

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

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EMILIO FERRERO

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Act. 519 TRASLADOINFORME Folios: 1

ASUNTO	Radicación	07-43820
	Trámite	11
	Evento	379
	Actuación	519
	Oficio	
	Folios	5 . 07799

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☐ Capítulo II ☒
No. Solicitud Internacional	PCT/US2005/035758		
Fecha de Presentación Internacional	6/Octubre/2005		

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 99 a 156	3/Mayo/2007
Reivindicaciones:	Folios 157 a 161	3/Mayo/2007
	Folios 254 a 257	30/Abril/2010
Dibujos:	Folios 163 a 172	3/Mayo/2007
Listado de Secuencias:	Folios 173 a 192	3/Mayo/2007
Prioridad:	US 60/616,782	7/Octubre/2004
	US 60/680,500	13/Mayo/2005
	US 60/700,297	19/Julio/2005

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio (Folios 254 a 257), porque no se amplía el objeto de la solicitud.

Expediente 07-43820 1er Art 45

4. Objeto de la invención

Título de la solicitud: "Agentes de transporte derivados de cupredoxinas y métodos de utilizar los mismos".

El problema planteado en esta solicitud consiste en la necesidad de suministrar fuentes alternativas de dominio de traducción de proteínas (PTD) para transportar cargas o compuestos farmacológicamente activos en las células de mamíferos.

Para resolver este problema técnico la solicitud en estudio proporciona un péptido aislado que comprende una secuencia truncada de una cupredoxina y la cual facilita la entrada de una carga enlazada a él en una célula de cáncer de mamífero.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 14/195, 14/82.

5. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1 – 10, 28 y 29 no se considera una invención, de acuerdo con el Art citado, porque hace referencia a material biológico existente en la naturaleza.

6. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 20 – 26 no es patentable de acuerdo con el Art citado.

7. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque hace referencia a materia no patentable.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio carece de concisión porque utiliza las expresiones "un truncamiento", "que tiene al menos aproximadamente 90% de identidad", "que tiene homología estructural", "un fragmento de la misma", "un polímero de residuos de aminoácidos", "una lipoproteína", "un polisacárido", "un ácido nucleico", "un pigmento", "una micropartícula", "una nanopartícula", "una toxina", "un fármaco", "un proteína fusión", "una sustancia detectable", "se modifica para prolongar o para optimizar la vida media del péptido en el torrente sanguíneo", lo cual dificulta determinar la extensión del objeto que se busca proteger.

Para las expresiones "que tiene al menos aproximadamente 90% de identidad", "que tiene homología estructural", "un fragmento de la misma", "un polímero de residuos de aminoácidos", "un ácido nucleico", "un proteína fusión", se recuerda al solicitante que un péptido y un ácido nucleico se caracterizan por sus secuencias de aminoácidos y nucleótidos, respectivamente.

Las expresiones "un polisacárido", "un pigmento", "una micropartícula", "una nanopartícula", "una toxina", "un fármaco", "una sustancia detectable", no definen de

forma inequívoca una molécula, la cual se debe definir por sus características técnicas esenciales o estructurales.

Las reivindicaciones 1, 10, 11 y 18 utilizan las expresiones “que facilita la entrada de una molécula enlazada en una célula de cáncer de mamífero”, “facilita la entrada del compuesto transportador en una célula de cáncer de mamífero”, “donde la sustancia es detectable por un método seleccionado a partir del grupo que consiste en fluorimetría, microscopía, rayos X CT, MRI y ultrasonido”, que son resultados a alcanzar y no definen a la molécula, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto.

La reivindicación 11 no es clara cuando utiliza el término “un complejo (...) donde el péptido se enlaza al compuesto transportador (...)”, porque no se define si el enlace entre el compuesto transportador y el péptido se hace a través de una proteína de fusión o producto de reacción química de acoplamiento. Se sugiere al solicitante hacer la aclaración respectiva.

La reivindicación 27 utiliza la expresión “se modifica para prolongar o para optimizar la vida media del péptido en el torrente sanguíneo”, corresponde a un resultado a alcanzar y no define al péptido, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto.

El capítulo reivindicatorio no es claro porque utiliza el término “aproximadamente”, el cual es impreciso y no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

La reivindicación 11 no es clara porque utiliza la expresión “un compuesto transportador”, cuando la traducción más apropiada es “una carga”, debido a que es la molécula transportada por el péptido derivado de cupredoxina. Se sugiere hacer la aclaración necesaria.

Se requiere al Solicitante hacer las aclaraciones necesarias.

8. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Octubre 7 de 2004 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	<u>Infection and Immunity. Vol. 70, No. 12, Pág. 7054-7052</u>	The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53.	Diciembre/2002

D1 <http://www.ncbi.nlm.nih.gov/pubmed?term=12438386> divulga que la cupredoxina azurina de *Pseudomonas aeruginosa* forma un complejo con la proteína supresora de tumores p53 (Resumen).

9. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 11 consiste en un complejo que comprende un compuesto transportador y un péptido, donde el péptido consiste en una secuencia que es un truncamiento de una cupredoxina tipo salvaje de longitud completa, donde el péptido se enlaza al compuesto transportador, y donde el péptido facilita la entrada del compuesto transportador en una célula de cáncer de mamífero.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Un complejo que comprende: a) un compuesto transportador y	Un complejo que comprende la proteína p53 y (Resumen)
b) un péptido, donde el péptido consiste en una secuencia que es un truncamiento de una cupredoxina tipo salvaje de longitud completa,	Azurina (Resumen)
c) donde el péptido se enlaza al compuesto transportador.	Donde la proteína p53 forma un complejo con azurina (Resumen)

Las características **esenciales** del complejo de la reivindicación 11 habían sido divulgadas previamente en D1, el cual enseña un complejo que comprende la proteína supresora de tumores p53 y la cupredoxina azurina de *Pseudomonas aeruginosa* (resumen).

En consecuencia, la reivindicación 11 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 12 – 18 no mencionan alguna característica que haga nueva al complejo de la reivindicación 11, por lo cual se considera que no son nuevas.

10. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Infection and Immunity. Vol 70, No. 12, Pág. 7054-7052	11 – 18	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso

de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



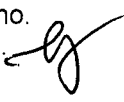
JOSÉ LUIS SALAZAR LÓPEZ

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 24 de mayo 2012

Elaborado por: Juan David Moncaleano.

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The Bacterial Redox Protein Azurin Induces Apoptosis in J774 Macrophages through Complex Formation and Stabilization of the Tumor Suppressor Protein p53

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Two redox proteins, azurin and cytochrome *c*₅₅₁ elaborated by *Pseudomonas aeruginosa*, demonstrate significant cytotoxic activity towards macrophages. Azurin can enter macrophages, localize in the cytosol and nuclear fractions, and induce apoptosis. Two redox-negative mutants of azurin have less cytotoxicity than does wild-type (wt) azurin. Azurin has been shown to form a complex with the tumor suppressor protein p53, a known inducer of apoptosis, thereby stabilizing it and enhancing its intracellular level. A higher level of reactive oxygen species (ROS), generated during treatment of macrophages with wt azurin, correlates with its cytotoxicity. Treatment with some ROS-removing antioxidants greatly reduces azurin-mediated cytotoxicity, thus demonstrating a novel virulence property of this bacterial redox protein.

Bacterial pathogens such as *Pseudomonas aeruginosa* (35) and many others (4, 29) produce a range of virulence factors that allow the bacteria to escape host defense and cause disease. Some of these virulence factors induce apoptosis in phagocytic cells such as macrophages to subvert the host defense (22, 45). Very little information is available, however, about the role of any purified bacterial virulence factor in triggering apoptosis in mammalian cells. An important inducer of mammalian cell apoptosis is the tumor suppressor protein p53 (2, 31, 40). A model for p53-induced apoptosis envisaged three critical steps: (i) the transcriptional induction of redox-related genes; (ii) the formation of reactive oxygen species (ROS); and (iii) the oxidative degradation of mitochondrial components, culminating in cell death (26). The release of cytochrome *c* from the mitochondria, resulting in complex formation with Apaf-1 and the activation of caspases such as caspase 9 (1), is a critical process in mammalian cell apoptosis, and p53-mediated apoptosis is known to involve caspase cascade activation (30, 31, 33). However, the level of p53 is quite low in normal mammalian cells due to its short half-life of a few minutes, primarily due to its degradation through the ubiquitin proteasome pathway (19). Stabilization and consequent higher levels of intracellular p53 can be achieved either through DNA-damaging agents such as UV radiation, which inhibits p53 ubiquitination (18), or through complex formation with specific proteins such as simian virus 40 T antigen (37) or with mammalian redox proteins such as NADH quinone oxi-

doreductase 1 (NQO1), which may act through accumulation of intracellular NAD⁺ (3).

In addition to stabilization of p53 by NQO1, which inhibits its degradation (3), redox proteins may contribute to the activation of p53 by stimulating its DNA-binding activity as a transcriptional activator. For example, the oxidized form of p53, which is inactive for DNA binding, is greatly stimulated in its DNA-binding activity in the presence of a dual-function redox and repair protein, Ref-1, particularly in the presence of reducing agents (13). Since p53 transcriptionally regulates the level of proapoptotic proteins such as Bax, NOXA, and P53AIP1 (25, 28), mammalian redox proteins such as Ref-1 play an important role in p53-mediated induction of apoptosis by enhancing the transcription of proapoptotic genes in mammalian cells (8).

Redox proteins such as azurin and cytochrome *c*₅₅₁ are involved in electron transfer during denitrification in *P. aeruginosa* (38). A great deal is known about the structure of these proteins, and many site-directed mutants are available (6, 7, 39). Azurin is a type I blue copper protein with a molecular mass of 14 kDa, while cytochrome *c*₅₅₁ (9 kDa) is a haem-containing cytochrome. Azurin possesses a relatively large hydrophobic patch close to the active site, and two residues in this hydrophobic patch, Met-44 and Met-64, are believed to be involved in its interaction with the redox partners cytochrome *c*₅₅₁ and nitrite reductase (39). Similarly, amino acid residues such as His-46 and His-117 are important for electron transfer, since the site-directed mutants His-46Gly (H46G) and His-117Gly have only 34 and 15% of the activity of the wild-type azurin (7). The double mutant Met-44Lys/Met-64Glu (M44K/M64E) is deficient in binding to its redox partners and demonstrates 3.4 and 3.3% of the electron transfer activity of wild-type azurin towards cytochrome *c*₅₅₁ and nitrite reductase, respectively (7). Although a great deal is known about these two redox proteins with regard to their electron transfer activity, nothing is known about any cytotoxicity of these two pro-

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teins towards phagocytic or other mammalian cells. In this article, we demonstrate that purified azurin and cytochrome c_{551} from *P. aeruginosa* exhibit cytotoxicity towards macrophages. We additionally demonstrate that azurin forms a complex with the tumor suppressor protein p53, generates reactive oxygen species (ROS), and induces apoptosis in macrophages. That the cytotoxicity is due to azurin and not due to contaminating cellular constituents such as cell wall lipopolysaccharides is clear from their absence in the purified wild-type or mutant azurin preparations and from the fact that several mutant azurins isolated by the same purification procedure as the wild type demonstrated very low cytotoxicity, even though they might have been contaminated by the same cellular constituents present in the wild type.

MATERIALS AND METHODS

Bacteria and growth media. *Escherichia coli* JM109 was used as a host strain for expression of the azurin-encoding gene (*azuA*) of *P. aeruginosa* strain PAO1. The recombinant *E. coli* strain was cultivated in 2YT medium containing 50 μ g of ampicillin/ml, 0.1 mM IPTG, and 0.5 mM CuSO_4 for 16 h at 37°C to produce azurin. *E. coli* JCB7120 was used as a host strain for expression of the cytochrome c_{551} -encoding gene of *P. aeruginosa*. The recombinant strain JCB7120 was cultivated under anaerobic condition at 37°C in the minimal medium as described previously (12).

Purification of wild-type and mutant azurins. *azuA* was amplified by PCR according to the method described by Kukimoto et al. (15). For expression of the *P. aeruginosa* cytochrome c_{551} gene, a plasmid, pkk223-3-PAC551, was used as described by Hasegawa et al. (10). Periplasmic fractions including azurin and cytochrome c_{551} from recombinant *E. coli* strains were obtained according to the method described by Cornelis et al. (5). Recombinant *E. coli* cells harvested from 5-liter cultures were washed with 1.2 liter of 10 mM Tris-HCl, pH 8.0, and centrifuged. The resultant cell pellets were suspended in 400 ml of 25% sucrose solution containing 0.1 mM EDTA and were shaken at room temperature for 15 min prior to centrifugation. The resultant cell pellets were suspended in 400 ml of ice-cold water and shaken again for 15 min prior to centrifugation. This supernatant was used as the periplasmic fraction. A mixture of 1 M Tris-HCl, pH 8.0, 1 M CuSO_4 , and 0.1 M potassium ferricyanide was then added to the periplasmic fraction containing azurin to adjust their final concentrations to 10, 1, and 0.1 mM, respectively. The fraction was mixed with a Q-Sepharose Fast Flow resin that had previously been equilibrated with 10 mM Tris-HCl, pH 8.0. The flowthrough fraction unbound to the Q-Sepharose gel was concentrated by Amicon YM-10. Purified azurin was obtained after gel filtration using a HiPrep 16/60 Superdex 75 column. The periplasmic fraction containing cytochrome c_{551} in 10 mM Tris-HCl, pH 8.0, was concentrated by Centrprep YM-3. The fraction was applied to a MonoQ column equilibrated with 10 mM Tris-HCl, pH 8.0. Proteins were eluted using a linear gradient from 0 to 0.15 M NaCl in 10 mM Tris-HCl, pH 8.0. The fraction showing redness was eluted with 0.05 M NaCl. This fraction was applied to a Superdex 75 column as described for azurin, and the red fraction was obtained as purified cytochrome c_{551} .

Macrophage culture and cytotoxicity assay. The J774 cell-line-derived murine macrophage cells were cultured in RPMI 1640 medium containing L-glutamine, buffered with 10 mM HEPES, and supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml at 37°C in a humidified incubator with 5% CO_2 . For measurement of the cytotoxic activity of azurin or cytochrome c_{551} , the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (23) was conducted as described previously (14). Then 10^5 cells of the macrophages per well were seeded onto 96-well culture plates in 200 μ l of RPMI 1640 medium. After overnight culture, the cells were washed with the same medium and were then replaced with fresh medium containing azurin. After 24 h treatment, 10 μ l of 5-mg/ml MTT solution was added to the culture and incubated for 2.5 h at 37°C. The MTT reaction was terminated by the addition of 40 mM HCl in isopropanol. The MTT formazan formed was measured spectrophotometrically as described earlier (23).

Measurement of apoptotic cells. To determine the extent of cytotoxicity due to induction of apoptosis, 1.5×10^5 cells were seeded per well into six-well culture plates in 3 ml of RPMI 1640 medium. After overnight culture, the cells were washed with the same media and then were replaced with 2 ml of new media containing 20 μ g of wild-type azurin or the mutant proteins per ml. After 16 h of treatment, flow cytometry analysis (Becton Dickinson) with the ApoAlert mito-

chondrial membrane sensor kit (Clontech) was used to determine the extent of apoptotic cells.

Preparation of macrophage cytosolic extracts for caspase assays. The macrophage cytosolic extracts were prepared as described by Zaborina et al. (43). Briefly, 20 ml of macrophage culture medium in a culture bottle was removed. Ten milliliters of ice-cold phosphate-buffered saline (PBS) (pH 7.2) was added, and adhered cells were lifted gently off the bottle. The cytosolic extract was then prepared as described previously (43). Caspase 9 and caspase 3 assays in the macrophage cytosolic extracts were conducted as previously described (43), using *N*-acetyl-Leu-Glu-His-Asp-p-NO₂-aniline and *N*-acetyl-Asp-Glu-Val-Asp-p-NO₂-aniline as substrates as described in the Fig. 2 legend.

Detection of DNA fragmentation by TUNEL assay. For the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, the ApoAlert DNA fragmentation assay kit (Clontech) was used; the assay was performed as recommended by the manufacturer. Briefly, macrophages were seeded at a density of 10^5 cells on Lab Tex chamber slides for 2 h. The macrophages were then treated with azurin and cytochrome c_{551} (50 and 25 μ g/ml) for different time intervals. Simultaneously, cells were treated with PBS (untreated) as a negative control. The cells were washed and fixed in 4% paraformaldehyde-PBS and permeabilized with prechilled 0.2% Triton X-100-PBS for 5 min on ice. The slides were washed with PBS and equilibrated with equilibration buffer. The tailing reaction was performed using the ApoAlert DNA fragmentation assay kit. Fifty microliters of equilibration buffer, 5 μ l of nucleotide mixture containing fluorescein-dUTP, and 0.5 μ l of terminal deoxynucleotidyltransferase enzyme were evenly spread on the treated area and incubated in a humid chamber at 37°C for 1 h. The reaction was terminated by incubating the slides with $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at room temperature. The cells were then stained with 1 μ g of propidium iodide per ml and washed. A drop of antifade solution (Vectashield; Vector Co.) was added, and the treated portion of the slide was covered with a coverslip with the edges sealed with clear nail polish. Slides were viewed within 2 h under an LSM 510 confocal laser microscope equipped with a 40 $\times$ objective using a dual-filter set for green fluorescence (488 nm) and red fluorescence (568 nm).

Gene array. To determine the gene expression profile of murine macrophages, the cells were incubated with 50 μ g of azurin/ml and 25 μ g of cytochrome c_{551} /ml or without any treatment as a control for various periods (0, 3, 6, and 12 h). Total RNA from macrophages was isolated by the RNeasy-Midi kit (Ambion). The mouse Apoptosis-1 GEArray kit (Super Array Inc., Bethesda, Md.) with 23 apoptosis-related genes, including the Bcl-2 family, caspase family, Fas, TRAIL, NF- κ B, and p53, was used to measure the level of gene expression. Membranes and a [α -³²P]cDNA probe were hybridized overnight. To compare the expressions of apoptotic genes in macrophage cells, signal intensity was measured by phosphorimager.

Subcellular fractionations of macrophages. Macrophages were treated with azurin (50 μ g/ml) and cytochrome c_{551} (25 μ g/ml) for 0, 3, 6, and 12 h. Mitochondria and cytosol fractions were prepared as described by Han et al. (9). After treatment, macrophages were suspended in buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EGTA, and 1 mM dithiothreitol [DTT]) containing 250 mM sucrose and proteinase inhibitor cocktail (Sigma). The resultant suspension was passed five times through a 26-gauge needle. After centrifugation at $1,000 \times g$ for 10 min, the supernatant was subjected to centrifugation at $10,000 \times g$ for 20 min at 4°C to separate the mitochondrial pellet, which was resuspended in 10 mM Tris-acetate, pH 8.0–0.5% NP-40–5 mM CaCl_2 buffer. For generating a cytosol fraction, the supernatant was centrifuged at $100,000 \times g$ for 1 h. The nuclear extracts were prepared as described by Rafto et al. (27). Macrophages were lysed by homogenization in hypotonic cell lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, and protease inhibitor cocktail). The nuclei were separated by centrifugation and were resuspended in isotonic-glycerol nuclear lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors). Monoclonal antibodies raised against p53, Bax, and cytochrome c (Santa Cruz Biotechnology) were used for immunoblotting. Blots were also probed for actin by using monoclonal antiactin antibody (Sigma) and mitochondrial membrane protein cytochrome c oxidase subunit IV (COX IV) with monoclonal anti-COX IV antibody (Molecular Probes) for checking cross-contamination and as internal controls. Protein bands were visualized using enhanced chemiluminescence reagents (Amersham Corp.).

Microscopy. Azurin was conjugated with the fluorescent chemical Alexa Fluor 468 (Molecular Probes) and was incubated with macrophages for 30 min. Entry of fluorescent chemically labeled azurin into the macrophages was observed by confocal microscopy. Morphological changes of macrophages were seen by phase-contrast microscopy with a 40 $\times$ objective.

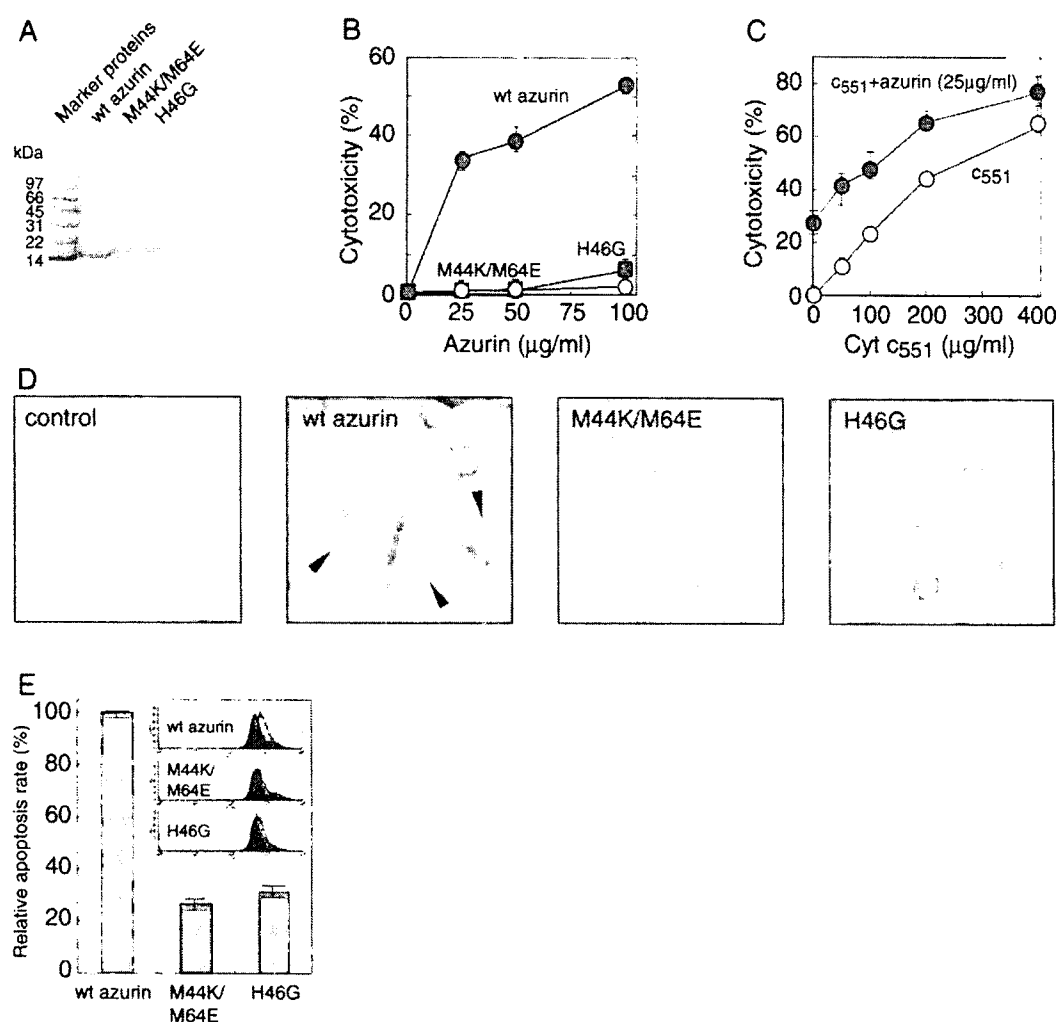


FIG. 1. Cytotoxicity and apoptotic activity of azurin and cytochrome c_{551} toward J774 cell-line-derived macrophage cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of wild-type (wt) and mutant azurins (A). Mutant azurins and wild-type azurin, as well as cytochrome c_{551} of *P. aeruginosa* PAO1, were hyperproduced in *E. coli*. The cytotoxicity of wild-type and mutant azurin proteins and cytochrome (Cyt) c_{551} toward macrophages was determined by MTT assay (B and C). Percent cytotoxicity is expressed as percent macrophage cell death as compared to that of untreated control (0% cytotoxicity). Phase-contrast pictures of untreated or azurin-treated macrophages were taken with an inverted microscope (Nikon Diaphot 200) equipped with a 40 $\times$ objective for checking the morphological changes (D). Arrowheads, morphological changes. The extent of cytotoxicity due to induction of apoptosis was determined by flow cytometry analysis (E). The relative cytotoxicity of M44K/M64E and H46G mutant azurins has been expressed as a percentage of that of the wild type, keeping the value of wild-type azurin as 100%.

Glycerol gradient centrifugation analysis. Complex formation between wild-type azurin, mutant azurin, and p53 was confirmed by glycerol gradient centrifugation analysis (24, 34, 42). Each purified protein, wild-type or mutant azurin, full-length glutathione *S*-transferase (GST)-p53, bovine serum albumin (BSA), or GST was used in 180-pmol amounts before loading. To allow complex formation, various combinations of proteins were incubated at 4°C overnight. The stepwise 5, 15, 20, 25, and 35% glycerol gradient was prepared as described by Shankar et al. (32). Sample proteins were mixed in a 5% glycerol solution (top layer). After centrifugation, fractions from each gradient were collected and tested by immunoblotting with antiazurin antibody and anti-p53 antibody.

Determination of p53 stability. To determine the stability of p53 in the presence of bacterial redox proteins, the macrophages were either treated with buffer (control) or treated with azurin (50 μg/ml) and cytochrome c_{551} (25 μg/ml) for

12 h. Cycloheximide (20 μg/ml) was then added to the culture media to prevent protein synthesis in the cells (18). p53 levels were measured in equal amounts of cell extract proteins by immunoblotting using a monoclonal anti-p53 antibody after various periods of cycloheximide addition. The intensity of bands at each time point after cycloheximide addition was measured densitometrically. The level of p53 was calculated at 0 to 6 h after cycloheximide addition. Because of the 12-h pretreatment of macrophages with azurin-cytochrome c_{551} , the level of p53 at 0 h of cycloheximide addition is higher in the extracts of treated macrophages than that in the control; however, the levels are plotted as a percentage of the p53 level (with 0-h cycloheximide at 100%) in each case.

Flow cytometry analysis of ROS. For the detection of ROS in wild-type azurin- and mutant azurin (50 μg/ml each)-treated J774 cell-line-derived macrophages, 2', 7'-dichlorodihydrofluorescein-diacetate (DCHF-DA; Sigma) was used as a

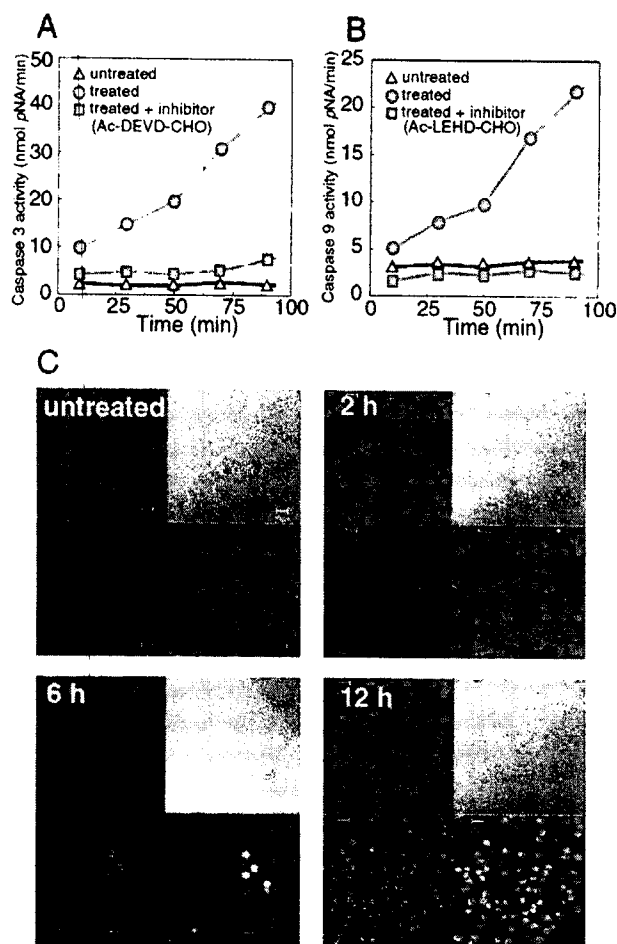


FIG. 2. Measurement of caspase activities in the cytosolic extracts of macrophages treated with or without azurin and cytochrome c_{551} (50 and 25 $\mu\text{g/ml}$, respectively). (A) Determination of caspase 3 activity involved N -acetyl-Asp-Glu-Val-Asp- p -NO₂-aniline as a substrate. Release of p -NO₂-aniline was measured spectrophotometrically at 405 nm from the caspase 3 substrate (200 μM) with or without the inhibitor N -acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) (used at 0.05 molar ratio [inhibitor/substrate ratio]) after various periods of incubation at 37°C with macrophage cytosolic extract. Fifty micrograms of macrophage cytosolic extract protein was used in each case. (B) Caspase 9 activity determination involved release of p -NO₂-aniline from 200 μM N -acetyl-Leu-Glu-His-Asp- p -NO₂-aniline as a substrate after various periods of incubation with cytosolic extract of buffer-incubated macrophages (untreated) or cytosolic extract of macrophages incubated overnight with 50 and 25 μg , respectively, of azurin and cytochrome c_{551} /ml (treated); the same extract from treated macrophages was also incubated with the substrate in the presence of the caspase 9 inhibitor, N -acetyl-Leu-Glu-His-Asp-CHO (Ac-LEHD-CHO). Fifty micrograms of macrophage cytosolic extract protein was used in all experiments. The TUNEL assay (C) was used for detection of apoptosis-induced nuclear DNA fragmentation using the ApoAlert DNA fragmentation assay kit (Clontech). J774 cell-line-derived macrophages were grown on Lab Tek chamber slides and incubated for 2, 6, and 12 h with azurin and cytochrome c_{551} (50 and 25 $\mu\text{g/ml}$). A negative control (untreated) without treatment (with buffer for 12 h) was also maintained. Macrophages viewed under both red and green channels as well as superimposed images are shown.

substrate (10 μM) by staining live cells for 30 min at 37°C and measuring the DCF (2',7'-dichlorofluorescein) green fluorescence by flow cytometry (16). Ten thousand cells were collected for flow cytometry analysis in each case. The protective effect of ROS-removing enzyme superoxide dismutase (SOD) or the vitamin E analogue Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic

acid; Sigma) on cytotoxicity was tested by incubating macrophages treated with 25 μg of azurin per ml in the absence or presence of various concentrations of these antioxidants. The MTT assay for cytotoxicity was conducted following 1-h preincubation with antioxidants and then exposure for 16 h to azurin and SOD or Trolox.

RESULTS AND DISCUSSION

Azurin- and cytochrome c_{551} -mediated cytotoxicity in macrophages. It was recently reported (43) that a column chromatographic (Q-Sepharose flowthrough) fraction enriched in azurin and cytochrome c_{551} from the growth medium of *P. aeruginosa*, as well as purified azurin and cytochrome c_{551} proteins (Sigma), demonstrated induction of apoptosis in macrophages. Murine peritoneal macrophages, J774 cell-line-derived macrophages, and primary peritoneal mast cells were all susceptible to azurin- and cytochrome c_{551} -induced apoptosis (43). To gain a better understanding of the role of enzymatic redox activity or the mode of their action, we cloned the azurin and cytochrome c_{551} genes of *P. aeruginosa* PAO1 in the expression vector pUC19 in *E. coli*. Since the role of specific amino acid residues in the redox activity of azurin has been well delineated (7, 39), we also constructed two site-directed mutants, H46G for mutation in the active site and the double mutant M44K/M64E, for mutation in the hydrophobic patch, as described previously (7, 39). All the proteins produced a single band on a sodium dodecyl sulfate-polyacrylamide gel (Fig. 1A) and demonstrated greater than 95% purity. We then determined the cytotoxicity of the hyperproduced wild-type azurin and the two mutant proteins, as determined by the quantitative MTT assay (23). Purified wild-type azurin demonstrated significant cytotoxicity towards the macrophages, while the two redox-defective mutant proteins had much less activity (Fig. 1B). Cytochrome c_{551} demonstrated cytotoxicity towards macrophages but only at relatively high concentrations (Fig. 1C). Interestingly, a mixture of azurin and cytochrome c_{551} had a higher level of cytotoxicity (Fig. 1C). Treatment of macrophages with wild-type azurin produced cells with drastic morphological changes, including swelling, membrane blebbing, and vacuolization (Fig. 1D). Control untreated cells had no such effect, while mutant azurins produced fewer cells with abnormal morphology (Fig. 1D). Flow cytometry analysis using Mitosensor dye demonstrated that the mutant azurin proteins had less apoptosis-inducing activity (Fig. 1E), as noted earlier with the MTT assay (Fig. 1B).

Induction of apoptosis by azurin and cytochrome c_{551} . To confirm that macrophage cell death, triggered by azurin and cytochrome c_{551} , is indeed due to induction of apoptosis, we measured caspase 3 and caspase 9 activities (44) in macrophages, incubated overnight either with PBS (untreated) or with a mixture of 50 μg of azurin and 25 μg of cytochrome c_{551} per ml (treated). Extracts of such macrophages were then assayed for caspase 3 and caspase 9 activities. It is clear that the extracts of treated macrophages, but not those of untreated macrophages, showed high activity for caspase 3 (Fig. 2A) or caspase 9 (Fig. 2B). Such activity was very low, either in the absence of azurin-cytochrome c_{551} treatment of the macrophages or in the presence of the specific inhibitors N -acetyl-Asp-Glu-Val-Asp-CHO for caspase 3 and N -acetyl-Leu-Glu-His-Asp-CHO for caspase 9, suggesting that treatment of macrophages with azurin-cytochrome c_{551} led to significant

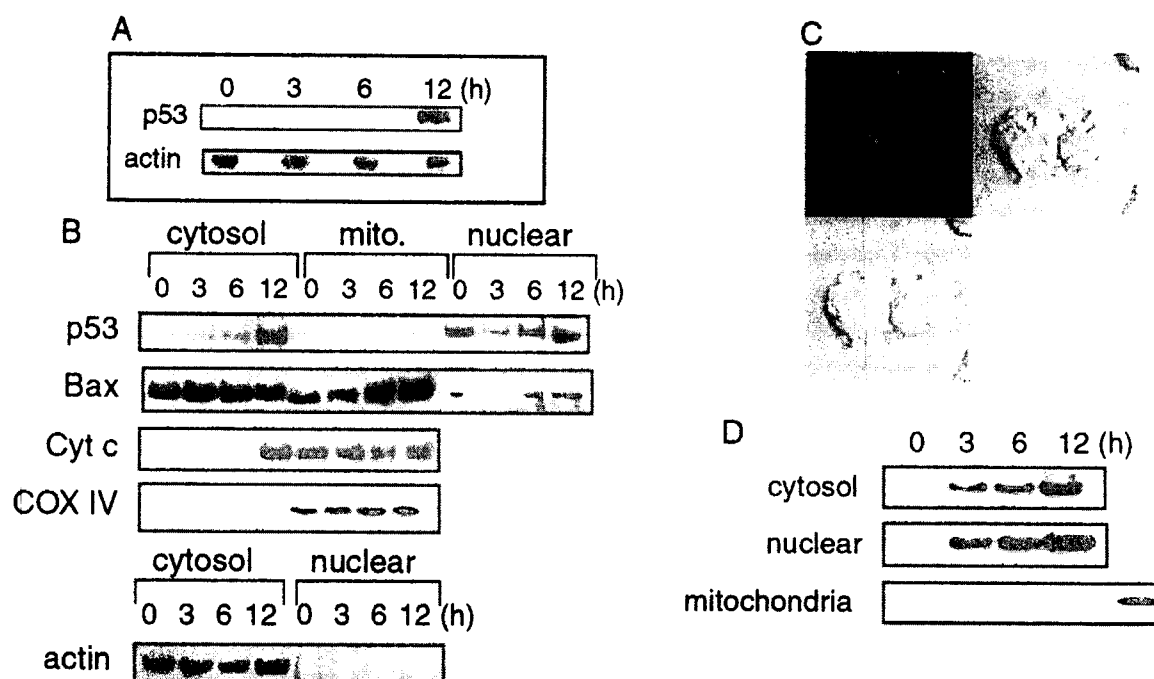


FIG. 3. (A) Immunoblot analysis of p53 protein level in whole-cell extracts of macrophages untreated (0 h) or treated for 3, 6, and 12 h with azurin-cytochrome c_{551} . (B) Subcellular fractionation of macrophage extracts treated as for panel A (B). p53, Bax, COX IV, actin, and cytochrome c protein levels were measured by immunoblotting in cytosolic (cytosol), mitochondrial (mito.), and nuclear fractions of macrophage cell extract. Fractionated samples were prepared as described in Materials and Methods. (C) Uptake of fluorescent chemical (Alexa Fluor 468)-labeled azurin in J774 cells was observed by confocal microscopy. (D) The localization of wild-type azurin in the subcellular fractions of macrophages either untreated (0 h) or treated with azurin for 3, 6, and 12 h was monitored by immunoblotting using antiazurin antibody (D).

activation of caspase 9 and caspase 3 activities, indicative of extensive apoptosis in such cells.

To determine a time course of the initiation of apoptotic activity when macrophages are subjected to azurin-cytochrome c_{551} treatment, we used the TUNEL assay to determine the extent of nuclear DNA fragmentation. The incorporation of fluorescein-conjugated dUTP in the fragmented nuclear DNA, leading to enhanced green fluorescence imparted by fluorescein and detected by confocal microscopy, demonstrated the initiation of apoptosis in macrophages at around 5 or 6 h and continuing beyond 12 h (Fig. 2C).

To determine if induction of apoptosis by azurin-cytochrome c_{551} might be due to transcriptional activation or repression of proapoptotic or antiapoptotic genes, we used DNA array chips harboring 23 such murine genes and determined the extent of expression of these genes as a function of azurin-cytochrome c_{551} treatment of murine macrophages for 3, 6, and 12 h. The data did not show significant (more than twofold) alteration in the expression of these genes following treatment with azurin-cytochrome c_{551} .

Subcellular localization of azurin and the tumor suppressor protein p53. In order to see if the bacterial redox proteins may trigger apoptosis in nonphagocytic cells, we tried several mammalian cell lines, including p53-positive and p53-null cell lines. We noticed that p53-negative cell lines were resistant to undergoing apoptosis in the presence of azurin-cytochrome c_{551} , while the p53-positive cell lines were more susceptible (42a). Since p53 is a known inducer of apoptosis (2, 31, 40), these observations indicated that p53 might play a role in the induction of apoptosis by azurin-cytochrome c_{551} . To see if treat-

ment with azurin-cytochrome c_{551} might alter the intracellular level of p53, we treated macrophages with azurin and cytochrome c_{551} (50 and 25 $\mu\text{g}/\text{ml}$, respectively) for 0, 3, 6, and 12 h and measured the level of p53 in the extracts of the treated (or untreated [0 h]) macrophages by Western blotting. We also measured the level of actin as an internal control (Fig. 3A and B). Significant elevation in the level of p53, but not of actin, was observed when the macrophages were treated with the redox proteins for 12 h (Fig. 3A and B).

To determine the subcellular localization of both azurin and p53 after treatment of macrophages with azurin-cytochrome c_{551} , the macrophage cell extract was further fractionated to obtain cytosolic, mitochondrial, and nuclear fractions and the levels of p53 and azurin were determined in such fractions. p53 is a positive transcriptional activator of the *bax* gene (28) and induces apoptosis via the mitochondrial apoptosome cytochrome c release pathway (1, 33). Thus, we were also interested in knowing the levels of Bax in various subcellular fractions and the extent of release of cytochrome c from the mitochondria to the cytosol during treatment of the macrophages for 0, 3, 6, and 12 h with azurin-cytochrome c_{551} . The level of p53 rose steadily in the cytosol and in the nuclear fractions during the 1-h period, but very little p53 was observed in the mitochondria (Fig. 3B). In contrast, the Bax level increased significantly in the mitochondria, particularly during 6 to 12 h when cellular apoptosis is initiated. The movement of Bax from cytosol to mitochondria during apoptosis of thymocytes, Cos-7 kidney epithelial cells, and L929 fibroblasts (11, 41) has been reported. There was a steady increase of cytochrome c in the cytosol during the 12-h period (Fig. 3B),

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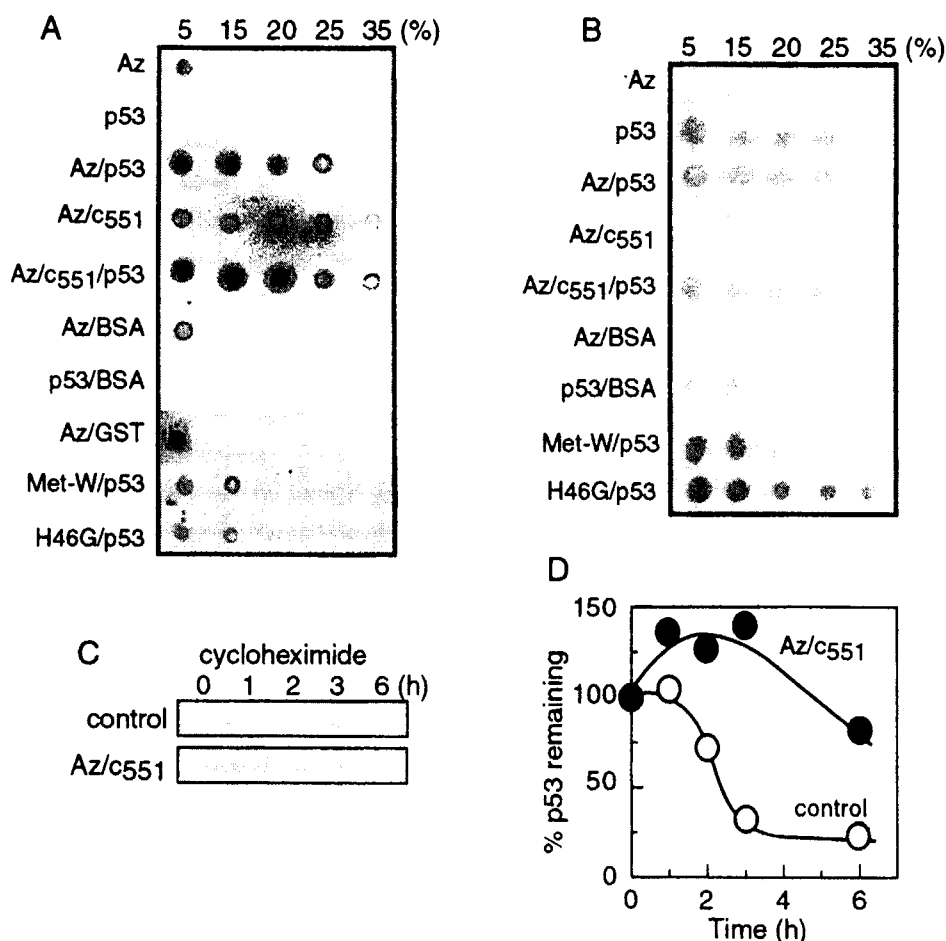


FIG. 4. Complex formation and stabilization of p53 with azurin. Complex formation between wild-type azurin, mutant azurins, and p53 was confirmed by glycerol gradient centrifugation analysis using antiazurin (A) and anti-p53 (B) antibodies as described in Materials and Methods. The stability of p53 in the presence of bacterial redox proteins was measured by immunoblotting using anti-p53 antibody after 12 h of azurin treatment and subsequent addition of cycloheximide (C). The intensity of p53 bands was determined densitometrically and plotted with the 0-h value at 100% and by measuring p53 stability up to 6 h after cycloheximide addition (D) in untreated (control) and azurin-cytochrome c_{551} (Az/c c_{551})-treated macrophages. Met-W, M44K/M64E.

suggesting a possible release of cytochrome *c* from the mitochondria to the cytosol. The COX IV subunit was detected only in the mitochondria and not in the cytosol, indicating that the cytosol was virtually free of the mitochondria. The actin was found in the cytosol but not in the nuclear fraction, suggesting that the nuclear fraction was significantly free of cytosolic contamination. Overall, azurin-cytochrome c_{551} treatment of the macrophages resulted in a steady accumulation of p53 in the cytosolic and nuclear fractions, while the Bax level increased mainly in the cytosolic and mitochondrial fractions. Significant release of mitochondrial cytochrome *c* to the cytosol occurred during 6 to 12 h of treatment.

To localize azurin during the treatment of macrophages with this protein, we used confocal microscopy of macrophages incubated for 30 min with chemically labeled (Alexa Fluor 468) green fluorescent azurin (Fig. 3C). Azurin was found to be located within the macrophage cell. Unlike azurin, another labeled protein, GST, did not show entry or intracellular accumulation (data not shown). We also examined various subcellular fractions for the presence of azurin by Western blotting using antiazurin antibody (Fig. 3D). Azurin was found to

be localized in the cytosol and the nuclear fractions. The concentration of internalized azurin increased with an increasing period of incubation (Fig. 3D). No azurin was found in mitochondria. Since the redox-negative mutant azurin proteins had low cytotoxicity (Fig. 1B), it was of interest for us to see if this was due to lack of internalization of mutant azurins or a different subcellular localization. Incubation of the macrophages with the wild-type and mutant azurin proteins showed localization of both types of azurin in the nuclear fraction and in the cytosol (data not shown). This suggested that the low cytotoxicity of mutant azurin proteins is not due to a lack of entry into the macrophages.

Azurin forms a complex with p53 and stabilizes it. As previously mentioned, mammalian redox proteins such as NQO1 (3) and Ref-1 (8, 13) modulate p53 level or its activity by binding to it and preventing its degradation or enhancing its DNA-binding activity. We thus wanted to determine if the bacterial redox protein azurin or cytochrome c_{551} could form complexes with p53 and elevate its level by stabilizing it. Glycerol gradient centrifugation to study complex formation among sigma factor-anti-sigma factor (42) or other interacting pro-

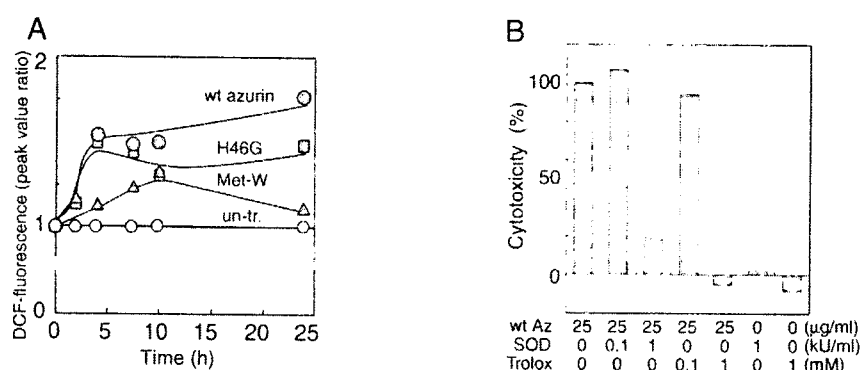


FIG. 5. ROS levels in live macrophage cells analyzed by flow cytometry (A). Macrophages were treated without (un-tr) or with wild-type (wt) or mutant azurins using DCHF-DA as a substrate, and the levels of ROS were measured as described in Materials and Methods (A). The extent of cytotoxicity in macrophage cells treated with or without azurins and/or antioxidants used at various concentrations as shown was determined by MTT assay (B). Met-W, M44K/M64E.

teins (24, 34) has previously been used. In this method, the proteins are sedimented by ultracentrifugation through a glycerol gradient, either singly or in combination. If the two proteins form a complex, then the complex sediments at a higher glycerol gradient than do the individual proteins. We used a 5 to 35% glycerol gradient to sediment wild-type azurin, p53 (a GST-p53 fusion protein was used), cytochrome c_{551} , BSA, GST, and mutant azurin proteins H46G and the double mutant M44K/M64E either singly or in various combinations. The presence of azurin in various glycerol gradient fractions was then detected by collecting samples from these fractions and running Western blots using antiazurin or monoclonal anti-p53 (or anti-cytochrome c_{551}) antibodies. Azurin by itself sedimented at 5% glycerol (Fig. 4A). When azurin and GST-p53 fusion protein were incubated before sedimentation, azurin could be detected at 5, 15, 20, and 25% glycerol fractions (Fig. 4A), suggesting the presence of a polydisperse complex. p53 is known to form aggregates (17) and was found to be present by itself in 5, 15, 20, and 25% glycerol gradient fractions as various aggregates (Fig. 4B). Incubation with azurin did not lead to its sedimentation at higher glycerol concentration, presumably because of the low molecular mass (14 kDa) of azurin. Azurin is known to associate with cytochrome c_{551} for its electron transfer reaction (38) and can be seen to form a polydisperse complex with cytochrome c_{551} , similar to that with p53 (Fig. 4A). Other proteins such as BSA and GST, however, had no ability to form complexes with azurin (Fig. 4A), suggesting the specificity of azurin-p53 complex formation. The inability of GST to form a complex with azurin strongly suggests that the complex formation of azurin with GST-p53 fusion protein is due to p53 and not due to GST. Interestingly, the two mutant azurin proteins had much less affinity for complex formation with p53. It is not known if the reduced cytotoxicity of the mutant proteins might be due to reduced affinity for this complex formation, rather than to loss of their redox activity. Addition of the M44K/M64E mutant azurin to wild-type azurin had no significant effect on its cytotoxicity, indicating a lack of competition between the two.

To determine if complex formation of p53 with azurin-cytochrome c_{551} might stabilize p53, thus accounting for its higher intracellular level, we used the method described by Maki and

Howley (18). We incubated macrophages either with buffer (untreated) or with azurin and cytochrome c_{551} (50 and 25 $\mu\text{g/ml}$) for 12 h, followed by the addition of 20 μg of cycloheximide per ml to prevent protein synthesis. The level of p53 in the macrophage extracts was then measured by Western blotting (Fig. 4C) using monoclonal anti-p53 antibody. The intensity of the bands was determined by using the enhanced chemiluminescence assay and was plotted as the percentage of p53 remaining of the initial cellular level at the time of addition of cycloheximide (0 h). The results (Fig. 4D) demonstrated that, while the level of p53 decreased steadily in untreated control cells in about 3 h, a substantial amount of p53 remained in the treated cells even after 6 h, suggesting its stabilization during azurin-cytochrome c_{551} treatment.

Correlation between cytotoxicity and ROS generation by azurin. Redox proteins such as PIG3 (26) and NQO1 (3) appear to be at least partly responsible for an increase in the formation of ROS in mitochondria, which has been implicated in the induction of apoptosis (21); however, ROS inhibitors have been reported to be unable to protect against p53-mediated apoptosis in some cases (31). On the other hand, antioxidants such as SOD have been shown to protect acute myeloblastic leukemia cells from undergoing apoptosis in the presence of the oxidative agent Etoposide (20). To see if the bacterial redox protein azurin might elicit a higher level of ROS formation in macrophages, we treated the macrophages either with buffer (untreated) or with 50 μg of wild-type azurin or mutant azurins H46G and M44K/M64E per ml. The level of ROS was then determined periodically for the next 24 h as DCF fluorescence generated from the substrated DCHF-DA (16). As opposed to buffer-treated macrophages, macrophages treated with wild-type azurin showed significant generation of ROS, while treatment with the mutant azurins generated a lower level of ROS, particularly during prolonged treatment (Fig. 5A). To see the effect of antioxidants such as catalase, SOD, and Trolox, a vitamin E analogue, on the azurin-induced cytotoxicity towards macrophage cells, macrophages were either not treated with azurin (0 $\mu\text{g/ml}$) or were treated with azurin at 25 $\mu\text{g/ml}$ with or without concomitant addition of SOD at 0.1 and 1.0 kU/ml or Trolox at 0.1 and 1.0 mM (Fig. 5B). Similar experiments were done with catalase. While cata-

lase had no effect on azurin-induced cytotoxicity, SOD at 1.0 kU/ml or Trolox at 1.0 mM significantly reduced the cytotoxicity exerted by 25 μ g of azurin/ml (Fig. 5B), thus demonstrating a correlation between the level of Trolox- or SOD-susceptible ROS and azurin-induced cytotoxicity in macrophages. The redox-negative mutants generated lesser quantities of ROS and had less cytotoxicity, confirming the role of ROS, at least partly, in azurin-mediated induction of apoptosis in macrophages.

The ability of bacterial redox proteins such as azurin-cytochrome c_{551} , which are secreted in the growth medium by pathogens such as *P. aeruginosa* (43) and which can be internalized in phagocytic cells such as macrophages, to allow complex formation and stabilization of tumor suppressor protein p53 and generation of enhanced level of ROS is an interesting example of how bacterial pathogens may subvert host defense through induction of host phagocytic cell apoptosis. Recent experiments indicate that some redox-negative mutant azurins can form complexes with p53, generate ROS, and induce macrophage apoptosis, suggesting that p53-mediated enhanced ROS generation is important for the induction of apoptosis. The role of mammalian redox proteins in the induction of p53-mediated apoptosis is a subject of considerable present interest (21). It remains to be seen if other microbial pathogens may elaborate similar redox proteins, either as virulence factors or as agents that facilitate microbial invasion and/or proliferation in eukaryotic organisms by complex formation with p53.

The enhanced cytosolic or mitochondrial Bax levels in murine macrophages during treatment with azurin-cytochrome c_{551} for prolonged periods (Fig. 3B) is most likely a reflection of high p53 levels in such cells. The reduced cytotoxicity of the two redox-negative mutant azurin proteins may similarly be correlated with their reduced ability to form complexes with p53 (Fig. 4A) and not to their loss of redox activity. The loss of the redox enzymatic functions in these two mutants, however, might also abolish their ability to activate p53-mediated binding to the response element of the *bax* genes (36) or to generate a significant amount of ROS. Further studies are under way to define clearly the role of enzymatic redox activity on azurin-mediated cytotoxicity and the ability of wild-type and redox-negative mutant azurins to modulate the DNA-binding activity of p53, as demonstrated for Ref-1 (8, 13).

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