potentials, and effects on the immune system, since Kvβs regulate lymphocyte Kv1.3 channels, whose blockade reduces the immune response[12,13].

It is unclear whether cortisone reaches high enough concentrations to serve as a physiological regulator of the Kv1/Kvβ complex; however, the discovery of this effect suggests that it is worth searching in the corticosteroid family and beyond for a physiologically relevant modulator of the complex. Such a molecule could act as a systemic signal to molecularly short-circuit the normally stable reciprocal regulation of voltage-gated K^+ channels and their specialized AKR enzyme partners. This result would uncouple the channel's regulation of membrane potential from the enzyme's sensing and regulation of the oxidative-reductive metabolic state of the cell. The upshot of this work is that a precedent has been set for ion channels: we should keep an eye open for other small molecules that may physiologically regulate subunit interactions that were previously thought to be inviolate, and we should consider seeking drugs that target not active or allosteric sites but protein interfaces from which docked regulators may be peeled off.

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Proteolytic needles in the cellular haystack

Mari Enoksson & Guy S Salvesen

The execution phase of cell death is driven by specific proteolytic signaling through cleavage of proteins by caspases. Within the mix of hundreds of newly identified caspase substrates lie the crucial proteolytic events whose sum defines the unique morphology known as apoptosis.

Like most post-translational protein modifications, proteolysis is of interest to the wide bioscience community primarily in the identity and consequence of its action on targets. Only when the protein target is known, and the modification site identified, can progress toward understanding the consequence of the modification evolve. Whereas it has been possible to define the proteases operating in signaling pathways, the identification of their targets has proved much more challenging. This problem is not specific to proteolysis but to all post-translational events, including ubiquitination, glycation/glycosylation and phosphorylation. The development of innovative chemistry and high-throughput mass spectrometry has recently influenced the search for the natural substrates of proteolysis, and previous systematic attempts to identify substrates of caspases, the executioners of apoptosis[1], have netted a couple of hundred[2]. Two new approaches have broadened the search[3,4], demonstrating the identity of hundreds of new proteins that serve as caspase substrates in dying cells. Together these studies also reveal some interesting insights into the nature of specific limited proteolysis in general.

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Mahrus *et al.*[3] present an intriguing approach to the problem of how to specifically label protein N termini and thereby discern the locations of protease cleavage sites. Jim Wells and colleagues previously engineered a form of the protease subtilisin able to efficiently reverse the cleavage of peptide bonds, demonstrating that this "subtiligase" can polymerize peptide chains given the amino acid raw ingredients[5]. Now they use this subtiligase to tag free N termini with a biotin peptide after induction of apoptosis, for efficient enrichment of proteolytic cleavage sites, and, using an LC-MS/MS approach coupled with database searching, identify over 300 putative caspase cleavage sites (**Fig. 1**, left).

In contrast, Dix *et al.*[4] use a more conventional gel-based method to interrogate a very similar apoptotic paradigm, thereby identifying a similar number caspase substrates. They separate protein samples from naive and apoptotic cells side by side on SDS-PAGE, slice the gel into 22 strips ordered by molecular weight, and digest the proteins in each gel slice with trypsin. The resulting peptide aliquots then undergo LC-MS/MS analysis. Peptides from a full-length protein are found at one molecular weight (one gel slice), and cleavage of a substrate will cause peptides from the protein to show up in a slice of lower molecular weight (**Fig. 1**, right). The presence of the same peptides at different molecular weights thus provides information about the identity of cleaved proteins. Perhaps more importantly, the spectral count of the different peptides (how many times the peptide is detected in each sample) gives a relative quantification of the fraction of the protein cleaved.

Some very interesting correlations are revealed by the recent analyses. The technique of Mahrus *et al.* does not distinguish, *per se*, between native protein N termini and N termini created by proteolysis. A rough calculation based on the data from Mahrus *et al.*, assuming that 80% of intracellular human proteins are naturally N-terminally blocked, leads to the conclusion that 15%–20% of all the proteins in an apoptotic cell are products of cleavage after aspartate residues, which is the defining feature of caspase action. This is a tremendous amount of proteolysis and, although the number may be subject to unknown methodological bias, will force us to revise the number of caspase substrates previously predicted. On the other hand, there have been several studies showing the activation of other proteolytic pathways, distinct from the caspase pathway, during apoptosis[6]. The data presented by Mahrus *et al.* thus open up an interesting field for discussion. Do caspases drive the only proteolytic events occurring during cell death signaling, or are other proteases also significant for the execution of apoptosis? Another interesting result presented by Mahrus *et al.* is the location of the cleavage sites. Dogma in the field predicts that most proteolytic cleavage sites will be found in disordered regions of proteins (unstructured loops or between domains) that have the flexibility to fit into the active site of the protease.

Therefore, it is noteworthy that many cleavage sites presented/predicted by Mahrus *et al.* are found in α-helical regions. Clearly, this finding requires revisiting the current dogma.

Proteins undergo degradation as a consequence of protein turnover, and it is believed that this process might be expedited as a result of proteolytic processing during apoptosis. Interestingly, Dix *et al.* demonstrate many examples of persistent fragments after proteolytic cleavage of apoptosis substrates. This observation makes it tempting to speculate that certain small protein domains that are sustained in the cell when the rest of the protein is undergoing degradation may have a biological function. The concept of gain-of-function cleavages during cell death has been suggested elsewhere[2], but clearly, many persistent domains have been overlooked since they would been very difficult to detect with the lower resolution methods before Dix *et al.*

The papers by Dix *et al.* and Mahrus *et al.* present quite unexpected results on the extent of proteolysis occurring during apoptosis. But these techniques are not restricted to apoptosis and should be totally transposable to other proteolytic signaling events (inflammation, coagulation, gastrulation, connective tissue remodeling and so forth). The current wave of protease-centered proteomics allows biologists to dissect protease specificity motifs, detect natural protease substrates and even begin to map out protease pathways. Each method has its advantages and disadvantages, and a combination of solution-based and gel-based methods would provide the twin necessities of location and quantitation of the degree of cleavage of the targets in a protease pathway.

However, several overarching problems remain that continue to confound interpretations of reported proteolytic cleavages. First, although many scientists in the field studiously avoid the issue, it is clear that we have yet to find a way to distinguish important participants from innocent bystanders.

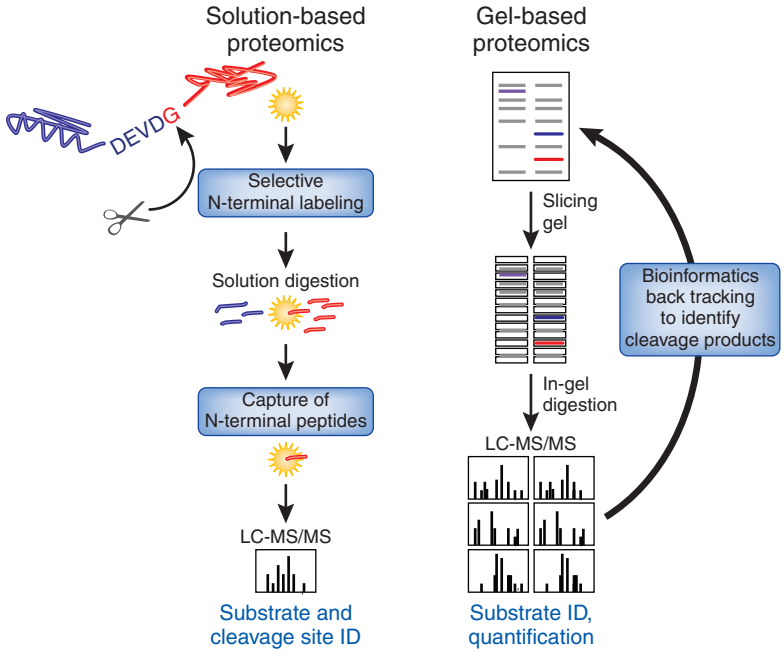

Figure 1 Detection of protease substrates by proteomics. The key to solution methodology, such as that described by Mahrus *et al.*[3] (left), is the specific labeling (yellow star) of protein N termini on a proteome-wide scale. This ultimately allows enrichment and identification of protease cleavage sites. The key to gel-based methods, such as the PROTOMAP technology described by Dix *et al.*[4] (right), is the identification of cleaved protein products by size changes on SDS-PAGE, deduced from backtracking the identity of proteins from size-ordered gel slices. Both methods have individual advantages, and their combination could prove powerful.

Second, as discussed previously[2], many proteolytic cleavages in the apoptotic pathway (and presumably other proteolytic pathways, such as coagulation) result in gain of function, and so even a small fraction being cleaved could lead to biological activity. In contrast, in a loss-of-function event, cleavage of large fractions is likely to be required to fully inactivate protein function. Thus, the ability to accurately detect small amounts of cleavage becomes very important. Finally, proteases with specificity much less well defined than the caspases pose a problem in assigning specific proteases to specific cleavages[6]. And so, as with most proteome-wide discovery methods, pinning down the culprits (in this case, the proteases) that deliver the outcome (in this case, the cleavage of protein substrates) is going to require a close matching between the chemical and bioinformatic technologies that are now emerging.

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