Forging a Path to Cell Death

Researchers are uncovering the internal signaling pathways that tell cells it's time to die, an event that is often necessary to maintain the well-being of the entire organism

In human society, suicide often seems an irrational and impulsive act. Not so in the society of cells in an organism. Like obedient soldiers making a personal sacrifice for the common good, excess cells, or those that pose a threat to the well-being of the organism, often commit suicide on command, via an orderly process called programmed cell death, or apoptosis.

How that command, received at the cell surface by specialized protein receptors, turns on the death machinery inside the cell was a complete mystery until just a little over a year ago. But in a recent burst of activity, a handful of labs around the world have uncovered a complex maze of internal signaling paths triggered by the receptors. While the picture is not yet complete, it seems that the particular routes to death that exist in different cell types give each type its own unique pattern of susceptibility to some death signals and immunity to others. "It is really an exciting story," says Vishva Dixit, whose laboratory at the University of Michigan Medical School in Ann Arbor has contributed to the findings. "I have never seen a field move this fast."

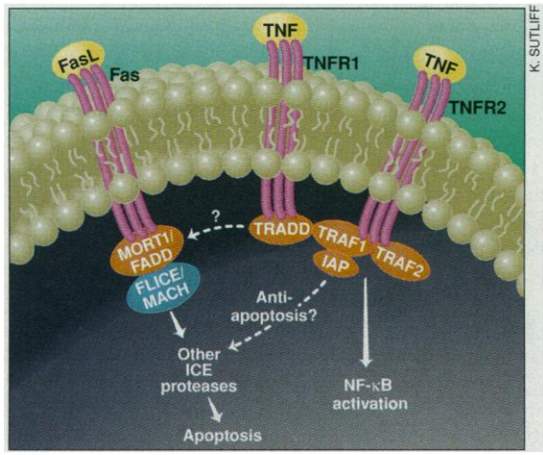
Researchers expect that the road map of signaling pathways they are now developing will improve their understanding of processes that involve cell death, such as embryonic development, or the normal operation of the immune system. The new insight into apoptosis may also lead to therapies for diseases in which cell death plays a role, such as cancers that may result from a failure of apoptosis, or neurodegenerative diseases, where abnormal apoptosis may lead to nerve cell loss.

"A couple of years ago people had real doubts about apoptosis being involved [in disease]," says immunologist and apoptosis researcher Craig Thompson, of the University of Chicago. "Now almost everyone is sold on the idea. The question is how is it regulated, and what is screwed up." The signaling pathways are likely to hold the answer to that question, and so may provide important targets for therapeutic drugs.

Researchers got their original glimmer of receptor-triggered cell death with the revelation in the mid-1970s that a mysterious factor made by cells of the immune system, dubbed tumor necrosis factor (TNF), could cause cells to die. Work from many labs led to the eventual identification of TNF and finally, in 1990, the cloning of the genes for two different TNF receptors, known as TNFR1 and TNFR2.

Meanwhile, another death receptor was emerging. In the late 1980s, two teams, one led by Peter Krammer of the German Cancer Research Center in Heidelberg and the other by Shin Yonehara of the Tokyo Metropolitan Institute of Medical Science, identified a protein, called Fas by the Japanese and APO-1 by the Germans, that is present in many cell types. Activation of Fas/APO-1 by a protein called the Fas ligand (FasL), found on some of the immune system's killer cells, causes cell death.

This interaction, and that of TNF with its receptors, helps the immune system eliminate potentially dangerous cells, such as those infected by viruses. Fas turned out to be related to the TNF receptors, and other receptors of the same family continue to be found, although not all cause death.



Meandering paths. Fas and the TNF receptors activate different proteins which interact within the cell to trigger cell death or NF-κB activation.

Shortly after the discovery of Fas and the TNF receptors, researchers placed a protein in the cell's interior squarely on the pathway to death. It is a protein-cutting enzyme, or protease, called ICE (for interleukin-1 β converting enzyme) that was originally identified as a key player in inflammation in mammals. In 1992, when Robert Horvitz's team at the Massachusetts Institute of Technology cloned the gene for a protein called CED-3, which causes cell death in the worm *Caenorhabditis elegans*, they found that CED-3 and ICE are structurally related, an indication that they might have similar functions. Soon after, Junying Yuan's team at Harvard Medical School proved that by showing that ICE activation causes cell death in cultured rat and chicken cells (*Science*, 11 February 1994, p. 754). ICE turned out to be one of a large family of proteases that help carry out apoptosis, presumably by activating proteins that kill the cell.

The trail to ICE

When ICE was first fingered as a killer molecule, researchers had no clue about how Fas and other cell surface receptors transmit their death orders to ICE family members inside the cell. To attack that enigma head-on, researchers began to blaze the trail inward from the cell surface, by searching for proteins in the cell that interact directly with Fas or the TNF receptors. Last year, four teams scored successes. David Wallach's group at Israel's Weizmann Institute found a protein they called MORT1 that links up to Fas; Dixit's team identified the same protein and called it FADD. At the same time, Brian Seed's team at Harvard Medical School reported a different Fas-binding protein, which they dubbed RIP; and David Goeddel's group at Tularik Inc., in South San Francisco, discovered a protein that binds to TNFR1, and named it TRADD.

The names TRADD and FADD stand for TNF receptor– or Fas associated–death domain protein, a moniker reflecting the fact that these proteins share a sequence of 80 amino acids that Shigekazu Nagata's group at the Osaka Biosciences Institute found in Fas itself in 1993. Later that year Goeddel's team found the sequence in TNFR1 and named it the "death domain" because it is necessary for the receptor to trigger the cell–death pathway.

Until last year, no one knew exactly what the death domain—which is also present in RIP—did. But the discoveries of the receptor-binding proteins made it clear, says Dixit, that "the death domain is nothing other than a protein–protein interaction domain." MORT1/FADD, TRADD, and RIP all use their own death domains to link up with other death domain–containing proteins.

To get a better fix on how the three proteins bring about cell death, researchers have artificially cranked up their expression in cells. In all three cases, this "overexpression" drives the cells to commit suicide. But when researchers overexpressed just parts of the proteins, they found striking disparities that pro-

vided clues to their functions in the apoptosis pathways.

MORT1/FADD's function proved easiest to understand. The key finding, made in several labs, was that overexpression of MORT1/FADD's death domain alone doesn't cause death, but the remainder of the protein, minus the death domain, does kill. That, says Goeddel, implies that MORT1/FADD is "one step downstream" from the death domain–dependent steps: The death domain receives the death signal from Fas, but the domain itself isn't needed to trigger the next step toward death.

With that information in hand, the race was on to find the next component of the pathway—the protein that MORT1/FADD associates with. And the end to that quest, which has unfolded within the past few months, proved stunning. Wallach's group and a collaborative team made up of Dixit's and Krammer's groups reported in the 14 June issue of *Cell* that, using different methods, they had closed in on the same MORT1/FADD binding protein. It turned out to be none other than an ICE protease family member.

The implication is that MORT1/FADD links Fas directly to the ICE-like enzyme, which Wallach's group called MACH, for MORT1 associated–CED-3/ICE homolog, and Dixit and Krammer called FLICE, for FADD-like ICE (because both have a similar protein-binding domain). "That is a really short pathway," marvels Chicago's Thompson. "It appears to be the most directly hooked-up pathway to get you right directly to ICE proteases."

What happens after activation of FLICE/MACH is less clear. Researchers suspect, however, that this enzyme activates other members of the ICE family. Activation is required because ICE-like enzymes begin life as part of inactive precursor proteins, which have to be cut at specific sites to release the functional enzymes—and the specific cut required is precisely the type of cut the ICE enzymes are specialized to make. If this supposition is true, activating one ICE could start a chain reaction in which that enzyme activates other family members, which in turn go on to clip other substrates, eventually leading to cell death. Indeed, says Goeddel, the fact that FLICE/MACH is physically linked to Fas suggests that the enzyme is at the top of such a cascade. Moreover, says Wallach, "we have indications, and others do as well, that the first substrates for MACH are other proteases of the CED-3/ICE family."

THE TNF RECEPTOR FAMILY

Receptor	Death domain	Interacting proteins	Signaling	Biological effects
Fas	yes	MORT1/FADD	ICE family	immune-mediated apoptosis
TNFR1	yes	TRADD	ICE family NF-κB	immune-mediated apoptosis, inflammation
TNFR2	no	TRAFs	NF-κB ICE family?	inflammation apoptosis?
CD30	no	TRAFs	NF-κB other?	stimulation, survival, and maybe death of lymphocytes
CD40	no	TRAFs	NF-κB other?	stimulation, survival, and maybe death of lymphocytes
OX40	no	TRAFs	??	lymphocyte stimulation
4-1BB	no	TRAFs	LCK or NF-κB	lymphocyte stimulation
LT β receptor	no	TRAFs	NF-κB other?	lymph node development? death of tumor cells
Low affinity NGF receptor	no	NRIF?	NF-κB other?	neuron survival or death?

While the pathway from Fas to cell death is remarkably short and straight, the pathways leading from TNF are more convoluted, with plenty of uncharted territory remaining. The path leading from TNFR1 goes through TRADD. But TRADD, unlike MORT1/FADD, requires its death domain both to receive the death signal from the receptor and to pass it along the pathway. This became apparent when researchers overexpressed TRADD's death domain and the rest of the protein separately, and got results that were the opposite of those obtained with FADD: The death-domain segment alone triggered cell death, while the rest of the protein had no effect. The finding led to a view of TRADD's death domain as an "adapter," linking TNFR1 to other proteins in the death pathway whose activities bring about the actual killing. (RIP's function is less clear. It behaves like TRADD, but Fas apparently doesn't need such an adapter, as it can bind directly to MORT1/FADD.)

Researchers now believe that those other proteins involved in killing by TNF include MORT1/FADD and FLICE/MACH. That conclusion comes from experiments showing that cell-killing by TNF is blocked when these proteins are altered so they can receive the death signal, but can't pass it on. "The easiest [explanation] is that FADD and MACH are both required for TNF death," says Goeddel. He cautions, however, that the simple explanation that TRADD forms a bridge directly connecting the TNF receptor to MORT1/FADD may not be true.

For one thing, he says, no one has shown that MORT1/FADD is linked via TRADD to TNFR1 when cells are triggered to die by TNF. Also, FasL takes mere hours to kill, while TNF can take a day or more. If TRADD were a direct link from TNFR1 to MORT1/FADD to FLICE, "then you would expect [death] to be much quicker" in response to TNF, says apoptosis researcher David Vaux, of the Walter and Eliza Hall Institute in Melbourne, Australia. That suggests there may be several unknown steps between TNFR1 and MORT1/FADD.

The TNF trail doesn't just meander on its way to MORT1/FADD; it forks as well. While TNFR1 sometimes signals death, it can also trigger a different signaling pathway that activates a regulator of gene transcription called NF-κB, which turns on genes active in producing inflammation. Goeddel's lab reported in the 26 January issue of *Cell* that TRADD is an essential part of both pathways, but TRADD, he says, is the "bifurcation point" after which the pathways diverge, so that each one can be blocked independently of the other.

If that were not enough convolution in the TNF pathway, there is yet another level of complexity: Both TNF receptors are present on most cells. TNFR2, like TNFR1, activates NF-κB, and in some cell types can also cause cell death. But TNFR2 differs from TNFR1 in that it has no death domain and doesn't directly bind TRADD. Instead it binds to two proteins called TRAF1 and TRAF2 (for TNF receptor–associated factor) that were discovered in 1994 by Mike Rothe in Goeddel's lab.

In their January *Cell* article, Goeddel's group showed that blocking TRAF activity blocks TNF's ability to trigger the NF-κB pathway via either TNF receptor. That suggested that the path from both receptors to NF-κB goes through the TRAFs, even though TNFR1 doesn't bind to the TRAFs directly. But Goeddel's group found the apparent link: TRAF2 binds to TRADD, and so the two together may bridge the NF-κB and apoptosis pathways. "The fact that [TRADD and TRAF2] interact means there are connections that would allow [both receptors] to talk to both pathways," says Mike Lenardo, of the U.S. National Institute of Allergy and Infectious Diseases, who studies the killing ability of TNFR2.

Choosing a path

With all that potential cross-talk, what determines how a given cell or cell type responds to TNF? "What you have is two receptors that can be expressed in differing ratios," says Goeddel, "and many signaling molecules that might have different expression levels." And how a cell responds may depend on how it is "primed" by those ratios: High levels of TRAFs and low levels of TRADD or MORT1/FADD, for example,

may bias a cell toward the NF-κB pathway as a response to TNF, rather than toward death.

Still another set of proteins that has been tied to the TNF pathways may play a role in determining whether a cell is primed for death. These are the mysterious IAPs, for inhibitors of apoptosis, discovered in 1993 by Rollie Clem, a graduate student with Lois Miller at the University of Georgia. Clem found the IAPs while searching for the genes an insect baculovirus uses to keep its host cells from dying before the virus replicates. But the IAPs have mammalian counterparts as well.

The first to turn up, a year and a half ago, was the neuronal apoptosis inhibitor protein, NAIP, which has some structural resemblance to the viral IAPs. It was discovered by Alex MacKenzie's group at the University of Ottawa, as the product of the gene mutated in some forms of spinal muscular atrophy, a fatal human disease in which motor neurons in the spinal cord die off during embryonic development. MacKenzie's team went on to show that the normal NAIP protein protects neurons from death.

Even closer relatives of the viral IAPs were discovered in fruit fly and mammalian cells by several labs. Some of these cellular IAPs block apoptosis, but researchers still don't know where or how they exert their effects. One clue, coming from Goeddel's lab, is the discovery that the IAPs form a complex with TRAF1 and TRAF2, suggesting that they act early in the pathway, but no one knows the biochemical consequences of the interactions.

Despite that uncertainty, some researchers suspect that the IAPs might act as a switch that can determine what path the TNF message takes. Vaux suggests the IAPs could bias cells toward the NF-κB pathway, perhaps by drawing TRADD to TRAF2, thus preventing TRADD from interacting with MORT1/FADD to trigger the death pathway. "That is a very attractive model," says Chicago's Thompson, but he notes that there is no evidence for it yet.

Even if that model turns out to be right, it is unlikely to be the whole story, for there are other factors that might influence a cell's propensity to suicide. These include the presence and levels in the cell of a protein called Bcl-2, whose mode of action is not yet known, but which also protects some cells from death.

One thing is certain: The vulnerability to different death signals and the ability to be saved from death by different inhibitors of apoptosis varies from cell type to cell type. Even among one general cell type such as lymphocytes, some subtypes succumb to Fas activation while others are oblivious, and some are killed by TNF while others are spurred to produce more inflammation.

These divergences have led researchers to conclude that while the general stations on the way to death—such as MORT1/FADD or the ICE enzymes—may be widely used, there are countless variations, side trails, or alternate routes that lead from death receptors to the agents of death in the cell. For example, each cell type may have its own constellation of ICE enzymes, each with its own unique profiles of activators and inhibitors, as well as its own characteristic levels of FADD, TRADD, TRAFs, and IAPs, that nudge the cells toward or away from death. "It is probably the balance [of all these things] that determines whether a cell will succumb or not," says Michigan's Dixit. And that means that the mappers of these pathways have their work cut out for them for years to come.

–Marcia Barinaga

PHYSICS

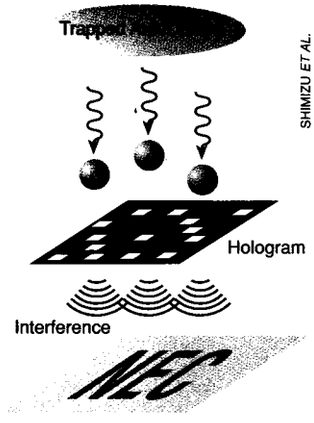
Painting Pictures With Atom Waves

In a famous haiku, the 18th-century poet Ryota wrote of the mysterious beauty of "cherry blossoms left unwatched." An echo of the phrase lingers in quantum mechanics, which says that matter acts like a wave as long as it is unobserved, then changes character when measured, collapsing into point particles. The echo may have gotten stronger with new work by a Japanese research group.

Using optical principles to control the unseen waves of neon atoms, researchers at the University of Tokyo and the NEC Fundamental Research Laboratories in Tsukuba coaxed the atoms to form a minute physical image. The work, says Mara Prentiss, a physicist at Harvard University, is "an elegant and beautiful demonstration" of quantum mechanical duality and could provide a new technique for etching intricate circuit patterns.

Reported 2 weeks ago in *Physical Review Letters* by Fujio Shimizu of Tokyo and his colleagues, the experiment "does with atoms what people have [ordinarily] done with light," as Wolfgang Ketterle of the Massachusetts Institute of Technology puts it. It mimics optical holography, in which an image is encoded in the interference pattern of light and dark patches created when two laser beams overlap—one reflected from the original object and another serving as a reference. When a "readout" beam passes through the holographic pattern, usually recorded on film or in a special crystal, an image of the object is regenerated.

Swapping light for atoms was not simple. The researchers knew that bouncing atom waves off an object to make a hologram was impractical—the analogy between atoms and light doesn't stretch that far. What's more, there are no crystals capable of recording atomic interference patterns. So the team simply calculated the hologram that would be needed to read out a particular image—in their case, the letters "NEC"—and then etched holes, analogous to the bright areas in the interference pattern, into a membrane, using a technique called electron-beam lithography. The result looks something like the computer-punched holes on old payroll checks.



SHIMIZU ET AL.

Quantum artistry. Interfering atom waves yield image.

The researchers then positioned the membrane beneath a source of neon atoms, whose random motions had been damped by bombarding them from all sides with laser light. When Shimizu and coworkers released these "cooled" atoms, they fell toward the membrane at almost the same speed, forming a coherent atom wave. As the wave diffracted through the holes, it interfered with itself and impinged on a fluorescent detector. There the individual atoms were resurrected, producing point-like flashes that, over a 2-hour exposure, summed to produce a fuzzy reproduction of the NEC logo, about a millimeter across.

That's far too coarse and slow for practical uses, but "it's a completely new way to generate a pattern of atoms," says Jabez McClelland of the National Institute of Standards and Technology in Gaithersburg, Maryland. And Shimizu thinks that with a more finely detailed hologram, his team could make atom-wave images with features as small as a few hundred nanometers—in principle, as small as the holes that can be etched on the mask. Because the barrage of atoms could etch away a substrate, the method might then be useful for making minute circuit patterns. The practical downside, says Harvard's Prentiss, is that the resolution can be no better than that of electron-beam lithography, which creates the holographic "templates."

But Shimizu's interest is aesthetic as well as practical. "It is fun to produce a pattern that cannot be imagined from the mask." And that approach might just raise the science of quantum imagery to an art form.

–James Glanz