MCF-7 cells with either WT or mutant azurin, however, had no effect on the cell-cycle progression (Fig. 3A). To examine whether the lack of activity of M44KM64E mutant azurin is due to a lack of uptake of the azurin, we labeled both WT and M44KM64E mutant azurins with Alexa Fluor 568 and examined their entry into MCF-7 cells. Both the azurin proteins were found inside the cells (Fig. 3B), suggesting that a lack of entry was not the reason for a lack of activity of the M44KM64E azurin in MCF-7 cells. Either WT or M44KM64E mutant azurin had very little effect on inhibition of cell-cycle progression in MDD-2 cells, which are dominant negative mutants in the p53 gene of MCF-7 (Fig. 3C).

M44KM64E Mutant Azurin Forms a Complex with p53 That Is Different from WT Azurin. We previously used both glycerol gradient centrifugation and GST pull-down assays to determine the nature of complex formation between azurin and p53 or its truncated derivatives (9, 10, 16). The glycerol gradient centrifugation is a widely used method, as it allows detection and isolation of the complex that sediments at a higher glycerol fraction than the individual proteins. When azurin was sedimented by centrifugation in a glycerol gradient comprising 5% to 25% glycerol, various gradient fractions collected, and each fraction Western blotted with anti-azurin antibody, azurin was

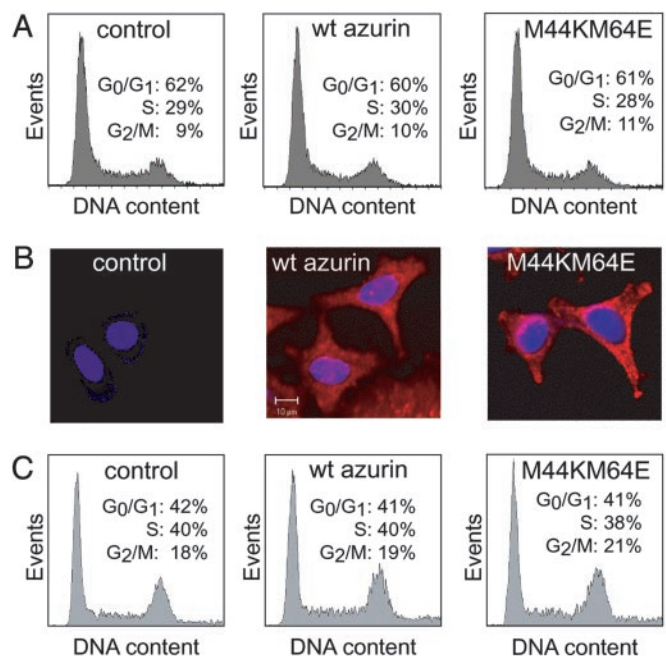


Fig. 3. (A) Cell-cycle analysis of human breast cancer MCF-7 cells. (B) Confocal microscopy using Alexa Fluor 568-conjugated azurins to demonstrate the entry of WT and M44KM64E mutant azurin in the MCF-7 cells. The azurins fluoresce red whereas the nucleus is stained blue with 4',6-diamidino-2-phenylindole (DAPI). (C) Cell-cycle analysis of MDD-2 cells having dominant negative p53 mutations.

detected only in 5% glycerol fraction (9, 15). When GST-p53 fusion protein was sedimented in a glycerol gradient, fractions were collected, and the presence of p53 in various fractions was tested by using a monoclonal anti-p53 antibody, essentially all fractions showed its presence because p53 is known to form oligomers that move in various fractions (9, 15). When WT azurin and GST-p53 fusion protein were mixed, incubated overnight at 4°C, and sedimented in a glycerol gradient, both azurin and p53 were found in all of the fractions, suggesting complex formation between WT azurin and oligomeric forms of GST-p53. Preincubation of WT azurin with GST alone under the same conditions and centrifugation in a glycerol gradient showed the presence of azurin only in 5% glycerol, suggesting that azurin and GST do not form complexes by themselves. When the M44KM64E mutant azurin and GST-p53 protein were incubated before glycerol gradient centrifugation, the mutant azurin, as well as the GST-p53, were found mostly in 5% glycerol with small amounts in 10% glycerol, indicating that the presence of M44KM64E azurin inhibited oligomer formation in GST-p53 (9, 15). It is clear from such experiments that the mutant azurin forms a different complex than the WT azurin that interferes in the oligomerization of p53.

M44KM64E Azurin Significantly Stimulates p21 Transcription. The enhancement of p21 levels in the extracts of J774 cells treated with M44KM64E azurin, as compared with WT azurin (Fig. 2), raised the question whether the mutant azurin stimulates p21 transcription because of a different type of complex formation with p53. We therefore measured the level of p21 transcripts by RT-PCR as a function of various times of treatment of J774 cells with WT and M44KM64E azurins (Fig. 4A). The results are shown graphically in Fig. 4B. In 6 h, the extent of p21 transcript formation was much higher when the cells were treated with the M44KM64E mutant azurin than with the WT azurin, suggesting that the mutant azurin specifically promoted p21

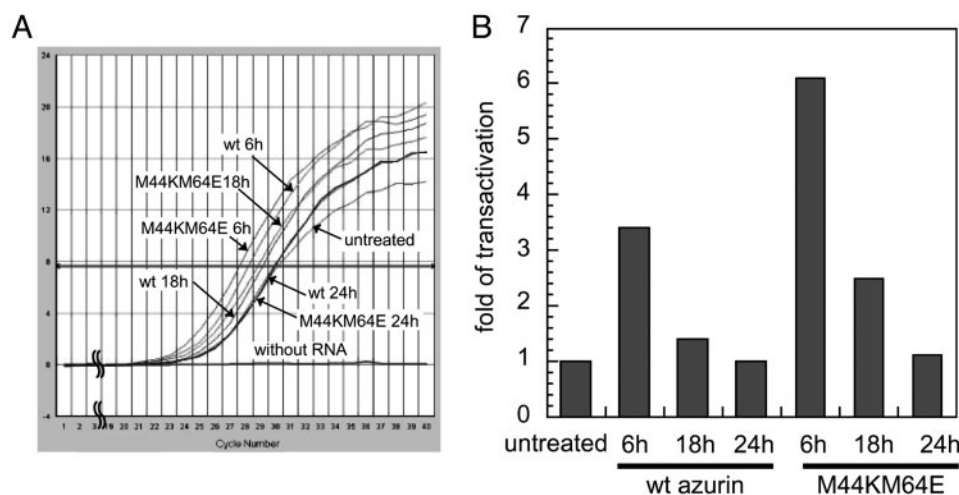


Fig. 4. (A) Amplification profiles of *p21* target gene by real-time PCR. PCR products were measured at every cycle. Target gene amplification was not found in the reaction without RNA. (B) Analysis of *p21* gene expression by RT-PCR with the $2^{-\Delta\Delta CT}$ method. The relative amount of *p21* transcript was normalized to the level of GAPDH transcript as a housekeeping gene.

transcription, presumably mediated by means of its complex formation with p53.

M44KM64E Mutant Azurin Significantly Reduces the Levels of Intracellular Cyclins and CDKs.

p21 is a 21-kDa protein, also known as Cip1 or Waf1, which is a strong inhibitor of CDK activity. CDKs are serine/threonine kinases that phosphorylate various substrates including the tumor suppressor protein Rb (retinoblastoma) and exist in complexes with cyclins such that different cyclin/CDK complexes are involved in the phosphorylation of Rb in different phases of the cell cycle. In early to late G_1 phase, CDK4/cyclin D and CDK6/cyclin D complexes allow phosphorylation of Rb. The D-type cyclins such as D1, D2, and D3 have short half-lives and are expressed only in presence of growth factors. These cyclins also physically interact with Rb, thereby inactivating it. Rb, a 105-kDa nuclear phosphoprotein, is active only when it is hypophosphorylated. In that form, it binds the transcription factor E2F and engages histone deacetylase at the promoters of E2F-regulated genes, thereby preventing the transcription of E2F-regulated genes encoding proteins such as topo1, DNA polymerase α , thymidylate kinase, cyclin D1, cyclin A, cyclin E, etc. Many of these genes are critical in the progression of the cell cycle from the G_1 phase to the S phase. CDK/cyclin complexes are important in the phosphorylation of Rb. Such CDK/cyclin-induced phosphorylation of Rb allows dissociation of Rb from the inactive Rb.E2F complexes, thereby releasing E2F in an active form to trigger transcription of the genes involved in S phase progression (4, 5, 22). The hyperproduction of *p21* in presence of M44KM64E mutant azurin therefore prompted us to look at the levels of the components of cyclin/CDKs during treatment of J774 cells with 1.0 mg/ml M44KM64E mutant azurin for 0, 6, 18, and 24 h. Even though M44KM64E mutant azurin had very little cytotoxicity, the levels of the cyclins and CDKs progressively decreased in J774 cells in the presence of M44KM64E mutant azurin (Fig. 5), and by 24 h, there was very little detectable cyclin/CDK components. This clearly suggested a role of the mutant azurin in inhibiting cell-cycle progression at the G_1 to S phase because of enhanced p53-mediated transcriptional activity of the *p21* gene. Because *p21* is a strong inhibitor of cyclin/CDK activity, and cyclin/CDKs are essential in cell-cycle progression, M44KM64E mutant azurin strongly inhibits cell-cycle progression in J774 cells by means of its effect on a change of transcriptional specificity of p53 toward activation of the *p21* gene (23).

The CDK/Cyclin Levels Are Unaffected in MCF-7 Cells in the Presence of Azurins.

It is known that dephosphorylation of Rb as well as an increase in *p21* levels and an increase in *p53* in concert with dephosphorylation of Rb are not enough to allow suppression of E2F activity in the MCF-7 breast tumor cells, suggesting that E2F activity is no longer susceptible to modulation by endogenous *p21* or Rb in such cancer cells (24, 25). MCF-7 cells also lack *p16^{Ink4a}*, which is a tumor suppressor protein that sequesters and inhibits CDK4 and CDK6 (26, 27). Absence of *p16^{Ink4a}* in MCF-7 cells allows high intracellular level of the CDKs and cyclin/CDK complexes. Moreover, overexpression of cyclin E in MCF-7 cells (28) results in elevated cyclin E/CDK2 complexes that can additionally contribute to the cyclinD/CDK4-CDK6/Rb loop, adding to the growth advantage of MCF-7 cells. We thus wanted to look at differential effects, if any, of WT and mutant azurins toward MCF-7 cells. The results in Fig. 6A show that the cyclins and CDKs are present at high levels in the extracts of MCF-7 cells, and treatment with either WT or M44KM64E mutant azurin has no significant effect, except for the level of CDK2, which is reduced slightly at 24 h. This shows that different cancer cells presumably exhibit different susceptibility based on various mutations in genes encoding tumor suppressors, cyclin/CDK or

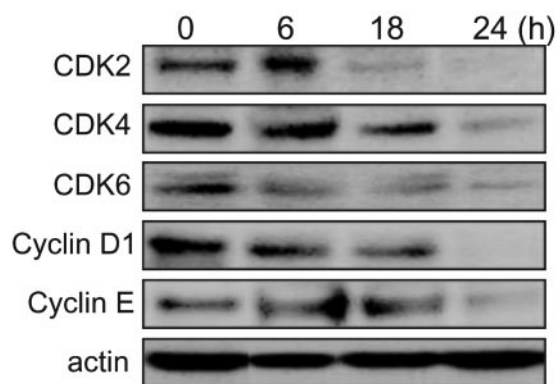


Fig. 5. Western blot analysis of cell cycle-related protein levels in J774 cells treated with 1.0 mg/ml M44KM64E mutant azurin for indicated times. In each lane, 30 μ g of protein from the extracts were loaded, and immunoblotting was conducted by using monoclonal antibodies against the various cyclins and CDKs, as well as actin as an internal control.

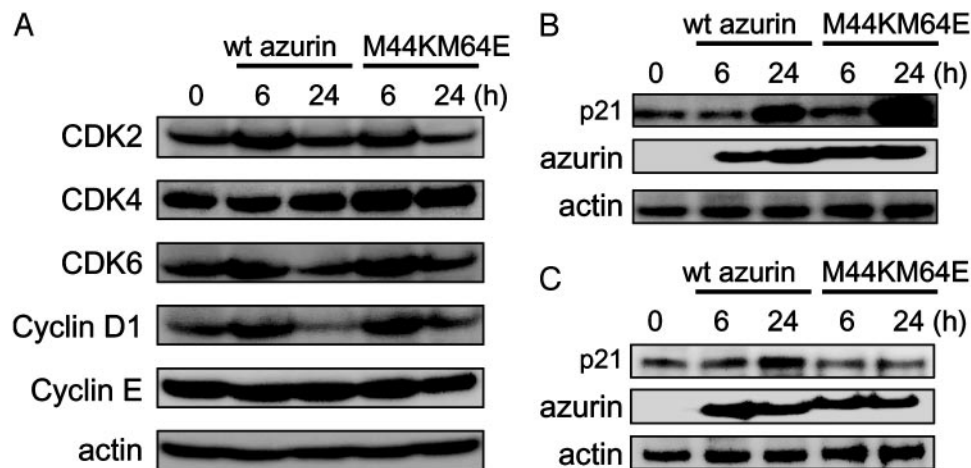


Fig. 6. (A) Western blot analyses of the levels of cyclins and CDKs in human breast tumor MCF-7 cells untreated (0 h) or treated with 1 mg/ml WT azurin or M44KM64E mutant azurin. The experimental details are similar to those described in the Fig. 5 legend. (B and C) Western blot analyses of the levels of p21, azurin, and actin as an internal control in MCF-7 (B) or MDD2 (C) cells untreated (0 h) or treated with 1 mg/ml WT azurin or M44KM64E mutant azurin.

other regulatory proteins. It should be noted that although MCF-7 cells are resistant to p16^{Ink4a} or p53-mediated growth arrest, they are susceptible to p53-mediated apoptosis by WT azurin, demonstrating significant *in vivo* cancer regression in nude mice (21). We plan to investigate various cancer cell lines on their susceptibility to growth arrest by M44KM64E mutant azurin or induction of apoptosis by WT azurin to examine the nature of mutations in such cells and their response to the azurins.

The Level of p21 Is Enhanced in p53-Positive MCF-7, but Not in p53-Negative MDD-2, Cells. To determine whether indeed M44KM64E mutant azurin induces inhibition of cell-cycle progression through p53-activated *p21* gene expression in J774 cells but not in the breast cancer cell line MCF-7, where the cyclin/CDK levels are high (Fig. 6A) because of mutations in the Rb activity domain (24–27), we measured the levels of p21 in cell extracts of MCF-7 cells treated with WT azurin and M44KM64E mutant azurin. We also used a p53 dominant negative mutant of MCF-7 (MDD2) to determine whether indeed p21 levels are primarily modulated by the p53-mediated activation of the *p21* gene promoter. To ensure that any alteration in the level of *p21* is not due to a lack of entry of either WT or mutant azurins in the MCF-7 or MDD2 cells, we also looked at the entry of the azurins in such cells. The results in Fig. 6B and C clearly indicate that, in p53-positive MCF-7 cells (Fig. 6B), WT azurin causes significant enhancement of p21 levels in 24 h whereas treatment with M44KM64E mutant azurin causes a much greater accumulation of p21. The level of actin was not significantly affected under such conditions. In p53-negative MDD2 cells (Fig. 6C), however, neither WT nor M44KM64E mutant azurin elicited a significant enhancement of intracellular p21 level, suggesting that the presence of p53 is important in the WT or M44KM64E mutant azurin-induced enhancement of p21 levels. WT or M44KM64E mutant azurin entered both MCF-7 and MDD2 cells equally well, suggesting that the lack of stimulation of p21 synthesis in MDD2 cells by WT or mutant azurin is not due to lack of entry of the azurins in such cells. Thus, the M44KM64E mutant azurin selectively stimulates p53-mediated activation of *p21* gene, even though the outcome on the inhibition of cell-cycle progression is different in J774 and the human breast cancer MCF-7 cells.

Discussion

An important unresolved problem in understanding p53 transcriptional specificity toward a multitude of genes, involved in

apoptosis or growth arrest, is how this specificity is dictated in various cells at various levels of stress (6, 20). In some cases, introduction or hyperproduction of certain viral or mammalian proteins, which are known to physically associate with p53, have been reported to direct p53 specificity toward genes involved in DNA damage repair or growth but not in apoptosis (7, 8). Wt azurin, which forms a stable complex at the N-terminal to mid-region of p53 (16) without affecting p53 oligomer formation, mostly directs p53 specificity toward activation of the *bax* gene, leading to apoptosis (9, 10). The hydrophobic patch of azurin is known to be involved in protein–protein interaction with its *in vitro* electron transfer partners (12–14). A replacement of two hydrophobic amino acids by polar amino acids in this region changes the way the M44KM64E mutant azurin interacts with p53, suppressing its oligomer formation. The oligomerization domain of p53 is in the center to the C-terminal region (2, 23). It is thus likely that the M44KM64E mutant azurin contacts p53 also in this domain, thereby interfering in oligomerization and changing the specificity of p53 toward *p21* gene transcription. This implies an interesting role of the azurin hydrophobic patch, not only in its physical association with partner electron transfer agents but also with p53. A detailed study of step by step replacement of the hydrophobic amino acids in the hydrophobic patch of azurin, either with polar or with more hydrophobic amino acids and determining the nature of p53-mediated complex formation and gene expression, may provide interesting clues as to how protein–protein interactions modulate p53 transcriptional specificity.

The resistance of human breast cancer MCF-7 cells toward M44KM64E azurin signifies that the cellular environment of the cancer cells will dictate whether or which cancer would be susceptible to potential azurin-type drugs of microbial sources. Because WT azurin mediates apoptosis of both human melanoma UIISO-Mel-2 (10) and breast cancer MCF-7 cells (21), thereby allowing their regression *in vivo* in nude mice, and M44KM64E mutant azurin causes growth arrest, it is likely that a combination of these two forms of azurin will demonstrate better regression if the cancer cells have both functional apoptotic and cell-cycle progression pathway. The unregulated growth of cancer cells is, however, often due to mutations in tumor suppressor proteins such as p53, Rb, p16^{Ink4a}, and various other genes involved in cellular growth regulation (1, 5, 20). Indeed, the MCF-7 cells are known not to be responsive to higher levels of p21, as they harbor mutations that lead to functional

E2F activity because of a reduced functionality of the Rb protein that allows E2F not to be modulated by Rb (24, 25). It would be interesting to see whether other bacterial redox proteins, particularly the cytochromes, will interact with other tumor suppressor proteins such as Rb or p16^{Ink4a}, thereby overcoming the

mutational alterations in cancer cells to prevent unregulated cell-cycle progression.

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1. Vogelstein, B., Lane, D. & Levine, A. J. (2000) *Nature* **408**, 307–310.
2. Prives, C. & Hall, P. A. (1999) *J. Pathol.* **187**, 112–126.
3. Schuler, M. & Green, D. R. (2001) *Biochem. Soc. Trans.* **29**, 684–688.
4. Dotto, G. P. (2000) *Biochim. Biophys. Acta* **1471**, M43–M56.
5. Sherr, C. J. (2000) *Cancer Res.* **60**, 3689–3695.
6. Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W. H., Tom, E., Mack, D. H. & Levine, A. J. (2000) *Genes Dev.* **14**, 981–993.
7. Majumder, M., Ghosh, A. K., Steele, R., Ray, R. & Ray, R. B. (2001) *J. Virol.* **75**, 1401–1407.
8. MacLachlan, T. K., Takimoto, R. & El-Deiry, W. S. (2002) *Mol. Cell. Biol.* **22**, 4280–4292.
9. Yamada, T., Goto, M., Punj, V., Zaborina, O., Kimbara, K., Das Gupta, T. K. & Chakrabarty, A. M. (2002) *Infect. Immun.* **70**, 7054–7062.
10. Yamada, T., Goto, M., Punj, V., Zaborina, O., Chen, M. L., Kimbara, K., Majumdar, D., Cunningham, E., Das Gupta, T. K. & Chakrabarty, A. M. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 14098–14103.
11. Ralph, P. & Nakoinz, I. (1975) *Nature* **257**, 393–394.
12. Rienzo, F. D., Gabdouliline, R. R., Menziani, M. C. & Wade, R. C. (2000) *Protein Sci.* **9**, 1439–1454.
13. Murphy, L. M., Dodd, F. E., Yousafzai, F. K., Eady, R. R. & Hasnain, S. S. (2002) *J. Mol. Biol.* **315**, 859–871.
14. Cutruzzola, F., Arese, M., Ranghino, G., van Pouderoyen, G., Canters, G. & Brunori, M. (2002) *J. Inorg. Biochem.* **88**, 353–361.
15. Goto, M., Yamada, T., Kimbara, K., Horner, J., Newcomb, M., Das Gupta, T. K. & Chakrabarty, A. M. (2003) *Mol. Microbiol.* **47**, 549–559.
16. Punj, V., Das Gupta, T. K. & Chakrabarty, A. M. (2003) *Biochem. Biophys. Res. Commun.* **312**, 109–114.
17. Asher, G., Lotem, J., Cohen, B., Sachs, L. & Shaul, Y. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 1188–1193.
18. Livak, K. J. & Schmittgen, T. D. (2001) *Methods* **25**, 402–408.
19. Javier, A., Satoshi, T., Jiexia, Q., Tsuyoshi, U. & Kaname, S. (2003) *Biochem. Biophys. Res. Commun.* **305**, 700–706.
20. Oren, M. (2003) *Cell Death Differ.* **10**, 431–442.
21. Punj, V., Bhattacharyya, S., Saint-Dic, D., Vasu, C., Cunningham, E. A., Graves, J., Yamada, T., Constantinou, A. I., Christov, K., White, B., *et al.* (2004) *Oncogene* **23**, 2363–2374.
22. Sherr, C. J. & Roberts, J. M. (1999) *Genes Dev.* **13**, 1501–1512.
23. Ahn, J. & Prives, C. (2001) *Nat. Struct. Biol.* **8**, 730–732.
24. Orr, M. S., Watson, N. C., Sundaram, S., Randolph, J. K., Jain, P. T. & Gewirtz, D. A. (1997) *Mol. Pharmacol.* **52**, 373–379.
25. Botos, J., Smith, R. & Kochevar, D. T. (2002) *Exp. Biol. Med.* **227**, 354–362.
26. Musgrove, E. A., Lilischkis, R., Cornish, A. L., Lee, C. S., Setlur, V., Seshadri, R. & Sutherland, R. L. (1995) *Int. J. Cancer* **63**, 584–591.
27. Zhou, J.-N. & Linder, S. (1996) *Anticancer Res.* **16**, 1931–1936.
28. Gray-Bablin, J., Zalvide, J., Fox, M. P., Knickerbocker, C. J. & DeCaprio, J. A. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 15215–15220.