we're trying to solve." Rothstein agrees. "We don't believe there's only one cause" of ALS, he says, but if the defect does turn out to contribute to ALS development, the finding could someday lead to a useful diagnostic test and perhaps to improved treatments.

Rothstein, postdoctoral researcher Glen Lin, and research associate Lynn Bristol discovered the EAAT2 defects while following up on earlier observations that many ALS patients have abnormally low levels of the protein in areas affected by the disease: the motor cortex and spinal cord. To track down the cause of that deficit, the Hopkins team examined the messenger RNAs (mRNAs), which provide the direct instructions for making the protein, in brain and spinal cord tissue obtained at autopsy from ALS patients. They found that those mRNAs were often edited incorrectly in precisely the same tissues where the low protein levels were seen.

Most genes of higher organisms contain non–protein-coding sequences, called introns, that have to be removed from the mRNAs before the proteins themselves are synthesized. But the Hopkins workers found that the machinery that removes the introns from the EAAT2 mRNAs made mistakes in 11 of the 20 sporadic ALS patients they tested. It either failed to remove an unneeded intron, or it deleted an essential protein-coding sequence, called an exon. As a result, the mRNAs make no EAAT2 at all or make a faulty version. Rothstein and his colleagues did not find the abnormalities in brain tissue from 12 normal subjects or in 16 patients who died of other neurodegenerative diseases, including Alzheimer's and Huntington's. This suggests that the EAAT2 defect is specific to ALS and not just a general consequence of nerve cell death.

Many questions remain unanswered. The researchers do not yet know what causes the mistakes in the mRNAs. It may be that a mutation in the EAAT2 gene gives the cell's RNA-processing machinery the wrong directions for removing the introns. Or the fault may lie in the processing machinery itself. Also unexplained, Rothstein says, is how the defect could be restricted to the motor cortex and spinal cord.

Nevertheless, says neurologist Wim Robberecht of the University of Leuven in Belgium, the evidence for a common denominator in at least some sporadic ALS cases is exciting. Even if the primary cause is "something completely unrelated" to glutamate metabolism—for example, the buildup of damaging free radicals from a malfunctioning SOD1 protein—Rothstein's findings "would finally give very conclusive evidence that an abnormality of glutamate metabolism is indeed involved" in ALS, if only as an important secondary mechanism.

–Gretchen Vogel

CELL BIOLOGY

Expanding the Eukaryote's Cast of Chaperones

Textbooks often depict cells as roomy compartments in which proteins go about their business relatively unimpeded. But in reality, the cell interior is more like a crowded marketplace, with proteins hustling from one job to the next, jostling and potentially interfering with one another along the way. In this rough-and-tumble milieu, proteins often need help to fold up into, and then maintain, the three-dimensional structures necessary for them to function normally. With three papers in this issue, it's becoming clear that, at least in higher organisms, a whole retinue of helper proteins is involved, and the task of helping—chaperoning—protein folding is far more complex than researchers had suspected.

In the past, the so-called heat shock proteins, in particular the two called Hsp90 and Hsp70, seemed to play the lead role in protein folding. Among other things, Hsp90 helps to prevent the clumping together of other proteins that have unfolded because of increased temperatures until they can refold into their proper conformation. It also keeps certain key components of the cell's internal signaling pathways, including steroid hormone receptors and kinase enzymes, folded in the right conformation to respond rapidly to their triggering signals. But the new findings bring a whole new set of chaperone proteins into the picture: a large protein family, found in organisms ranging from bacteria to mammals, called the immunophilins.

"There might be a whole slew of chaperones in eukaryotic cells that we just don't know about yet."

—Susan Lindquist

One report (p. 1713) provides the evidence that, in yeast, an immunophilin cooperates with Hsp90 in maintaining the signaling pathways. And results from the other two studies (pp. 1715 and 1718), done in the test tube, suggest that the immunophilins by themselves can keep proteins on the right track for folding. "These proteins themselves have the properties of a molecular chaperone," says F. Ulrich Hartl, a cellular biochemist at Memorial Sloan Kettering Cancer Center in New York City. "They play a role, somehow influencing the conformation [of proteins]. Previously it was rather mystical what these proteins would do."

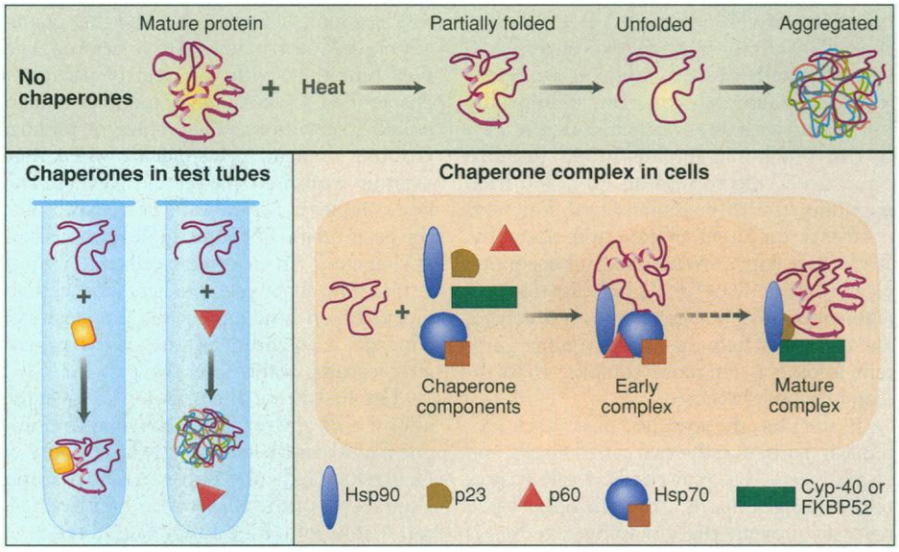
Besides expanding the cast of characters in protein folding, the new findings add to the intricacy of the plot. They suggest that each of the many chaperones could play a different role in a molecular assembly line that gradually moves a protein toward its final form. And they hint that, with the help of particular chaperones, proteins may sometimes remain poised in an intermediate stage of folding, enabling them to react quickly when they meet up with the right protein partner. The new complexity, say investigators, may be just the beginning. "There might be a whole slew of chaperones in eukaryotic cells that we just don't know about yet," predicts yeast cell biologist Susan Lindquist of the University of Chicago, a co-author of one of the papers, after learning of the other two papers.

The first hints of this complexity came almost a dozen years ago, when cell biologist David Smith, now at the University of Nebraska Medical Center in Omaha and David Toft of the Mayo Clinic in Rochester, Minnesota, found that Hsp90 tends to be accompanied by an entourage of a half-dozen other proteins when it chaperones a steroid receptor. Among them were molecules called immunophilins, primarily cyclophilins, then best known as the targets of immunosuppressive drugs, such as cyclosporin. The appearance of the immunophilins in such a wide range of organisms had already suggested to Smith and others that they have an essential function in the everyday life of the cell. Now, their link to Hsp90 hinted that their crucial role could be protein folding.

To test this possibility, Lindquist, geneticist Richard Gaber of Northwestern University in Evanston, Illinois, and their colleagues first showed that both of the immunophilins in yeast (*Saccharomyces cerevisiae*), which are called Cpr-6 and -7, bind to yeast Hsp90. They also found that Cpr-7, at least, is necessary for normal cell growth. When they knocked out the gene for that protein, the yeast cells grew much more slowly than normal yeast, which led the researchers to wonder whether Cpr-7, like Hsp90, is crucial for proper intracellular signaling. Further experiments suggested that it might be.

When they inserted the gene for the

mammalian glucocorticoid receptor into either normal yeast cells or yeast cells lacking Cpr-7, they found that the receptor activity in the cells without the immunophilin was only one-fifth of that in the normal yeast. Similar experiments with the mammalian estrogen receptor yielded comparable results. "[In the cell], Cpr-7 is important for signal transduction and for Hsp90-associated events," Lindquist concludes.



Protein preparedness. Without helper molecules called chaperones *(top)*, proteins unfold when heated and aggregate into useless clumps. Lab experiments depicted at lower left show that some chaperone components (yellow) prevent this aggregation, while others (red) do not. But in the cell *(lower right)*, these components appear to work together in shifts to enable a protein to refold into the right shape.

Smith describes that finding as "very novel, important." The link between the immunophilins and signal transduction could eventually help explain the immunosuppressive effects of drugs that target them, although to date no one really knows exactly how those drugs work. And it may suggest new ways to prevent immune system activation or combat side effects seen in people receiving these medications.

It does not, however, prove that Cpr-7 is a chaperone that guides protein folding. But the other two teams gathered more direct evidence for the involvement of immunophilins and other proteins associated with Hsp90 in this process by studying them in the test tube. For their studies, biochemists Brian Freeman and Richard Morimoto of Northwestern University and Toft used the enzyme β-galactosidase as a model protein.

Normally, when a protein like β-galactosidase is "denatured," that is, exposed to heat or other treatments that cause it to unfold in the test tube, the unfolding molecules clump together and form an insoluble precipitate. But as the researchers report on page 1718, they found that either of two Hsp-associated proteins, the immunophilin called Cyp-40 or the protein p23, which is not an immunophilin, can prevent that, just as Hsp90 can.

Biochemical analyses indicated that each of these two proteins grabs onto the β-galactosidase, maintaining it in a partially folded state. By itself, neither p23 nor Cyp-40 could restore the enzyme to its completely folded state, but adding an additional component, Hsp70, to the mixture did have that effect—a result suggesting that the proteins cooperate in the refolding effort.

In a similar set of experiments with a different test enzyme, citrate synthase, Johannes Buchner from the University of Regensburg in Germany and his colleagues also found that p23 and another Hsp90-associated immunophilin, FKBP52, protect a partially denatured protein from further disintegration. "When we learned about each other's results, it was quite striking and reassuring," Buchner says about the U.S. group. "We're using quite different [proteins], yet in both we see the very same results."

Not only do these experiments expand the cast of helper molecules that interact with an unfolded protein; they also help pinpoint the helpers' roles. When the two groups analyzed the molecular complexes that formed after they added various combinations of these new "chaperones" to denatured protein, they realized that each one interacts with protein at different stages of unfolding. "We once thought proteins were just folded or unfolded," Morimoto explains. "Now we see there are intermediate states." That fits in with growing evidence that maintaining the proper intermediate state is likely to be critical to normal functioning for many cell proteins, including the steroid receptors, says Morimoto. Indeed, improper protein folding may even lead to diseases such as Alzheimer's characterized by the abnormal protein deposits known as amyloid (*Science*, 15 March, p. 1493).

The existence of intermediate states—and of chaperones for each of those states—lends credence to the idea that there is a chaperone assembly line, Nebraska's Smith suggests. Each of the associated proteins could be taking a turn, in conjunction with an Hsp90 molecule, tweaking the unfolded protein a slightly different way. "I view Hsp90 as a molecular potato head," he explains. "Stick different proteins on it, and it gives it different [roles]."

Lindquist, too, likes the idea that there may be different protein subcomplexes for different stages of folding. She envisions the process less as a linear assembly line than as a very dynamic set of pathways in which, say, a steroid receptor goes back and forth—perhaps between various Hsp90 complexes—in various states of being folded and unfolded because it's such a labile structure. It might be difficult, energetically, to stay in the right conformation for accepting, say, a steroid, and so it may not stay poised for very long. As it loses its shape, the receptor might then be picked up by one of the Hsp90 complexes and refolded. Thus, these chaperones help ensure there's always some steroid receptor poised in the partially folded state its steroid needs.

Still, even Smith is not convinced that the test tube assays reflect what the Hsp-associated proteins are doing in living cells. "I wonder if the proteins will behave in the same manner in the much more complex environment of the cell," he says. Stripping Hsp90 from p23 and the two immunophilins may simply have given the proteins the ability to link with molecules they normally could not bind. Buchner doesn't think that's the case, however. He points out that neither his team or Morimoto's saw any indication of chaperoning by another of Hsp90's partners—the p60 protein—when they stripped away the heat shock protein and tested p60 by itself.

It will take more experiments to decide whether the complexes formed by Hsp90 and its associated proteins play as big a chaperoning role as has sometimes been suggested. But if in vivo studies confirm that the cell has many molecular folding specialists, each of which takes a protein part of the way to its final form, the intracellular bazaar will start looking even livelier.

Proteins will have to be seen as existing in many forms, each of which may serve a different role in the chemistry of life. Far from being just another wrinkle on protein folding, says Morimoto, these intermediates "are going to turn out to be a very important concept in biology."

–Elizabeth Pennisi