CELL BIOLOGY

Superoxides Relay Ras Protein's Oncogenic Message

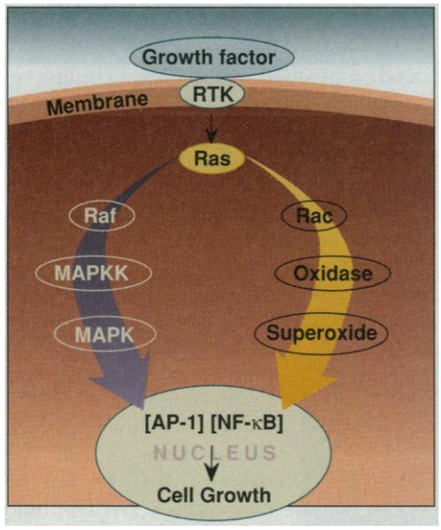
For years, health-conscious consumers have been eating green vegetables as much for their antioxidants as for their fiber and vitamin content. Those extra helpings of broccoli are supposed to reduce the risk of cancer and other diseases by helping rid the body of oxygen free radicals, highly reactive molecules thought to contribute to cancer development by damaging the DNA. Now, there's another reason to think that suppressing free radical production might help protect against cancer.

On page 1649, the team of Pascal Goldschmidt-Clermont, a cardiologist now at Ohio State University in Columbus, reports new results suggesting that superoxide—a free radical consisting of an oxygen molecule with an extra electron—helps a protein called Ras transmit growth-stimulating messages to the cell interior. The work adds to the growing evidence that reactive oxygen species, traditionally viewed only as toxins, can also play a normal role in the cell's signaling pathways. That makes it a "really timely" result, says Sue Goo Rhee, a biochemist at the National Heart, Lung, and Blood Institute (NHLBI) in Bethesda, Maryland.

It is also the first evidence that puts superoxide firmly into the Ras pathway, which is one of the cell's most important growth-stimulating pathways. Indeed, predicts Roger Davis, a cell biologist at the University of Massachusetts Medical Center in Worcester, the linking of oxygen free radicals to Ras-activated growth "will cause a lot of people to rethink the mechanisms of growth control." What's more, the Ras pathway can contribute to cancer development if it becomes overactive, which may happen as a result of mutations in *ras*, the gene that encodes Ras, or changes in other oncogenic proteins that also send their signals through the Ras pathway. Consequently, says cell biologist Marc Symons at Onyx Pharmaceuticals in Richmond, California, "the elements in [this] pathway are all good [anticancer] drug targets."

Other work has suggested that cells make reactive oxygen species for signaling, but pinning down exactly what the superoxide radical does has been difficult, partly because cells normally contain only very low concentrations of the molecules. "They are hard to get a handle on," says Bernard Babior, a biochemist at the Scripps Research Institute in La Jolla, California. Goldschmidt-Clermont and his colleagues succeeded by using two sophisticated methods to assess superoxide levels in fibroblast cells into which they had introduced a known oncogenic form of the *ras* gene. One was electron paramagnetic resonance spectroscopy, which enabled the researchers to measure a derivative of the chemical dimethyl pyrroline-*N*-oxide that forms in the presence of superoxide. The other was lucigenin-enhanced chemiluminescence, which assesses the glow given off by the dye lucigenin when it is oxidized as an indicator of superoxide levels.

These measurements showed that cells



Ras gets radical. Ras sends its growth-stimulating message to the nucleus through superoxide, as well as through the MAP kinases (MAPK and MAPKK).

with the oncogenic *ras* gene, which is perpetually active, make more superoxide than cells with only normal *ras*. "The more Ras molecules you express, the more superoxide you produce," says Goldschmidt-Clermont, who did this work with Kaikobad Irani, a cardiologist at Johns Hopkins University School of Medicine in Baltimore. And when the researchers chemically blocked the protein, superoxide concentrations fell.

Goldschmidt-Clermont and his colleagues also found evidence that the superoxide production has the functional consequences expected if it carries the Ras growth signal. When they treated the cells with an antioxidant that reduces the amount of superoxide generated in response to Ras, this reduction decreased by a proportional amount the DNA synthesis that has to occur before a cell divides. "The data are compelling that [stimulating DNA synthesis] is a role for reactive oxygen species," says J. Sylvio Gutkind, a biochemist with the National Institute of Dental Research in Bethesda, Maryland.

The researchers then tried to trace how Ras stimulates superoxide production. Although the cell's mitochondria produce reactive oxygen species as a byproduct of their energy-producing reactions, the team found that blocking the mitochondrial oxidase that makes these molecules had no effect on the cell's superoxide levels. It seems instead that the superoxide is produced by the same enzyme, NADPH oxidase, that phagocytic immune cells use to generate reactive oxygen species to attack invading pathogens. Evidence for this came from experiments in which a molecule that works with this oxidase to generate superoxide was inhibited. This reduced the amount of superoxide produced in response to Ras.

The next step was to try to figure out how Ras might trigger the enzyme to make superoxide. The team knew that in order to stimulate cell growth, Ras has to interact with at least two related proteins, one called Raf and the other, Rac. "Ras is the protein in this family that seems to be on top of this hierarchy," Goldschmidt-Clermont explains. But their experiments indicated that the Ras interaction with Raf apparently has little to do with superoxide production.

It's the Rac branch that seems to spur superoxide production, Goldschmidt-Clermont's team shows. When the researchers introduced a mutant *rac* gene that causes normal cells to make excess Rac protein, the cells produced levels of superoxide approaching those in the cells with the oncogenic Ras. And when the researchers caused cells with oncogenic Ras to make a defective Rac, the levels of superoxide dropped. "We have discovered what seems to be a new signaling pathway downstream of Rac," Goldschmidt-Clermont concludes.

What apparently happens is that Rac, after it is activated by Ras, forms a molecular complex that includes the membrane oxidase. In this complex, the oxidase becomes activated and begins to add electrons to oxygen molecules to produce the superoxide.

Just how the superoxide carries the Ras signal, ultimately leading to DNA synthesis, is still uncertain. One standard mechanism by which the cell turns key proteins on and off is by adding and removing phosphate groups, and it "might use [oxidation-reduction reactions] in a parallel way," says NHLBI cardiologist Toren Finkel, another of the paper's co-authors. The reactive oxygen species might, for example, oxidize cysteine, removing hydrogen from its sulfur group and inducing the formation of cysteine-cysteine disulfide bonds that alter a protein's structure and turn it either on or off.

That idea fits with other evidence that cells may often rely on active oxygen species to regulate their activities. "They're popping up in a lot of different pathways," notes Larry

Feig, a biochemist at Tufts Medical School in Boston. Already, several research teams have demonstrated that oxidizing agents affect key transcription factors, some of which help regulate cell growth. They activate one called nuclear factor κB, which turns on the genes for a variety of molecules leading to inflammation. And they shut down another factor, AP-1, which controls some genes involved in growth and development; antioxidants restart it. In late 1995, Finkel and his colleagues also showed that hydrogen peroxide is involved in the signaling pathway of platelet-derived growth factor, which causes the smooth muscle cells of blood vessels to proliferate (*Science*, 13 October 1995, p. 296).

To Goldschmidt-Clermont, the story of reactive oxygen species is beginning to look like that of nitric oxide, once considered an environmental pollutant and now recognized as key to cell communication in the brain, arteries, immune system, liver, pancreas, lungs, and uterus. "In parallel to the nitric oxide system, low concentrations can be signals, but if you have lots of it, it can damage cells," he explains.

Still, Rhee and Symons point out that there are some uncertainties in the superoxide work. They note that the researchers don't yet know if superoxide works by itself or through some other reactive oxygen species, such as hydrogen peroxide. Nor do they know whether superoxide participates in Ras signaling in cells other than fibroblasts. "It will be interesting to see whether these results can be extrapolated to other cell types," says Symons. Finkel is confident they can, and thinks oxygen radicals are likely to turn up in other signaling pathways as well. If so, maybe even former President George Bush will think twice about refusing to eat broccoli.

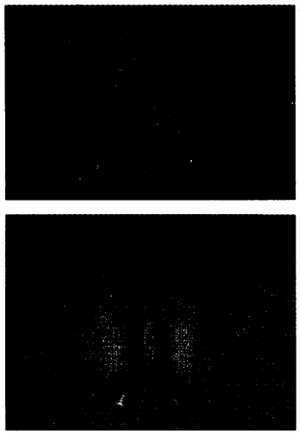
–Elizabeth Pennisi

HUMAN GENETICS

HOX Gene Links Limb, Genital Defects

Ever since they were shown to be conserved between vertebrates and arthropods in 1984, the large family of so-called HOX genes has provided a master key to unlocking the intricacies of development. Originally found in the fruit fly *Drosophila melanogaster*, where they help lay down the insect's head-to-tail pattern, HOX genes were soon shown to be crucial for the development of animal species ranging from lowly nematodes to mammals.

But the elucidation of the role of HOX genes has lagged in one important organism: humans. Although developmental biologists think these genes are important in humans—after all, each of us carries 39 of them arranged in four roughly parallel sets—the only way to know for sure is to find HOX mutations in people and see what kind of abnormalities they cause. But such human mutations have proved scarce, possibly because the genes are so important that many mutations prove fatal. The first such mutation, which causes abnormal hands and feet, was discovered only last year. Now, University of Michigan pediatric geneticist Jeffrey Innis and graduate student Douglas Mortlock have uncovered a second window into human HOX gene function, a mutation that causes genital, as well as limb, abnormalities.



JEFFREY INNIS

Foxy feet and fingers. HOX gene mutation causes shorter thumbs shifted down the hand, and similar abnormalities in big toes.

Developmental biologists are pleased by the finding, described in last month's issue of *Nature Genetics*, because it ties in with previous work showing that inactivation of the comparable gene in mice causes similar defects. "This report is tremendous because it demonstrates that what we've seen in mice is valid for humans," says HOX gene expert Denis Duboule of the University of Geneva. "This has profound evolutionary implications." The link between limbs and genitals may help explain why even apparently nonvital HOX genes—and the parts of the body plan they govern—are so strongly conserved in evolution, he says.

The work began when Mortlock and Innis identified and published a mutation causing limb and genital anomalies in the mouse *hoxa13* gene last year. Then, a colleague alerted them to the presence of similar malformations in a Michigan family. The family's hereditary abnormalities included thumbs and big toes that are both shorter than normal and shifted slightly toward the elbow and knee. The limb anomalies do not cause any apparent hardship to family members, who refer to their differences as "foxy feet" and "butterfly fingers." But three women in the family also have uterine abnormalities that led one to be infertile and the others to have difficulty conceiving. Mortlock and Innis sampled the family's DNA and found that they indeed carried a mutation in the human form of the *HOXA13* gene.

On closer study of the mutated gene, Mortlock and Innis found that one of its codons for the amino acid tryptophan was replaced by a stop codon. So the HOXA13 protein is incomplete, missing 20 amino acids. This may eliminate or reduce the protein's ability to bind to DNA, presumably altering the transcription of target genes and so somehow altering morphology, says Innis.

A link between a HOX gene mutation and both limb and genital abnormalities had already shown up in mice several years ago. Duboule's team had inactivated the closely related gene, *hoxd13*, in mice and found that the animals had smaller digits and many males had malformed penises. Then last year, a team led by cell biologist Bjorn Olsen of Harvard Medical School found that mutations in the *HOXD13* gene in a human family led to limb defects—but the researchers did not report whether family members had genital defects (*Science*, 26 April 1996, p. 548).

Now that the limb-genital link has shown up in a different human HOX gene, Duboule says, it may explain why even nonvital HOX genes appear to be conserved across evolutionary history. Mutations in genes needed for normal genital development wouldn't be tolerated because they lessen the likelihood that mutated individuals could reproduce. Duboule speculates that this link may even help explain one of life's great mysteries: why so many vertebrates have five digits on their hands and feet—a number that has been favored for over 300 million years of evolution, notes paleontologist Michael Coates of University College, London.

The HOX genes play a pivotal if poorly understood role in determining the number of digits, and if they are needed in reproductive function, then conservation of the digit number may have been secondary to that, an idea that gets cautious support from Innis and Coates. Put simply, says Coates, "stability in the distal part of the limb is favored because otherwise you mess up the genitalia. That's a very nice argument," which "appears to be strengthened by the new findings."

There's little proof yet, however, that HOX genes owe their stability to the genital connection. Answers may have to wait for more details on genes activated by the HOX genes and how they alter the body plan. Despite the new human family and the ongoing work in mice, "we still don't know what these genes are actually doing," says Duboule. "It's remarkably mysterious."

–Steven Dickman

Steven Dickman is a science writer in Cambridge, Massachusetts.