[Cell Cycle 3:6, 752-755; June 2004]; ©2004 Landes Bioscience

Perspectives

Regulation of Mammalian Cell Growth and Death by Bacterial Redox Proteins

Relevance to Ecology and Cancer Therapy

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Received 04/09/04; Accepted 04/14/04

Previously published online as a *Cell Cycle* E-publication: http://www.landesbioscience.com/journals/cc/abstract.php?id=922

KEY WORDS

cell cycle checkpoint, cupredoxins, cytochromes, apoptosis, growth arrest, cancer, environmental predators, tumor suppressors

ACKNOWLEDGEMENTS

This investigation is supported by a Public Health Service grant ES-04050-18 by the National Institute of Environmental Health Sciences.

We thank Dr. Beatrix Schlarb-Ridley for samples of purified plastocyanin and cytochrome f, Dr. Christopher Dennison for samples of pseudoazurin and Dr. Kazuhiro Sasaki for the cloned rusticyanin gene.

Because of space constraints, many relevant publications could not be cited. We sincerely apologize for this lapse on our part.

ABSTRACT

Recent evidence indicates that bacterial redox proteins such as cupredoxins and cytochromes, that are normally involved in electron transfer during respiration, can enter mammalian cells and induce either apoptosis or inhibition of cell cycle progression. Such proteins have also been shown to demonstrate a good deal of specificity for entry and induction of cytotoxic effects in cancer cells, allowing both in vitro cell death and in vivo inhibition of cancer progression. An alteration in the hydrophobicity of the bacterial redox proteins can lead to a switch from apoptosis to growth arrest and vice versa through modulation of the intracellular levels of tumor suppressors. The preferential entry and cytotoxicity of these redox proteins in cancer cells raises interesting questions about the presence of other bacterial proteins that may affect cell cycle at the G_2/M phase, thereby potentially arresting cancer growth. The intracellular localization of the bacterial redox proteins in nonpathogenic soil bacteria similarly raises questions about their possible role in allowing various nonpathogenic soil bacteria to defend themselves from environmental predators by inducing cytotoxicity when engulfed in large numbers. A new role of the redox proteins in soil bacteria in maintaining an ecological balance among the predators and preys is proposed.

CUPREDOXINS AND CYTOCHROMES: THEIR NEWLY-FOUND ROLES

Aerobic microorganisms produce a variety of enzymes that carry out oxidation-reduction (redox) reactions during aerobic metabolism by shuttling electrons from various substrates to molecular oxygen. Cupredoxins are a family of low molecular weight, water soluble, copper-containing proteins involved in electron transfer during various metabolic processes including denitrification, oxidation of metals such as Fe^{2+} or during photosynthesis. Their electron transfer partners are often cytochromes, which are iron (haem)-containing proteins that are part of the bacteria's electron transport pathway.[1,2] An important feature of the cupredoxins and cytochromes is their involvement in diverse reactions. For example, azurin and cytochrome c_{551} are involved in electron transfer during denitrification by *Pseudomonas aeruginosa*[3] while rusticyanin is a principal component in the iron respiratory electron transport chain of the acidophilic chemolithotropic bacterium *Thiobacillus ferrooxidans*[4] (now called *Acidithiobacillus ferrooxidans*). *Thiobacillus ferrooxidans* is well adapted to grow at pH values 1.6 to 3.5 and is able to derive all its energy through the biological oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) where rusticyanin plays a role.[5] Because of its unique ecological niche, *T. ferrooxidans* is nonpathogenic and lacks well-known toxins. Pseudoazurin is also a type 1 blue copper protein found in denitrifying bacteria such as *Achromobacter cycloclastes* where it acts as an electron donor to nitrite reductase.[6] Plastocyanin and cytochrome f are redox partners in the photosynthetic electron-transfer chain of cyanobacteria and plants. The cyanobacterial plastocyanin functions during photosynthesis to shuttle electrons between the cytochrome bf complex and photosystem I or cytochrome oxidase.[7] It is localized in the thylakoid lumen of chloroplasts and the periplasmic space of the cyanobacteria. Like *T. ferrooxidans*, cyanobacteria such as *Phormidium laminosum* are nonpathogenic. Thus various cupredoxins and cytochromes occur in diverse bacteria carrying out such diverse functions as denitrification, metal oxidation, photosynthesis, etc.[1,2]

While the ability of the cupredoxins and cytochromes to act as redox partners and transfer electrons has been well known, very little was known until recently about their role in entering mammalian cells and induce apoptosis or cause growth arrest. A few redox proteins, primary among them are mitochondrial cytochrome c and the apoptosis inducing factor (AIF), are known to induce apoptosis when released from the intermembrane space

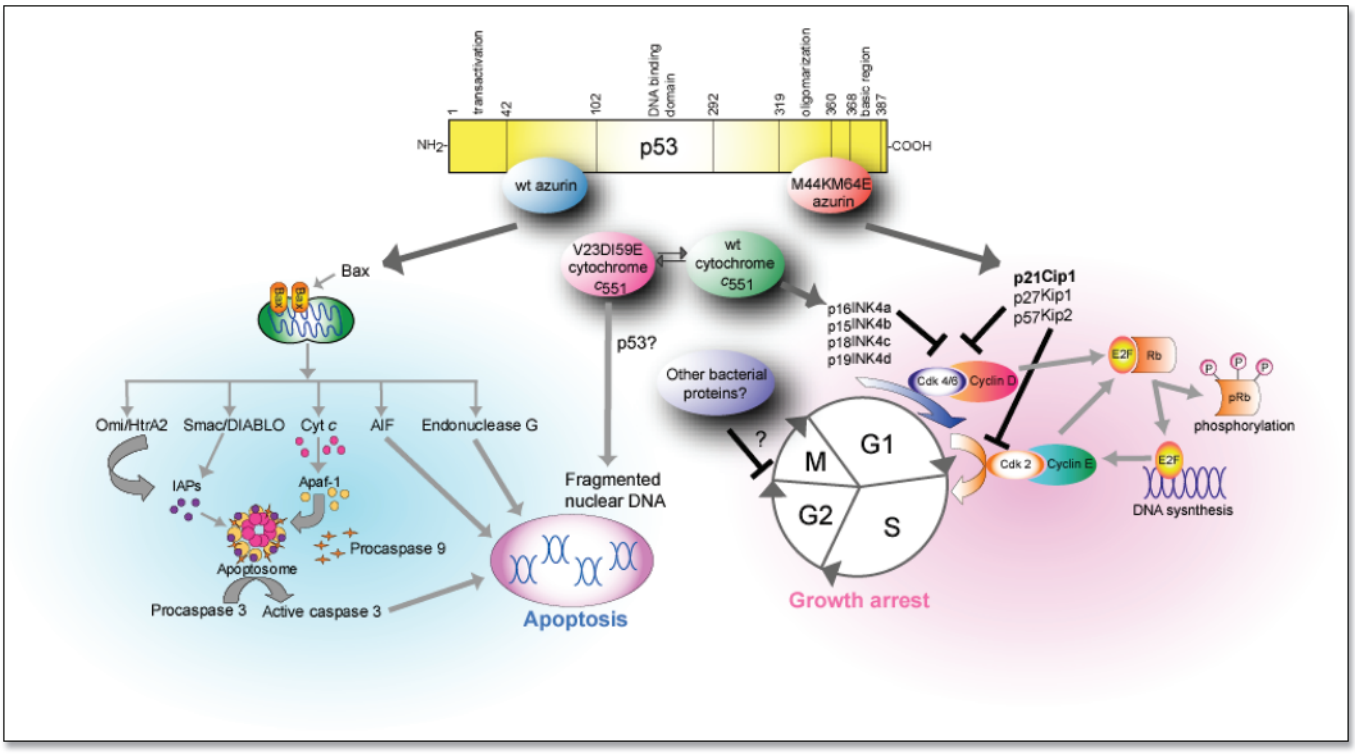

Figure 1. Modulation of apoptosis or growth arrest by wt and mutant forms of azurin and cytochrome c_{551}. In either case, a change in the hydrophobicity of the protein leads to a switch to an altered physical association with a tumor suppressor resulting in either apoptosis or an inhibition of cell cycle progression at the G_1 to S phase.[15,18] Whether other bacterial proteins may allow growth arrest through inhibition of cell cycle at the G_2/M phase is not known at present.

of the mitochondria to the cytosol in the presence of death signals.[8,9] Their ability to enter mammalian cells and trigger cell death has, however, been less widely known. The ability of purified azurin and cytochrome c_{551} from *P. aeruginosa* to enter J774 cells, which are derived from murine reticulum cell sarcoma,[10] was first reported by Zaborina et al.[11] Yamada et al.[12,13] subsequently demonstrated that azurin could not only enter J774 cells and induce apoptosis,[12] but it could also enter cancer cells such as human melanoma UISO-Mel-2 cells and cause cell death.[13] In both instances, azurin appeared to form a complex with the tumor suppressor p53, thereby stabilizing it and raising its intracellular level. High levels of p53 then triggered apoptosis in such cells through enhanced Bax formation and the release of mitochondrial cytochrome c to the cytosol.[12,13] Most interestingly, azurin demonstrated high cytotoxic effect in vivo in UISO-Mel-2-bearing immunodeficient mice, allowing inhibition of tumor growth without any major effects on normal cells or demonstrating visible toxicity.[13] A similar effect has recently been shown in human breast cancer MCF-7 cells where azurin induced significant cytotoxicity in vitro in MCF-7 cells, causing inhibition of in vivo tumor growth in nude mice, but not showing any major effects on normal tissues.[14]

In contrast to the cupredoxin azurin, cytochrome c_{551} showed a more subtle effect. It had much reduced cytotoxicity than azurin but appeared to enhance azurin-mediated cytotoxicity when used in combination with azurin.[11] More recently, Hiraoka et al.[15] demonstrated that while cytochrome c_{551} has very little cytotoxicity towards J774 cells, it strongly inhibits cell cycle progression at the G_1 to S phase. On entry into J774 cells, cytochrome c_{551} promotes accumulation of the tumor suppressor protein $p16^{Ink4a}$, an inhibitor of cell cycle progression at the G_1 to S phase because of its ability to sequester CDK4/CDK6 into binary CDK-Ink4 complexes.[16] Indeed the intracellular levels of cyclin D and CDKs were greatly reduced when J774 cells were treated with cytochrome c_{551} for 4 to 24 h.[15] Most interestingly, however, Hiraoka et al.[15] also demonstrated that not only *P. aeruginosa* cytochrome c_{551} but mammalian cytochromes such as horse or bovine cytochrome c could enter J774 cells, when added exogenously, and induced apoptosis in a p53-independent manner. Yeast cytochrome c, which is incapable of inducing apoptosis because it lacks Apaf-1 binding sites, was also incapable of inducing apoptosis in J774 cells.[15]

CUPREDOXIN/CYTOCHROME C SURFACE HYDROPHOBICITY AND A SWITCH IN TUMOR SUPPRESSOR SPECIFICITY

The cupredoxins and cytochromes such as azurin and cytochrome c_{551} have hydrophobic amino acids on their surfaces that are important for their interactions as electron transfer partners.[3,17] Having found a physical association between azurin and p53,[12-14] it was of interest to us to examine if the hydrophobicity of the azurin or cytochrome c_{551} surface plays a role in their protein: protein interaction with p53. In a recent paper, Yamada et al.[18] have demonstrated that wildtype (wt) azurin, that appears to form a complex primarily in the N-terminal to the middle core region of p53,[14,19] induces Bax hyperproduction, leading to mitochondrial cytochrome c release in the cytosol and triggering an apoptotic response (Fig. 1). In contrast, the M44KM64E mutant azurin with reduced surface hydrophobicity, appears to form a complex at a site in the C-terminal of p53 that interferes in p53 oligomerization and enhances p53-responsive p21 gene transcription, leading to inhibition of cyclin/CDK formation and consequently cell cycle progression (Fig. 1).

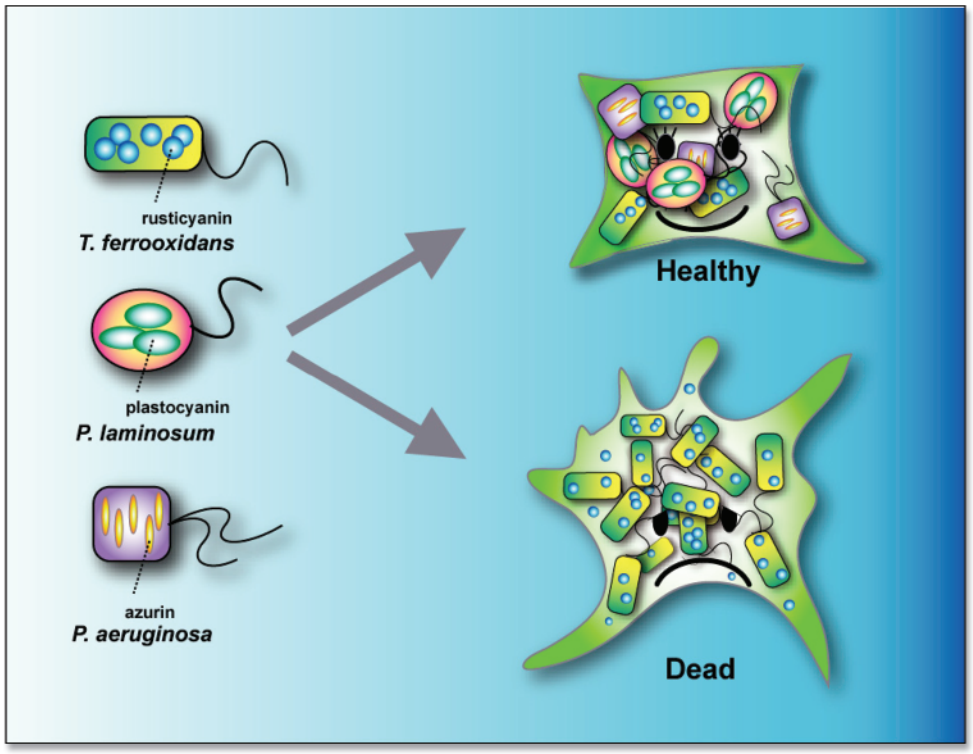

Figure 2. A model depicting how soil bacteria, both pathogenic and nonpathogenic, may use cupredoxins for defense against eukaryotic predators in an open environment. Cupredoxins are mainly periplasmic proteins, although for artistic simplicity, they are shown as cytoplasmic. Apart from the fact that the cupredoxins, which can enter eukaryotic cells, may be released from the bacteria in response to the presence of the eukaryotic predators for their defense, even the engulfed bacteria may release the cupredoxins inside the eukaryotic cells. This will enable nonpathogenic soil bacteria without known toxins to intoxicate the predators, when consumed in large numbers, inducing severe toxicity or fatality (lower right hand panel). However, since different cupredoxins may act differently, consumption of low numbers of a variety of bacteria will prevent acute toxicity because of the low concentrations of individual cupredoxins (upper right hand panel). Curpedoxins may be substituted by cytochromes in some cases.

Hiraoka et al.[15] replaced two hydrophobic amino acids, a valine in position 23 and an isoleucine in position 59 in cytochrome c_{551} with two charged amino acids aspartic and glutamic acids. These two amino acids were earlier shown to be involved in protein: protein interaction between azurin and cytochrome c_{551} during electron transfer.[17] Unlike wt cytochrome c_{551} which enhanced intracellular tumor suppressor $p16^{Ink4a}$ levels but showed no detectable interaction with p53, the V23DI59E mutant cytochrome c_{551} was shown to physically associate with p53 and enhanced apoptosis in both J774 and human breast cancer MCF-7 cells (Fig. 1) in a p53-dependent manner.[15] Thus both in case of azurin and cytochrome c_{551}, an alteration in key hydrophobic residues led to altered interaction not only with their electron transfer partners but also with the mammalian tumor suppressors. In case of azurin, the replacement of two hydrophobic methionine residues with two polar amino acids led to a change in the transcriptional specificity of p53. It would be of great interest to replace all the hydrophobic amino acid residues of azurin either with polar or with more hydrophobic amino acids (for example M44VM64V) and examine their nature of p53 interactions with biophysical tools such as BIAcore or NMR as well as their ability to activate various p53-responsive genes such as *mdd2, bax, p21*, etc. Likewise, the hydrophobic residues in cytochrome c_{551} may be replaced by less or more hydrophobic amino acid residues, introduced in mammalian cells and assessed for their biological effect in stabilizing or activating various tumor suppressors.

HOW DO CUPREDOXINS/ CYTOCHROMES ENTER MAMMALIAN CELLS?

We previously demonstrated that azurin, cytochrome c_{551} and mammalian cytochromes can enter J774 or cancer cells.[12,13,15] Other cupredoxins such as plastocyanin, rusticyanin and pseudoazurin are also known to enter mammalian cells (Yamada T, Punj V, Bratescu L, Das Gupta TK, Chakrabarty AM, manuscript in preparation). We have some preliminary evidence that there is a short segment of azurin, termed a protein transduction domain, that can act as a vehicle to transport inside mammalian cells other cargo proteins that cannot normally enter mammalian cells. Azurin entry also shows some specificity for cancer cells which potentially can make azurin an interesting vehicle for targeting cancer cells with various toxins (Yamada T, et al., manuscript in preparation). Given the fact that many cupredoxins and prokaryotic/eukaryotic cytochromes have now been shown to enter mammalian cells, one can ask whether the entry mechanism is the same or different, if there would be host cell specificity for entry of each redox proteins or whether a comparison of the protein transduction domains can provide important insights regarding the structural features of such domains.

CUPREDOXINS AND CYTOCHROMES IN CANCER THERAPY

One of the most interesting developments in recent times is a renewed interest in the use of microorganisms or their products in cancer therapy.[19,20] The fact that azurin allows in vivo regression of both melanoma and breast cancer in nude mice without producing toxicity or significant death of normal cells[13,14] makes azurin, and hopefully other cupredoxins, attractive model anticancer compounds. Azurin primarily acts by inducing apoptosis in cancer cells and has no effect on cell cycle. An important property of a potential anticancer agent is if the agent can induce both growth arrest and cell death of cancer cells. While the M44KM64E azurin inhibits cell cycle progression in J774 cells, it has very little effect on cancer cells such as MCF-7 because cancer cells often harbor mutations in genes that encode tumor suppressor or other regulators of cell cycle check point. Normal cellular growth regulations are overridden in cancer cells because of such mutations, allowing the cells to grow indefinitely.[16] Thus an ability of M44KM64E mutant azurin[18] or wt cytochrome c_{551}[15] to allow cell cycle inhibition at the G_1 to S phase through cyclin/CDK depletion does not work for growth inhibition of cancer cells because of unregulated E2F release.[18]

If a pathogenic bacterium such as *P. aeruginosa* considers cancer cells as adversaries, perhaps because of their altered overwhelming growth rate, and secretes redox proteins for induction of apoptosis in

such cells, could they also secrete other proteins that may operate at G_2/M phase preventing cell division even in cells that allow unregulated DNA replication (Fig. 1)? Preliminary experiments involving fractionation of the cell extracts and filtered cell free growth media of *P. aeruginosa* demonstrated the presence of a fraction which on incubation with J774 cells appeared to inhibit cell cycle at the G_2/M phase (Fig. 1). A combination of proteins or small peptides derived from them and capable of inducing both apoptosis and growth arrest may have formidable anticancer activity. Careful and purposeful attempts in characterizing new and interesting compounds or molecules with anticancer activity, including a range of cupredoxins and cytochromes, may give rise to a potential microbial anticancer industry similar to the present day antibiotic industry.

CUPREDOXINS AND CYTOCHROMES IN MICROBIAL ECOLOGY?

Aside electron transfer, the roles of cupredoxins and cytochromes in microbial life cycles are little understood. While azurin and cytochrome c_{551} are known to be periplasmic and have been detected in the growth media of *P. aeruginosa*, an opportunistic pathogen, some of them are known to be membrane-associated and may very well be intracellular. Typical soil bacteria such as *T. ferrooxidans* have no known virulence factors that might help kill eukaryotic predators, perhaps other than a highly acidic environment or the presence of cell wall LPS (endotoxin). So how do such nonpathogenic bacteria defend themselves from predators in the soil? Given its cytotoxicity against mammalian cells, is there some protective function of rusticyanin against eukaryotic predators? It is interesting to note that biofilms from highly acidic acid mine drainage have been reported to harbor 4% eukaryotes,[21] although it is not clear if they are members of the community or predators. We have some preliminary evidence that different cupredoxins and cytochromes have different modes of cytotoxic action. Thus at suboptimal intracellular concentrations, a combination of them may prove to be harmless even though they provide boundless food supply (Fig. 2). On the other hand, high concentrations of a single cupredoxin or cytochrome might prove to be toxic or lethal (Fig. 2). Even if other cupredoxins such as plastocyanin and rusticyanin, similar to azurin, demonstrate preferential entry to cancer cells, they will still exert their cytotoxicity once engulfed by the predators. Thus an intriguing possibility is that the redox proteins act as poison pills to prevent predators such as amoebae or grazing protozoa to consume large numbers of individual bacteria. The analogy is similar to some toxic fruits and berries that when consumed in large amounts will produce a toxic reaction and consumption of such large amounts is usually avoided by birds and small animals that feed on them. While bacteria in a biofilm or an open environment are 'sitting ducks' for predators, the predators know that they are better off consuming mixtures of bacteria, each at a dose that's nontoxic. Consumption of significant amounts of single species of bacteria with intracellular cytochromes or cupredoxins will produce a toxic symptom while the same amount of cell proteins from different bacteria will not because of different modes of cytotoxic actions by cupredoxins and cytochromes (Fig. 2). Having a balanced diet is thus as important for environmental predators as it is for man. Because the bacteria have fast growth rates, any surviving bacteria can replenish the lost population quickly and a balanced, dynamic equilibrium can be maintained in nature.

The above concept, if true, is not only experimentally verifiable but may allow a measure of protection to genetically-engineered bacteria designed for environmental release during bioremediation. For example, such bacteria may additionally be equipped with genes encoding specific cupredoxins or cytochromes under a strong, constitutive promoter. The environmental survival of such bacteria can then be monitored in the open environment or in an experimental environment artificially seeded with eukaryotic predators. If such bacteria are found to be less vulnerable than their parents to environmental predators, then the concept of poison pill will be on a firmer ground. It will, of course, be important to monitor the fate of the predators as well, since they may not be able to differentiate such bacteria from the indigenous ones and get poisoned, disturbing the ecological balance in nature. The ease of hyperexpression of cupredoxins and cytochromes in bacteria will allow an evaluation of their roles in maintaining the normal ecosystem structure and function.

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