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Report

Rusticyanin, a Bacterial Electron Transfer Protein, Causes G_1 Arrest in J774 and Apoptosis in Human Cancer Cells

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ABSTRACT

During acid mine drainage, *Acidithiobacillus ferrooxidans*, a nonpathogenic, acidophilic, lithotrophic bacterium, utilizes rusticyanin to transfer electrons for the oxidation of Fe^{2+} to Fe^{3+} for deriving its energy. No other function of rusticyanin is known. We demonstrate that purified rusticyanin enters mammalian cells inducing either inhibition of cell cycle progression or caspase-8 mediated apoptosis. Treatment of human melanoma cells with rusticyanin allowed significant generation of reactive oxygen species and active caspase-8, leading to cell death. The ability of rusticyanin to modulate mammalian cell death might be relevant to a role of this cupredoxin in protecting *At. ferrooxidans* from eukaryotic predators in the environment.

INTRODUCTION

Acidithiobacillus ferrooxidans, previously called *Thiobacillus ferrooxidans*, is a major agent for bioleaching[1] where the bacteria dissolve insoluble sulfides of metals such as copper, uranium, etc., to a soluble form for their extractive recovery. *Acidithiobacillus ferrooxidans* (*At. ferrooxidans*) is an acidophilic, chemolithotropic, Gram-negative bacterium capable of growing at pH values 1.6 to 3.5. As an autotroph, *At. ferrooxidans* can fix nitrogen and CO_2 from the air and derive its energy through biological oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) which can then allow oxidation of insoluble metal sulfides in an acidic environment to their soluble oxidized forms.[2,3] The *At. ferrooxidans* genome of about three million base pairs containing more than 2700 identified open reading frames has been sequenced, although not fully, and candidate genes in many physiological processes in *At. ferrooxidans* are now being characterized.[4] One of these genes encodes a copper-containing redox protein rusticyanin which has been shown to be involved in shuttling electrons from a cytochrome C2 to another cytochrome C4 during oxidation of Fe^{2+} to Fe^{3+}. This electron transfer occurs in the periplasmic space of *At. ferrooxidans* at a pH of about 3.0 where rusticyanin is localized and is biologically active.[5,6] The gene for rusticyanin (*rus*) is part of a gene cluster with the genes for cyc2 and cyc4 which encode the two partner cytochromes. All these genes have been shown to be cotranscribed[6] and during active iron oxidation, the level of intracellular rusticyanin may reach as high as 5% of the total cellular proteins.[7]

The only known function of rusticyanin is in the iron respiratory chain of *At. ferrooxidans* where it is believed to shuttle electrons from high molecular weight cytochrome via cytochrome c_{552} to cytochrome oxidase.[3,6,8] Rusticyanin is a member of a family of blue copper-containing proteins called cupredoxins, and shows homology to the other members in its C-terminus. Unlike other cupredoxins such as azurin, plastocyanin and stellacyanin, rusticyanin has the highest redox potential (680 mV) while stellacyanin has a value of about 185 mV and azurins and plastocyanins have typical values of 305–395 mV.[9] Unlike other cupredoxins, rusticyanin has high acid stability and is biologically functional at pH below 1.0.[10] The Ser 86 residue is believed to contribute to the acid stability and high redox potential of rusticyanin.[11] The extreme acid stability of rusticyanin is important since *At. ferrooxidans* carries out rusticyanin-mediated electron transfer during Fe^{2+} oxidation at pH 1.5 to 3.5, the optimum growth pH of this organism for metal leaching.[1,2]

While rusticyanin is involved in iron oxidation, other members of the cupredoxin family participate in other diverse biological functions. For example, azurin is involved during denitrification by *Pseudomonas aeruginosa* where nitrate is sequentially reduced to nitrite, nitric oxide, nitrous oxide and dinitrogen.[12,13] Another cupredoxin, plastocyanin, and a cytochrome, cytochrome f, are redox partners in the photosynthetic electron transfer chain of cyanobacteria such as *Phormidium laminosum* and plants.[14,15] The cyanobacterial plastocyanin functions during photosynthesis to shuttle electrons between the cytochrome bf complex and photosystem I or cytochrome oxidase,[14,15] a function very different from those where azurin or rusticyanin is involved.[16] Other cupredoxins are involved in other dehydrogenation reactions.[16]

We have recently reported that azurin, a cupredoxin from *P. aeruginosa*, can enter J774 cells which are derived from murine reticulum cell sarcoma[17] or various human cancer cells such as UISO-Mel-2 melanoma or MCF-7 breast cancer cells.[18-20] Upon entry, azurin induces apoptosis in such cells through complex formation and stabilization of the tumor suppressor protein p53, a known inducer of apoptosis.[18-20] We have also recently demonstrated that the hydrophobicity of the azurin surface is very important for its ability to modulate p53 function. A double-mutant of azurin M44KM64E where two hydrophobic methionine residues from the hydrophobic patch of azurin[12,13] were replaced by two polar amino acids aspartic and glutamic acids, has been shown to modulate p53's specificity from the induction of apoptosis to the induction of inhibition of cell cycle progression at the G_1 to S phase, causing growth arrest of J774 cells.[21] Not only cupredoxin azurin, but a cytochrome, cytochrome c_{551}, which is an in vitro electron transfer partner of azurin during denitrification, has also been reported to enter J774 cells. Upon entry, wt cytochrome c_{551} induces inhibition of cell cycle progression at the G_1 to S phase because of enhancement of the intracellular level of the tumor suppressor protein $p16^{Ink4a}$ in the treated cells.[22] Interestingly, a double mutant V23DI59E of cytochrome c_{551}, where two hydrophobic valine and isoleucine residues on the hydrophobic patch of cytochrome c_{551} were replaced by two polar amino acids, demonstrates a reduced ability to induce inhibition of cell cycle progression, but an enhanced ability to trigger apoptosis in J774 cells, suggesting a role of surface hydrophobicity that is also important for cytochrome c_{551} to interact with its electron transfer partner azurin.[23]

The ability of azurin and cytochrome c_{551} to modulate mammalian cell growth and death depending on their surface hydrophobicity raised an interesting question: is such an effect unique to azurin/cytochrome c_{551} present in an opportunistic pathogen *P. aeruginosa* where the redox proteins may act as virulence factors? Since *At. ferrooxidans* has a unique ecological niche very different from human/animal body, and is nonpathogenic, an important question was if rusticyanin would demonstrate a similar ability to modulate the growth and death of mammalian cells. In this report, we demonstrate that indeed rusticyanin, as a purified protein, can enter both J774 and human cancer cells, similar to azurin, and induce either inhibition of cell cycle progression or apoptosis. This is a unique trait of rusticyanin never known before and may signify a more general role of cupredoxins in modulating mammalian cell growth and death.

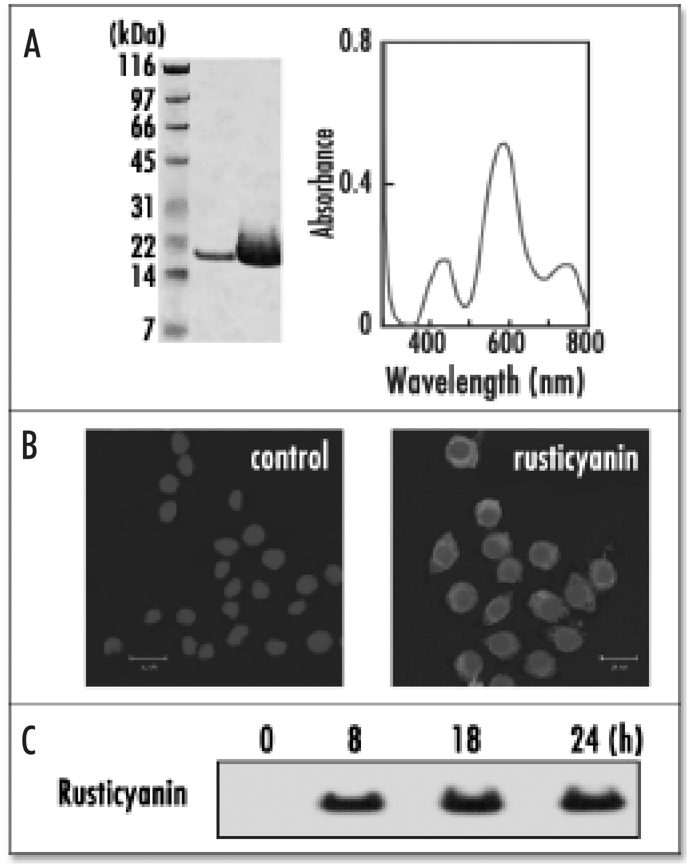

Figure 1. Purification and internalization of rusticyanin in mammalian cells. (A) Rusticyanin derived from *Acidithiobacillus ferrooxidans* was isolated after hyperexpression of the *rus* gene in *E. coli* BL21. Purified rusticyanin was loaded on SDS-PAGE and visualized by Coomassie Blue staining. UV-Vis spectrum showed characteristic rusticyanin absorbance spectrum. (B) Rusticyanin was chemically labeled with red fluorescing Alexa fluor 568 (Molecular Probes). J774 cells were treated with chemically labeled-rusticyanin for 1 h at 37°C. After fixation with methanol at -20°C, the nucleus was stained blue with DAPI. Cells were observed by LSM510 confocal microscopy (Carl Zeiss). (C) Entry of rusticyanin was confirmed by Western blotting. J774 cells were treated with 200 μg/ml of rusticyanin for 0,8,18 and 24 hours. Whole cell lysates were run on SDS-PAGE and rusticyanin was detected using anti-rusticyanin antibody.

MATERIALS AND METHODS

Bacterial Culture and Isolation of Rusticyanin. *Escherichia coli* BL21 was used as a host strain for hyperproduction of rusticyanin. After induction with 0.4 mM IPTG at early log-phase, rusticyanin was isolated from the soluble fraction using ion-exchange and gel filtration columns.

Cell Culture. The J774 cells, human melanoma and breast cancer cells were cultured as described before.[18-20]

MTT Assay. MTT [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)] assay was used for the measurement of the cytotoxicity of rusticyanin as described previously.[18,19]

Flow Cytometric Analysis of Cell Cycle Progression. The J774 or melanoma cells were incubated with rusticyanin at various concentrations for 24 h. Samples for flow cytometric separation were described before.[21,22] Briefly, rusticyanin-treated cells were washed twice with phosphate-buffered saline (PBS) and fixed with 70% ethanol at -20ºC. Fixed cells were washed twice with PBS and stained by 50 μg/ml of propidium iodide in PBS containing 20 μg/ml of RNase A. For determination of DNA content at various phases in the cells, flow cytometry (Becton Dickinson, USA) was used. The percentage of cells in different phases of cell cycle was determined by MODIFIT LT software. At least, ten thousand cells were collected in each experiment.

Western Blotting. J774 cells were cultured with rusticyanin for 0, 8, 18 and 24 h. Whole cell lysate was prepared as described previously.[21] UISO-Mel-2 cells were treated with rusticyanin for 0, 8 and 18 h at 37°C. After treatment, mitochondrial, cytosolic and nuclear fractions were prepared.[19] Monoclonal antibodies raised against caspases (Cell Signaling Technology), p53, Bax (Santa Cruz Biotechnology), p16, p21, p27, CDKs and cyclins (BioSource) were used for immunoblotting. Monoclonal anti-actin antibody (Sigma) and anti-COX IV antibody (Molecular Probes) were also applied for checking cross-contamination and as internal controls. Protein bands were visualized using ECL reagents (Amersham Corp.), as described earlier.[18,19]

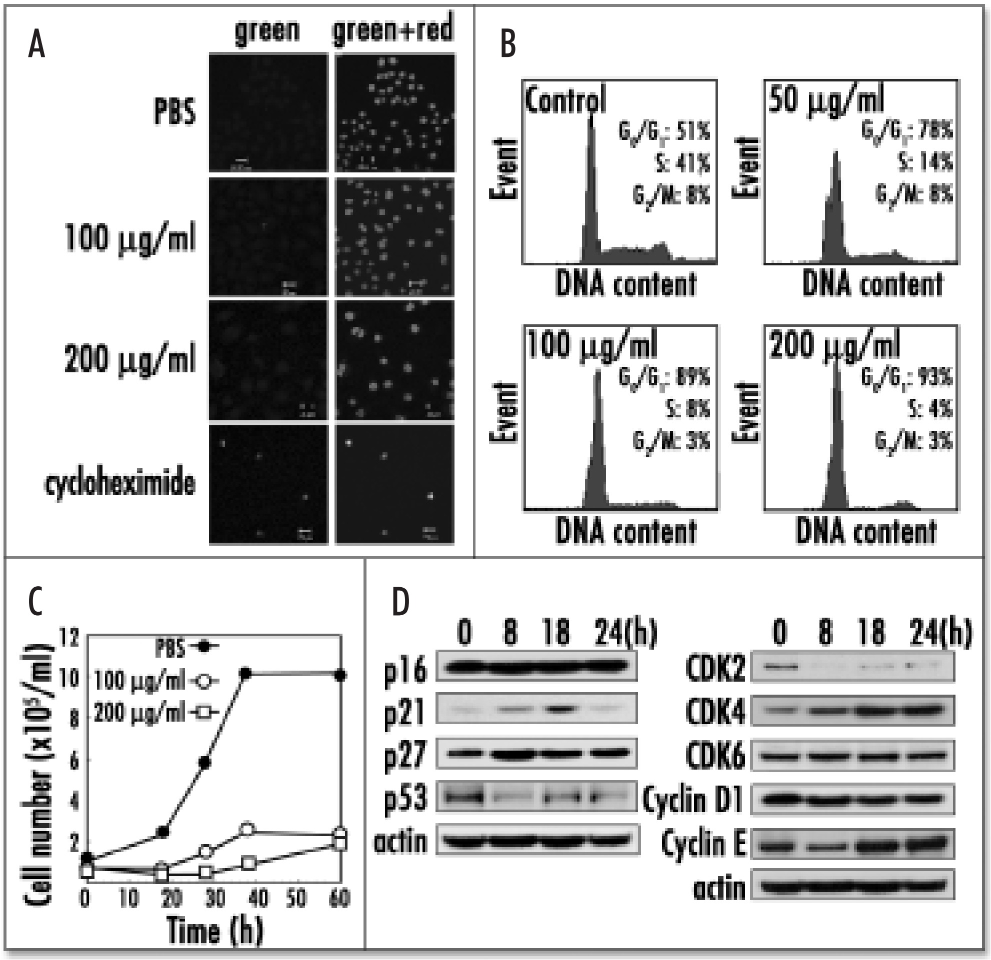

Figure 2. Rusticyanin inhibits cell cycle progression in J774 cells by depleting CDK2 and causes growth arrest. (A) J774 cells were incubated with various concentrations of rusticyanin for 24 h. Cycloheximide was used as a positive control for induction of apoptosis. After treatment, TUNEL assay was performed[18] using ApoAlert TUNEL assay kit (Clontech). Confocal microscopy was used for taking pictures (x 40). Red cells are healthy; green cells are apoptotically dead. The low number of cells in cycloheximide treated sample is due to reduced attachment of apoptotically dead cells. (B) J774 cells were cultured with various concentrations of rusticyanin for 24 h. Cell cycle progression on J774 cells was analyzed by flow cytometry as described previously.[21,22] (C) J774 cells were seeded into 6-well plates at 1 x 10^5/ml. Cells were incubated with fresh media containing various concentrations of rusticyanin. Total cell numbers were counted at various time periods using a hematocytometer. (D) J774 cells were incubated with 200 µg/ml of rusticyanin for indicated times. Whole cell lysates (30 µg protein from each sample) from rusticyanin treated and nontreated J774 cells were loaded on SDS-PAGE. After electrophoretic separation, immunoblotting was performed for determination of the levels of proteins involved in cell cycle regulation.

Detection of ROS Generation. UISO-Mel-2 and UISO-Mel-6 cells (5 x 10^5 cells) were treated without or with 500 µg/ml of rusticyanin for 2, 4, 8, 16 and 24 h. After treatment, the cells were trypsinized and washed once with PBS. The washed cells were stained with 10 µM 2',7'-dichlorodihydrofluorescein-diacetate (DCHF-DA) for 30 min at 37°C and the 2',7'-dichlorofluorescein (DCF) green fluorescence was detected by flow cytometry as described previously.[18]

RESULTS AND DISCUSSION

To determine if rusticyanin can enter mammalian cells and exert cytotoxicity, we hyperexpressed the cloned *At. ferrooxidans rus* gene under the *T7* promoter in *E. coli*. Rusticyanin was shown to appear as a single homogenous protein band in SDS-PAGE gel with a molecular size of about 16.5 kDa and an authentic absorption spectrum (Fig. 1A). Rusticyanin was then conjugated with Alexa fluor 568, a red-fluorescing dye (Molecular Probes). Confocal microscopic examination demonstrated the entry of red-fluorescing rusticyanin in J774 cells treated for 1 h at 37°C with 200 µg/ml (11.7 µM) of the conjugated rusticyanin (Fig. 1B). The nucleus in the J774 cells was stained blue with DAPI (4',6-diamidino-2-phenylindole). We also used Western blotting to detect the internalization of rusticyanin. J774 cells were treated with 200 µg/ml rusticyanin for 0 (no treatment), 8, 18 and 24 h. The cells were washed extensively with phosphate-buffered saline (PBS) to remove external rusticyanin, lysed and the whole cell lysates were loaded and electrophoresed on SDS-PAGE. After transfer to polyvinylidene fluoride (PVDF) membrane, Western blotting was performed using anti-rusticyanin antibodies (Fig. 1C). Rusticyanin was detected inside the J774 cells under such conditions.

We previously reported[18] that another cupredoxin, azurin, from the pathogen *P. aeruginosa* induced apoptotic cell death in J774 cells. To examine if rusticyanin may similarly induce apoptosis in such cells, we conducted TUNEL assays[18] using ApoAlert TUNEL assay kit (Clontech). Very little induction of apoptosis was noted under such conditions (Fig. 2A), contrasting the mode of action of rusticyanin from azurin. However, a mutant form of azurin, the M44KM64E azurin, elicited little induction of apoptosis in J774 cells but triggered inhibition of cell cycle progression.[21] To examine if rusticyanin may mimic M44KM64E azurin mode of action, we used flow cytometry to follow the cell cycle progression in J774 cells treated for 24 h with various concentrations of rusticyanin. With increasing concentrations, rusticyanin elicited increasing levels of cell cycle inhibition at the G_1 to S phase (Fig. 2B). Corresponding to such inhibition of cell cycle progression, rusticyanin at concentrations of 100 to 200 µg/ml significantly arrested the growth of J774 cells over a period of 60 h (Fig. 2C).

To obtain an understanding of how rusticyanin might inhibit cell cycle progression in J774 cells, we incubated such cells with 200 µg/ml of rusticyanin for varying periods of time, the cells thoroughly washed to remove rusticyanin, lysed and the levels of various intracellular tumor suppressors and other proteins involved in cell cycle were determined by Western blotting using antibodies against such proteins as described previously for M44KM64E azurin[21] or wt cytochrome c_{551}.[22] The levels of p21, an inhibitor of cyclin/CDKs,[24,25] enhanced slightly during 8 to 18 h of incubation (Fig. 2D). More pronounced was the elevation of the intracellular level of p27 (Fig. 2D), a member of the cip/kip family that is a potent inhibitor of CDK2.[24] Indeed, the intracellular level of CDK2, a key cyclin dependent kinase involved in cell cycle transition from the G_1 to the S phase,[24,25] was drastically reduced during treatment of J774 cells with rusticyanin (Fig. 2D). The levels of other cyclins and CDKs were not substantially altered, suggesting that rusticyanin treatment led to enhanced accumulation of p27 in J774 cells, leading to inhibition of CDK2 and a corresponding inhibition of cell cycle at the G_1 to S phase.

We previously reported that the *P. aeruginosa* cupredoxin azurin not only induced apoptosis in J774 cells[18] but also in human melanoma UISO-Mel-2[19] and in human breast cancer MCF-7 cells.[20] In the case of cancer cells, azurin allowed in vivo 60 to 85% regression of the melanoma and breast cancers in athymic mice.[19,20] To examine if rusticyanin could enter and induce either inhibition of cell cycle or apoptosis in UISO-Mel-2 or MCF-7 cells, we examined the entry of Alexa fluor 568-conjugated rusticyanin in UISO- Mel-2 and MCF-7 cells by confocal microscopy. Rusticyanin was shown to be internalized in such cells (Fig. 3A). Unlike the J774 cells, however, rusticyanin had no significant effect on cell cycle progression, either in UISO-Mel-2 or in MCF-7 cells (data not shown). This is not surprising as it is known that such cancer cells often harbor mutations in tumor suppressors such as pRb (retinoblastoma) that override cell cycle checkpoint and allow DNA replication even in presence of inhibitors of cyclin/CDKs.[21] In contrast to J774 cells, the p53-positive UISO-Mel-2 and MCF-7 cells were susceptible to rusticyanin-mediated cytotoxicity (Fig. 3B). A p53-negative mutant form of MCF-7, MDD2,[20] was less susceptible as was a p53-null cell line UISO-Mel-6,[19] suggesting that p53 might play a role in rusticyanin-induced cytotoxicity in cancer cells. The susceptibility of UISO-Mel-6 cells to rusticyanin, however, indicated that p53 was not essential for such cytotoxicity. That the cytotoxicity was primarily due to induction of apoptosis was demonstrated by TUNEL assay where UISO-Mel-2 cells, exposed to increasing concentrations of rusticyanin (200 and 400 μg/ml), underwent apoptosis as determined by the fragmented nuclear DNA of apoptotic UISO-Mel-2 cells (Fig. 3C).

Apoptosis in mammalian cells is normally mediated by two primary pathways, extrinsic and intrinsic. Extrinsic pathways involve cell surface death receptors which are a family of transmembrane proteins that belong to the tumor necrosis factor receptor superfamily with a 'death domain' in their cytoplasmic tails that is required for apoptotic signaling.[26] Fas/CD95 receptor and its ligand, along with adaptor proteins such as FADD, allow recruitment of procaspase-8 and its subsequent activation to caspase-8, thus inducing apoptosis through activation of executioner caspases such as caspase-3. In contrast, the intrinsic pathways are triggered by stress signals that involve changes in mitochondrial outer membrane permeability leading to the release of mitochondrial cytochrome c from the intermembrane space and formation of complexes with Apaf-1 and binding of ATP/dATP. This allows oligomerization of the complex to form an apoptosome which recruits procaspase-9 and allows its activation to form caspase-9. Caspase-9 then cleaves other procaspases to produce caspase-3 and the other downstream caspases,[27] leading to apoptosis.

We previously reported that azurin triggers apoptosis in both UISO-Mel-2 and MCF-7 cells through release of mitochondrial cytochrome c in the cytosol.[19,20] To determine if rusticyanin may induce apoptosis in UISO-Mel-2 cells by a similar mechanism, we incubated such cells in presence of 500 μg/ml of rusticyanin for 0, 8 and 18 h and looked for the localization and levels of tumor suppressor p53, the proapoptotic protein Bax and the mitochondrial intermembrane space protein cytochrome c. We also examined the localization of rusticyanin in cytosolic, nuclear and mitochondrial fractions. p53, which has cytosolic and nuclear localizations, was found in significant amounts also in the mitochondria during 8–18 h incubation (Fig. 3D). The Bax level did not change in the

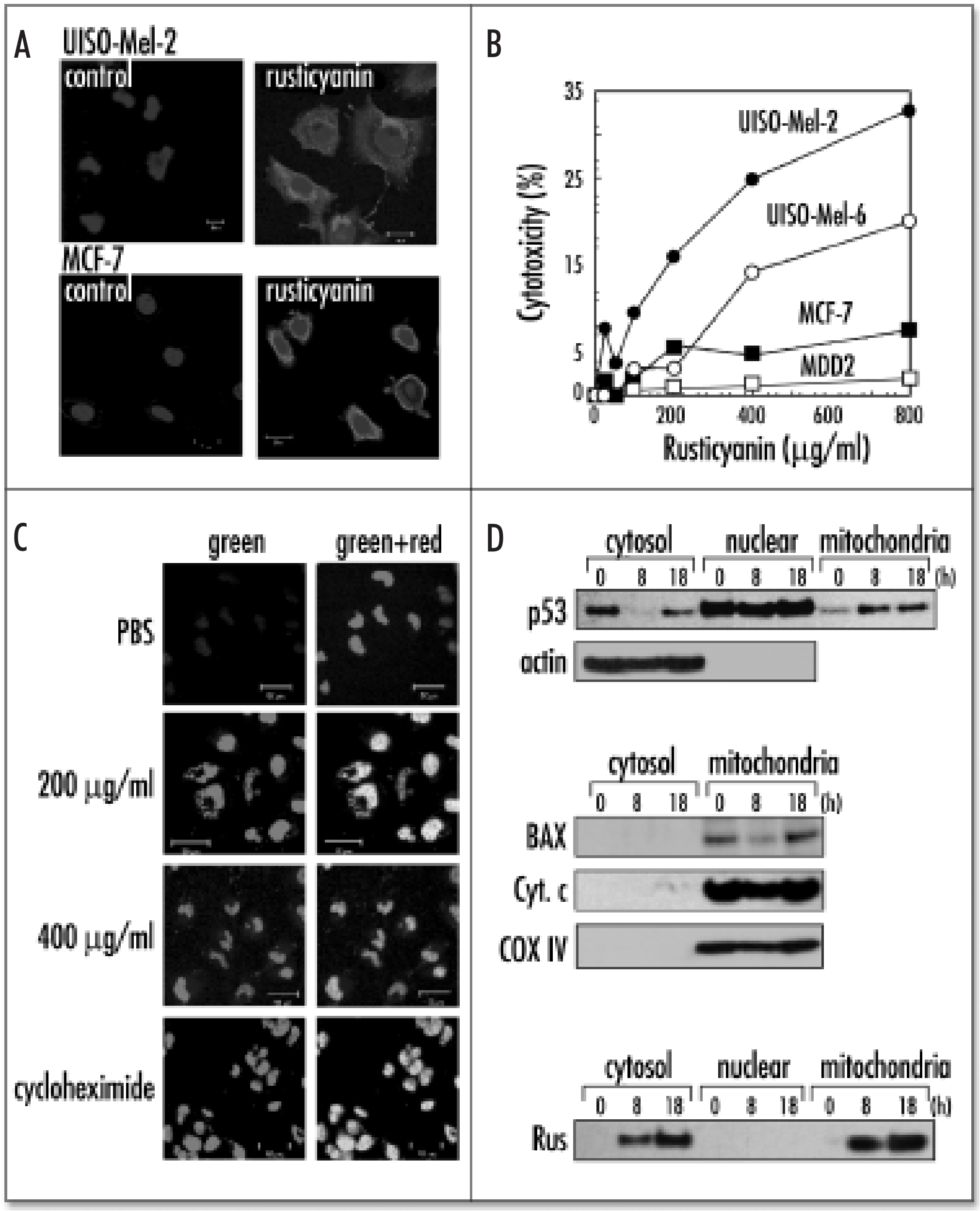

Figure 3. Effect of rusticyanin on cancer cells. (A) UISO-Mel-2 and MCF-7 cells were treated with 500 μg/ml of Alexa fluor 568-conjugated-rusticyanin for 1 h at 37°C. Cells were then observed by LSM510 confocal microscopy. (B) MTT [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)] assay was performed for the measurement of the cytotoxicity of rusticyanin as described before.[18,19] (C) UISO-Mel-2 cells were cultured with various concentrations of rusticyanin for 24 h. TUNEL assay was carried out using ApoAlert TUNEL assay kit (Clontech). Healthy cells are red; apoptotic cells are green because of frequent incorporation of FITC-conjugated deoxyribonucleotides.[18,19] Apoptotically dying cells are yellow when the red and green channels are merged. (D) Subcellular fractionations and mitochondrial localization of rusticyanin and p53. UISO-Mel-2 cells were treated with 500 μg/ml of rusticyanin for 0, 8 and 18 h. Cells were then washed with PBS twice and fractionated.[18,19] Same amount of proteins from each fractions were applied on SDS-PAGE and Western blotting was performed using antibodies against p53, Bax and mitochondrial cytochrome c. Anti-actin and anti-COX IV antibodies were used for checking cross-comtamination and as internal controls.

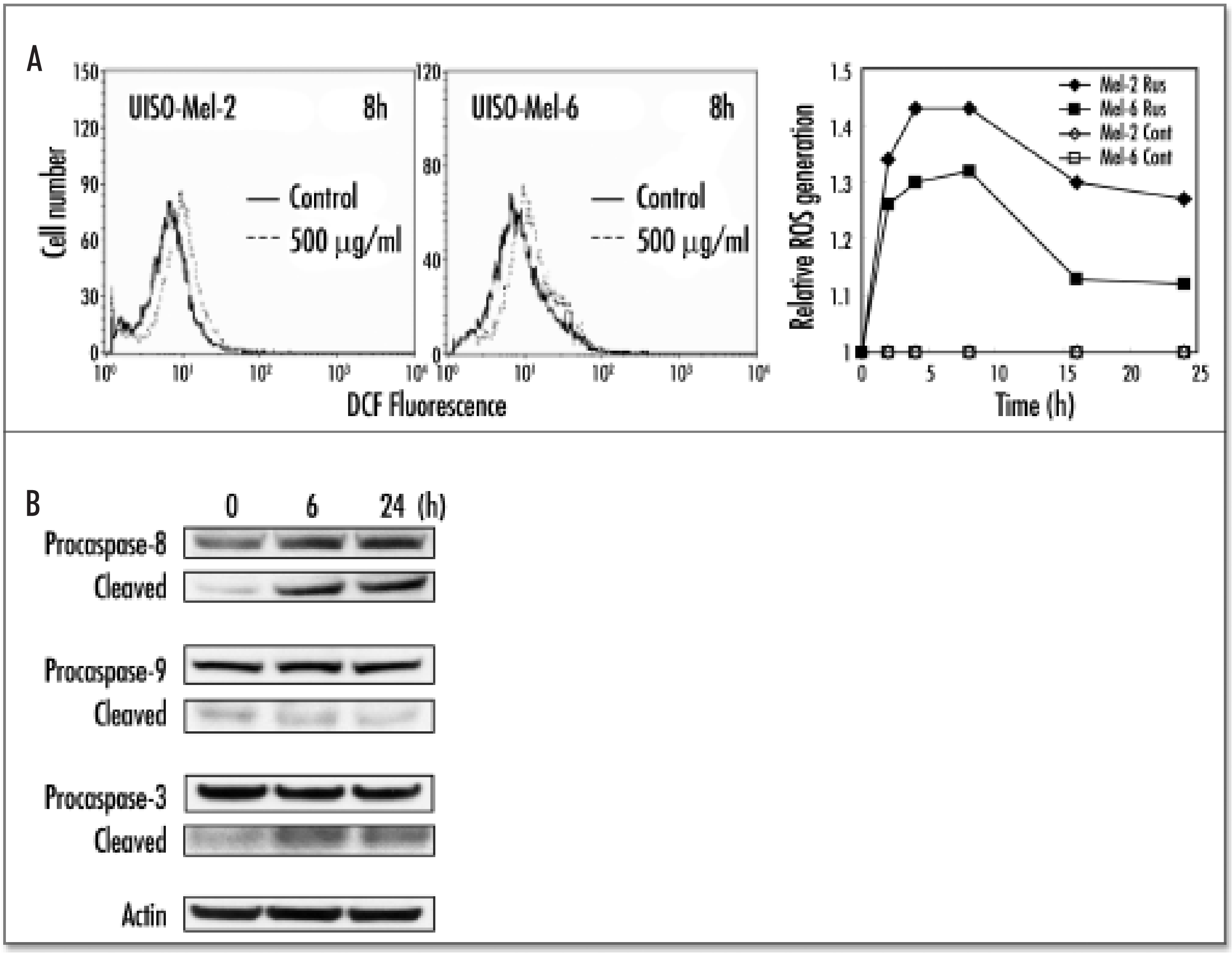

Figure 4. Rusticyanin generates significant levels of ROS and induces caspase-8 mediated apoptosis in UISO-Mel-2 cells. (A) UISO-Mel-2 and UISO-Mel-6 cells were treated without (Control) or with 500 μg/ml rusticyanin for various time periods. ROS levels in live UISO-Mel-2 and UISO-Mel-6 cells were analyzed by flow cytometry using DCHF-DA as a substrate as described previously.[18] (B) Immunoblot analysis of caspase-8, caspase-9 and caspase-3 levels in UISO-Mel-2 cells. UISO-Mel-2 cells were treated with 500 μg/ml rusticyanin for indicated time periods. At the end of rusticyanin treatment, cells were washed and whole cell extracts were prepared. Pro- and cleaved caspase-8, caspase-9 and caspase-3 were detected by Western blotting using specific monoclonal antibodies.

mitochondria, and unlike the azurin effect, there was very little mitochondrial cytochrome c released in the cytosol (Fig. 3D). Interestingly, unlike azurin that was also found in the nuclear fractions,[18,19] there was very little rusticyanin in the nuclear fraction, but similar to p53, significant amount of rusticyanin was localized both in the cytosol and in the mitochondria (Fig. 3D).

The simultaneous localization of p53 and rusticyanin in mitochondria implied a role of the mitochondria in the induction of rusticyanin-mediated apoptosis, though not through the release of mitochondrial cytochrome c to the cytosol. p53 is known to have a direct apoptogenic role at the mitochondria where targeting p53 to mitochondria in tumor cells is sufficient to induce apoptosis.[28] As a zinc protein, p53 is known to activate genes responsible for the production of reactive oxygen species (ROS)[29,30] or repress genes responsible for ROS scavenging.[31] It has also been reported that rapid apoptosis of neutrophils requires NADPH oxidase-derived ROS generated during phagocytosis.[32] This phagocytosis-induced ROS promotes cleavage/activation of procaspase-8 to caspase-8, a key player in the extrinsic (death receptor) mediated pathway of apoptosis. Caspase-8 but not caspase-9 was required for neutrophil apoptosis.[32] We, therefore, measured the extent of ROS generation when the p53-positive UISO-Mel-2 and p53-null UISO-Mel-6 cells were treated with 500 μg/ml of rusticyanin for various periods of time. The p53-positive UISO-Mel-2 cells showed a much higher level of ROS than the p53-null UISO-Mel-6 cells or untreated control cells (Fig. 4A), particularly between 2 and 8 h. Subsequently, the ROS levels decreased in both but the UISO-Mel-2 cells always maintained a higher level than UISO-Mel-6 cells (Fig. 4A), suggesting that p53 plays a stimulatory role in rusticyanin-mediated ROS generation. To determine if such ROS generation triggers an extrinsic pathway, rather than an intrinsic pathway that involves release of mitochondrial cytochrome c to the cytosol and activation of caspase-9, we measured the levels of the cleavage products of both procaspase-8 and procaspase-9 during treatment of UISO-Mel-2 cells with 500 μg/ml of rusticyanin for 0, 6 and 24 h. While there was very little cleavage of procaspase-9 to active caspase-9 during rusticyanin treatment, significant cleavage of procaspase-8 to active caspase-8 was seen under such conditions (Fig. 4B), suggesting that unlike azurin, rusticyanin triggers apoptosis in human melanoma UISO-Mel-2 cells by an extrinsic pathway that involves ROS generation and caspase-8 activation.

Acidithiobacillus ferrooxidans is a nonpathogenic autotrophic bacterium unable to grow in the physiological pH and hostile environment of human or animal bodies. The ability of rusticyanin to enter mammalian cells and trigger ROS generation and apoptosis therefore begs the question regarding any additional role of this redox protein outside its known role as an electron transfer agent. It is interesting to note that soil bacteria have to deal with eukaryotic cells as they are easy prey to environmental predators such as amoebae, nematodes or grazing protozoa. Indeed, isolation of biofilms from highly acidic acid mine drainage demonstrates the presence of eukaryotic cells comprising of 4% of the population,[33] although it is not clear if they are components of the biofilm or are predators seeking their prey or both. Either the entry of any rusticyanin released from the periplasmic space of *At ferrooxidans* to eukaryotic predators or the release of rusticyanin during engulfment would limit the number of bacteria the predator would like to consume since high concentrations of released rusticyanin would induce cell death. Thus rusticyanin and similar cupredoxins may act as defense mechanisms of soil bacteria against eukaryotic environmental predators.[34]

Both azurin produced by *P. aeruginosa* and rusticyanin produced by *At. ferrooxidans* are periplasmic cupredoxins with similar electrostatic properties,[16] although rusticyanin is characterized by the presence of an N-terminal extension, high standard reduction potential and extraordinary acid stability. Azurin has been shown to allow in vivo tumor regression in immunodeficient mice.[19,20] The ability of rusticyanin to trigger in vivo tumor regression is under investigation. The acid stability of rusticyanin may allow its use in the treatment of stomach cancer where stability to low pH is important. It is also interesting to note that azurin and rusticyanin not only perform very diverse functions, *viz*., denitrification and metal oxidation, as redox proteins but also exhibit very different mode of action with regard to their ability to kill cancer cells in vitro. It would be exciting to see if a mixture of these two bacterial cupredoxins will promote higher levels of cancer cell death because of their different modes of action.

References

1. Brierley CL. Bacterial leaching. CRC Crit Rev Microbiol 1978; 6:207-62.
2. Rawlings DE, Silver S. Mining with microbes. Bio/Technology 1995; 13:773-8.
3. Yamanaka T, Fukumori Y. Molecular aspects of the electron transfer system which participates in the oxidation of ferrous ion by *Thiobacillus ferrooxidans*. FEMS Microbiol Rev 1995; 17:401-13.
4. Barreto M, Quatrini R, Bueno S, Arriagada C, Valdes J, Silver S, et al. Aspects of the predicted physiology of *Acidithiobacillus ferrooxidans* deduced from an analysis of its partial genome sequence. Hydrometallurgy 2003; 71:97-105.
5. Bengrine A, Guiliani N, Appia-Ayme C, Jedlicki E, Holmes DS, Chippaux M, et al. Sequence and expression of the rusticyanin structural genes from *Thiobacillus ferrooxidans* ATCC 33020 strain. Biochim Biophys Acta 1998; 1443:99-112.
6. Appia-Ayme C, Guiliani N, Ratouchniak J, Bonnefoy V. Characterization of an operon encoding two c-type cytochromes, an aa_3-type cytochrome oxidase, and rusticyanin in *Thiobacillus ferrooxidans* ATCC 33020. Appl Environ Microbiol 1999; 65:4781-7.
7. Ingledew WJ, Cobley JG. A potentiometric and kinetic study on the respiratory chain of ferrous iron grown *Thiobacillus ferooxidans*. Biochim Biophys Acta 1982; 590:141-58.
8. Cobley JG, Haddock BA. The respiratory chain of *Thiobacillus ferrooxidans*: The reduction of cytochromes by Fe^{2+} and the preliminary characterization of rusticyanin, a novel 'blue' copper protein. FEMS Lett 1975; 60:29-33.
9. Dodd FE, Hasnain SS, Hunter WN, Abraham ZH, Debenham M, Kanzler H, et al. Evidence for two distinct azurins in *Alcaligenes xylosoxidans* (NCIMB 11015): Potential electron donors to nitrite reductase. Biochemistry 1995; 34:10180-6.
10. Blake II RC, Shute EA. Respiratory enzymes of *Thiobacillus ferrooxidans* J Biol Chem 1987; 262:14983-9.
11. Hall JF, Kanbi LD, Harvey I, Murphy LM, Hasnain SS. Modulating the redox potential and acid stability of rusticyanin by site-directed mutagenesis of Ser86. Biochemistry 1998; 37:11451-8.
12. Murphy LM, Dodd FE, Yousafzai FK, Eady RR, Hasnain SS. Electron donation between copper containing nitrite reductases and cupredoxins: The nature of protein-protein interaction in complex formation. J Mol Biol 2002; 315:859-71.
13. van de Kamp M, Silvestrini MC, Brunori M, Van Beeumen J, Hali FC, Canters GW. Involvement of the hydrophobic patch of azurin in the electron-transfer reactions with cytochrome c_{551} and nitrite reductase. Eur J Biochem 1990; 194:109-18.
14. Schlarb-Ridley BG, Bendall DS, Howe CJ. Role of electrostatics in the interaction between cytochrome f and plastocyanin of the cyanobacterian *Phormidium laminosum*. Biochemistry 2002; 41:3279-85.
15. Crowley PB, Otting G, Schlarb-Ridley BG, Canters GW, Ubbink M. Hydrophobic interactions in a cyanobacterial plastocyanin-cytochrome f complex. J Am Chem Soc 2001; 123:10444-53.
16. Rienzo FD, Gabdoulline RR, Menziani MC, Wade RC. Blue copper proteins: A comparative analysis of their molecular interaction properties. Protein Sci 2000; 9:1439-54.
17. Ralph P, Nakoinz I. Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. Nature 1975; 257:393-4.
18. Yamada T, Goto M, Punj V, Zaborina O, Kimbara K, Das Gupta TK, et al. The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53. Infect Immun 2002; 70:7054-62.
19. Yamada T, Goto M, Punj V, Zaborina O, Chen ML, Kimbara K, et al. Bacterial redox protein azurin, tumor suppressor protein p53, and regression of cancer. Proc Natl Acad Sci USA 2002; 99:14098-103.
20. Punj V, Bhattacharyya S, Saint-Dic D, Vasu C, Cunningham EA, Graves J, et al. Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. Oncogene 2004; 23:2367-78.
21. Yamada T, Hiraoka Y, Ikehata M, Kimbara K, Avner BS, Das Gupta TK, et al. Apoptosis or growth arrest: Modulation of tumor suppressor p53's specificity by bacterial redox protein azurin. Proc Natl Acad Sci USA 2004; 101:4770-5.
22. Hiraoka Y, Yamada T, Goto M, Das Gupta TK, Chakrabarty AM. Modulation of mammalian cell growth and death by prokaryotic and eukaryotic cytochrome c. Proc Natl Acad Sci USA 2004; 101:6427-32.
23. Cutruzzola F, Arese M, Ranghino G, van Pouderoyen G, Canters G, Brunori M. *Pseudomonas aeruginosa* cytochrome c_{551}: Probing the role of the hydrophobic patch in electron transfer. J Inorg Biochem 2002; 88:353-61.
24. Sherr CJ. The Pezcoller lecture: Cancer cell cycles revisited. Cancer Res 2000; 60:3689-95.
25. Dotto GP. $p21^{waf1/cip1}$: More than a brake to the cell cycle? Biochim Biophys Acta 2000; 1471:M43-56.
26. Ashkenazi A, Dixit VM. Death receptors: Signaling and modulation. Science 1998; 281:1305-8.
27. Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2001; 15:2922-33.
28. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, et al. p53 has a direct apoptogenic role at the mitochondria. Mol Cell 2003; 11:577-90.
29. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53 induced apoptosis. Nature 1997; 389:300-5.
30. Tan M, Li S, Swaroop M, Guan K, Oberley LW, Sun Y. Transcriptional activation of the human glutathione peroxidase promoter by p53. J Biol Chem 1999; 274:12061-6.
31. Drane P, Bravard A, Bouvard V, May E. Reciprocal down-regulation of *p53* and *SOD2* gene expression: Implication in p53 mediated apoptosis. Oncogene 2001; 20:430-9.
32. Zhang B, Hirahashi J, Cullere X, Mayadas TN. Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis. J Biol Chem 2003; 278:28443-54.
33. Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, et al. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 2004; 428:37-43.
34. Yamada T, Hiraoka Y, Das Gupta TK, Chakrabarty AM. Regulation of mammalian cell growth and death by bacterial redox proteins: Relevance to ecology and cancer therapy. Cell Cycle 2004; 3:752-5.