Resource

Functional Interplay between Caspase Cleavage and Phosphorylation Sculpts the Apoptotic Proteome

Melissa M. Dix,1,3 Gabriel M. Simon,1,3 Chu Wang,1 Eric Okerberg,2 Matthew P. Patricelli,2 and Benjamin F. Cravatt1,*

1The Skaggs Institute for Chemical Biology and Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA
2ActivX Biosciences, La Jolla, CA 92307, USA
3These authors contributed equally to this work
*Correspondence: cravatt@scripps.edu
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SUMMARY

Caspase proteases are principal mediators of apoptosis, where they cleave hundreds of proteins. Phosphorylation also plays an important role in apoptosis, although the extent to which proteolytic and phosphorylation pathways crosstalk during programmed cell death remains poorly understood. Using a quantitative proteomic platform that integrates phosphorylation sites into the topographical maps of proteins, we identify a cohort of over 500 apoptosis-specific phosphorylation events and show that they are enriched on cleaved proteins and clustered around sites of caspase proteolysis. We find that caspase cleavage can expose new sites for phosphorylation, and, conversely, that phosphorylation at the +3 position of cleavage sites can directly promote substrate proteolysis by caspase-8. This study provides a global portrait of the apoptotic phosphoproteome, revealing heretofore unrecognized forms of functional crosstalk between phosphorylation and caspase proteolytic pathways that lead to enhanced rates of protein cleavage and the unveiling of new sites for phosphorylation.

INTRODUCTION

Proteolysis and phosphorylation are two of the most pervasive forms of protein posttranslational modification, playing essential roles in the majority of (patho)physiological processes, including tissue development, cancer, and cell death (Kurokawa and Kornbluth, 2009; López-Otín and Hunter, 2010). Apoptosis, or programmed cell death, is orchestrated by a family of cysteine proteases called caspases, which cleave their protein substrates after aspartic acid residues (Crawford and Wells, 2011; Fuentes-Prior and Salvesen, 2004; Thornberry and Lazebnik, 1998). Recent advances in global protease substrate identification technologies have generated a large inventory of proteins that are cleaved by caspases during apoptosis, demonstrating that as much as 5% of the proteome is subject to caspase-mediated proteolysis (Amtzen and Thiede, 2011; Crawford and Wells, 2011).

Protein kinases are prominently represented among caspase substrates and, in some cases, cleavage activates these kinases so that they can perform important functions in apoptosis (Kurokawa and Kornbluth, 2009). Caspase-mediated activation of Rho-associated kinase 1 (ROCK1), for instance, promotes the characteristic membrane blebbing associated with apoptosis (Coleman et al., 2001). Kinases can also be inactivated by caspase-mediated cleavage to block their activity during apoptosis (Kurokawa and Kornbluth, 2009). The crosstalk between caspases and kinases also includes the phosphorylation of caspases to either enhance or suppress their activity (Kurokawa and Kornbluth, 2009). Likewise, the phosphorylation of some caspase substrates, notably BID phosphorylation on Thr59 (which is the P2 residue of the caspase-8 cleavage site) blocks caspase cleavage (Degli Esposti et al., 2003). These findings suggest that caspase and kinase pathways interact in intricate ways to influence the balance between cell survival and death (Janes et al., 2005). Nonetheless, whether a more global relationship between proteolysis and phosphorylation exists in apoptosis has not been investigated.

We recently introduced a proteomic method termed PROTOMAP (short for Protein Topography and Migration Analysis Platform) that can be used to characterize proteolytic events in cells by detecting shifts in protein migration through a combination of SDS-PAGE and mass spectrometry (MS)-based proteomics (Dix et al., 2008). Using this approach, we identified over 250 cleaved proteins in apoptotic cells, including 170 proteins that were not previously known to be cleaved by caspases. In the current study, we sought to create an advanced, quantitative version of PROTOMAP that enables simultaneous analysis of proteolytic and phosphorylation processes in cells, such that phosphorylation sites could be directly integrated into the topographical maps of cleaved proteins during apoptosis. We applied this method to study the intrinsic apoptotic cascade in Jurkat T cells, resulting in the identification of more than 700 cleaved proteins and 5,000 sites of phosphorylation. The integration of these global data sets revealed that phosphorylation events are enriched on cleaved proteins and...
are clustered around sites of caspase cleavage. We further identified a cohort of previously unreported phosphorylation sites that were specific to apoptotic cells, suggesting the existence of a cell-death-related phosphorylation network. We show using activity-based proteomic methods that at least a part of this network is driven by caspase-mediated activation of DNA-dependent protein kinase (DNA-PK) at early stages during the time course of apoptosis. Finally, we interrogated the functional relationship between proteolysis and phosphorylation, uncovering multiple forms of crosstalk that include the caspase processing of proteins to expose new sites for phosphorylation and the phosphorylation of proteins at the +3 (P3) position of caspase recognition sequences to dramatically enhance proteolysis by caspase-8.

**RESULTS**

**Quantitative Proteomic Analysis of Phosphorylation and Proteolysis by qP-PROTOMAP**

The proteomic measurement of dynamic posttranslational modifications, like phosphorylation, requires quantification of individual peptides, and we therefore sought to combine PROTOMAP with stable isotopic labeling methods (SILAC; Ong et al., 2002) for this purpose. We also needed to incorporate a phosphopeptide enrichment step without sacrificing the protein size and topography information provided by the SDS-PAGE step of the original PROTOMAP method. The workflow for the resulting quantitative phospho-PROTOMAP (or qP-PROTOMAP) platform was therefore as follows (Figure 1A): Control and

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**Figure 1. Quantitative Profiling of Phosphorylation and Proteolytic Pathways in Apoptosis by qP-PROTOMAP**

(A) General features of qP-PROTOMAP method as described in the main text. Peptides are colored red and blue to represent signals detected in healthy control (light) and apoptotic cells (heavy), respectively.

(B) Number of cleaved proteins detected using the original PROTOMAP method (Dix et al., 2008) versus qP-PROTOMAP as described in this study. See Table S1 for peptographs of cleaved proteins identified by qP-PROTOMAP.

(C) Distribution of phosphorylation events identified in control and apoptotic cells by qP-PROTOMAP. Phosphorylation events were designated “control specific” or “apoptosis specific” if they showed light/heavy SILAC ratios of >2 or <0.5, respectively (corresponding to log2 values of 1 or −1). Phosphorylation events displaying light/heavy ratios between these values were designated as “static.” See also Table S2.
apoptotic cells were grown in media containing isotopically light and heavy amino acids, respectively. Equal quantities of each cell proteome were then combined and separated by SDS-PAGE. Next, as in the original PROTOMAP method, gel lanes were sliced into 22 evenly spaced bands that were digested in-gel with trypsin to extract peptides. Phosphopeptides were then enriched via immobilized metal-affinity chromatography (IMAC) and subjected to reverse-phase liquid chromatography and MS analysis on an LTQ-Velos Orbitrap. Flow-through from the IMAC step (containing unphosphorylated peptides) was also analyzed, and the combined SILAC ratios of unphosphorylated and phosphorylated peptides were integrated into quantitative peptographs to provide a complete picture of protein phosphorylation and proteolysis.

Peptographs for qP-PROTOMAP experiments display detected peptides from left-to-right based on their position in the primary sequence of their proteins, and from top-to-bottom depending on the gel band in which they were detected (the vertical dimension thus represents molecular weight) (Figure 1A). Phosphopeptides are marked by a circle. For the purposes of quantitation, each peptide is assigned a color on a continuum from red to blue reflecting the light/heavy ratio: peptides exhibiting no change (1:1 ratio) are displayed in purple; control- and apoptosis-specific peptides are shown in red and blue, respectively. To facilitate visual interpretation of these quantified peptide data, a box plot is provided in the middle panel (to the right of the peptograph) that displays the distribution of ratios found in each band. Spectral-count information is displayed in a third panel to enable estimation of the relative abundance of each protein isoform. Most importantly, because qP-PROTOMAP integrates phosphorylation sites into the topographical maps of cleaved proteins, the approach can determine the precise protein isoforms that possess individual phosphorylation events. Thus, we are able to identify phosphorylation events that may occur exclusively on full-length proteins or, alternatively, on fragments of these proteins generated during apoptosis. We present an example peptograph and more details on its interpretation in Figure S1 (available online).

For our assessment of crosstalk between proteolysis and phosphorylation, we induced the intrinsic apoptotic pathway in Jurkat T cells with staurosporine (“STS”). Although it might initially seem counterintuitive to use a broad-spectrum kinase inhibitor like STS to study phosphorylation events in apoptosis, we hypothesized that this drug, through the inhibition of many kinases, might simplify the phosphoproteome to facilitate the characterization of phosphorylation events that were important for programmed cell death. Indeed, we, and others, have shown that STS induces a highly efficient apoptotic cascade in Jurkat T cells that is essentially complete by ~6 hr (Dix et al., 2008; Na et al., 1996; Stolzenberg et al., 2000), and we therefore expected that any kinase pathways relevant for this rapid cell death process would necessarily be insensitive to this drug. We therefore analyzed STS-treated Jurkat cells at an “early” (2 hr) and “late” (4 hr) stage of apoptosis by qP-PROTOMAP. In total, 4,521 proteins were detected across both time points, and 5,034 sites of phosphorylation were quantified on serine, threonine, or tyrosine residues from 1,624 of the proteins (36% of all proteins detected). Peptographs were generated for each protein at both time points, enabling rapid visual interpretation of their (1) cleavage status, (2) cleavage magnitude, and (3) phosphorylation status on individual protein isoforms in a time-dependent manner.

### Quantitative Analysis of Cleaved Proteins in Apoptotic Cells by qP-PROTOMAP

We initially evaluated the performance of qP-PROTOMAP as a global method for characterizing cleaved proteins, which were expected to: (1) possess multiple peptides in the parental protein band that substantially deviate from the 1:1 SILAC ratio observed for uncleaved proteins, and/or (2) display persistent protein fragments selectively in apoptotic cells. We developed abundance thresholds for quantifying the cleavage state of proteins, and, out of 2,867 proteins that met these thresholds, 744 of them (26%) showed strong evidence of cleavage (Table S1; see the Extended Experimental Procedures for details). A majority of the cleaved proteins identified in our original PROTOMAP study were also observed to be cleaved by qP-PROTOMAP (77%, Figure 1B), and we detected 349 proteins that had not been previously described in the literature as caspase substrates (Table S1). Considerably more proteins were found to be cleaved than has been previously reported, both in absolute number and as a percent of the proteome. This can be ascribed to the higher accuracy and sensitivity that is achievable using SILAC quantitation, enabling high-confidence assessments of lower-abundance proteins. As reported previously (Dix et al., 2008), the majority of cleaved proteins (67%) displayed one or more persistent fragments (Table S1).

### Quantitative Analysis of the Phosphoproteome in Apoptotic Cells by qP-PROTOMAP

We next assessed the performance of qP-PROTOMAP as a global method for characterizing phosphorylation events in apoptotic cells. Phosphorylation events that showed >2-fold SILAC ratios in control or apoptotic cells were defined as “control specific” or “apoptosis specific,” respectively. We should note the potential for these phosphopeptide SILAC ratios to be influenced by the cleavage of proteins. For instance, reductions in protein abundance during apoptosis could indirectly cause a loss of phosphopeptide signals. Conversely, the stochastic nature of peptide detection in individual data-dependent MS runs could result in the identification of a static phosphorylation event exclusively on one isoform of a protein. We attempted to address at least some of the complexities by performing numerous replicates for our phosphoproteomic experiments (see Extended Experimental Procedures for details), which yielded rapidly diminishing returns for unique phosphorylation-site identification with each replicate (Figure S2).

A global analysis of SILAC ratios for the 5,060 phosphorylation events identified in our combined qP-PROTOMAP data sets led to several important discoveries. First, a majority of phosphorylation events (>85%) either showed no change or were elevated in control cells (Figure 1C). This is not a surprising result given that we induced apoptosis with the broad-spectrum kinase inhibitor STS. Even with this mode of inducing apoptosis, however, we still identified 531 phosphorylation sites that were...
apoptosis specific (Figure 1C; Table S2). Striking examples included the pSer347 and pSer882 events in the retinoblastoma protein (RB1) (Figure 2A, features “1” and “2,” respectively), a well-studied tumor suppressor that is known to be cleaved at two distinct caspase sites following the induction of apoptosis (Fattman et al., 2001). We identified 18 additional sites of phosphorylation on RB1, virtually all of which were control specific (Figures 2A and 2B). Most of these control-specific phosphorylation events occurred at well-characterized sites identified in numerous (>10) independent studies as determined by searches of the PhosphoSite database (Hornbeck et al., 2011) (Figure 2B); interestingly, however, the apoptosis-specific pSer347 and pSer882 events had only been reported rarely in previous phosphoproteomic analysis (≤3 times each; Figure 2B) and, in the case of pSer347, only in Jurkat cells treated with pervanadate, a known proapoptotic stimulus (Hehner et al., 1999). These initial data suggested that apoptosis might activate a special phosphorylation network that is distinct in content from other cellular processes. We more systematically assessed this possibility by comparing static/control-specific and apoptosis-specific phosphorylation events, as estimated by the absence of annotation of these events in the PhosphoSite database. Strikingly, close to half of the apoptosis-specific phosphorylation sites were previously unreported in the literature, whereas less than 15% of the static/control-specific phosphorylation sites fell into this category (Figure 2C). Apoptosis-specific phosphorylation events were also underrepresented among phosphorylations that were frequently detected in the literature (≥5 citations) (Figure 2D). We conclude from these data that apoptosis leads to the activation of a specific set of kinases (and/or inactivation of phosphatases) to create a rare pool of phosphorylation events that are not observed in healthy cells.

A closer examination of the apoptosis-specific phosphorylation sites on RB1 uncovered another provocative feature—both of these events are proximal to known sites of caspase cleavage (green lines in Figure 2A) that generate persistent fragments detectable by qP-PROTOMAP and western blotting (Figure 2A). The pSer347 event, for instance, occurs just two residues upstream (the P3 position) from the scissile aspartate (Fattman et al., 2001). The pSer882 event is located four residues upstream of a known caspase cleavage (at the P5 position) from the scissile aspartate (Fattman et al., 2001) and was identified by qP-PROTOMAP on a half-tryptic peptide ending at this residue, indicating that the phosphorylation event resides on a caspase-cleaved fragment of RB1 (in this case, the cleavage event is not expected to produce a shift in gel migration of RB1 because cleavage occurs near the C terminus of the protein). These observations led us to wonder whether such cleavage-site-proximal phosphorylation events were unique to RB1, or whether they might represent a more general phenomenon that occurs during apoptosis.

**Systems-wide Crosstalk between Proteolysis and Phosphorylation during Apoptosis**

To evaluate how phosphorylation events that occur during apoptosis might globally intersect with caspase-mediated proteolysis, we compiled all of the known sites of caspase cleavage, including 75 sites that were identified in the current study (Table S3), to give 679 total sites on 566 distinct proteins. Four hundred and thirteen of these proteins were detected in our analysis of Jurkat T cells, and we aligned their sequences such that they were all anchored around the scissile P1 aspartate residue. We then searched for phosphorylation events in our data sets that were located 200 residues up- or downstream of the P1 residues, resulting in the discovery of ~675 such phosphorylation events on 210 proteins. These phosphorylation sites were strikingly clustered in the region immediately surrounding the scissile aspartate, in particular from the P6 to P6′ residues (shaded region in Figure 3A; also see Table 1). This clustering was evident not only for apoptosis-specific phosphorylation events, but also for static and control-specific phosphorylation events (Figure S2). We furthermore found that known caspase substrates were more likely to be phosphorylated in Jurkat T cells than were uncleaved proteins (Figure 3B).

We next asked whether the phosphorylation events that occurred in apoptotic cells were catalyzed by a specific set of kinases. To discover kinases that might be activated during apoptosis, we employed a functional proteomic platform termed KiNativ. The KiNativ technology uses active site-directed chemical probes containing biotin-conjugated electrophilic analogs of ATP or ADP for covalent capture of ATP-binding proteins from proteomes. Conserved lysines in kinase active sites react with the probes and are then enriched and quantified with streptavidin chromatography and targeted MS analysis, respectively (Patricelli et al., 2011; see Extended Experimental Procedures for details). The majority of kinases showed reduced KiNativ signals in apoptotic cells (Figure 3C and Table S4), likely reflecting inhibition by STS. However, a handful of kinases showed stronger KiNativ signals in STS-treated cells, the most dramatic of which was DNA-dependent protein kinase (DNA-PK) (Figure 3C). DNA-PK is known to preferentially phosphorylate serines and threonines that are located before glutamine residues on proteins ([S/T]-Q motif; Kim et al., 1999). Consistent with the activation of DNA-PK during apoptosis, a motif-x analysis (Schwartz and Gygi, 2005) revealed that S-Q phosphorulations were the most overrepresented motifs among the apoptosis-specific phosphorylation events in our data sets (Figure 3D and Table S4). No such enrichment of S-Q motifs was observed for static or control-specific phosphorylation events (Table S4). These proteomic data were confirmed by western blotting using an antibody that recognizes p[S/T]-Q motifs, which showed a time-dependent increase in p[S/T]-Q-immunoreactive proteins in apoptotic cells compared to control cells that peaked at 2 hr post-STS treatment (Figure 3E).

The [S/T]-Q substrate motif is utilized by other kinases, most notably ATM and ATR, which, along with DNA-PK, are important regulators of genome stability and the DNA-damage response (Kim et al., 1999). No change in ATM or ATR activity was seen in our KiNativ data (Table S4), but this finding does not rule out a contribution of these kinases to phosphorylation events in apoptosis. We more directly tested for this possibility by treating Jurkat T cells with selective inhibitors of DNA-PK (NU-7441 and NU-7426), ATM (KU55733), or ATM/ATR (CGK733) for 1 hr prior to induction of apoptosis. Western blotting revealed a near-complete block of p[S/T]-Q events upon treatment with
Figure 2. Identification of a Cohort of Apoptosis-Specific Phosphorylation Events

(A) Western blot and quantitative peptograph for RB1 at 4 hr post-STS treatment. Two apoptosis-specific phosphorylation events were identified at Ser347 (1) and Ser882 (2). To the right of the peptograph, representative MS1 chromatographs for phosphorylated and unphosphorylated versions of these peptides are shown in which light (control) and heavy (apoptotic) signals are colored red and blue, respectively. Green lines mark known sites of caspase cleavage (Fattman et al., 2001) that likely generate the observed N-terminal and internal fragments visible on the peptograph and, in the case of the internal fragment, by western blotting. Asterisk denotes a nonspecific band on the blot.

(B) Shown are the 39 phosphorylation sites on RB1 listed in the PhosphoSite database, along with the number of literature references for each site. Note that the two apoptosis-specific phosphorylation events (blue) have been rarely reported in previous studies, in contrast to control-specific phosphorylation events (red), which were, in general, detected in many previous studies. Below each phosphorylation site, we show the corresponding SILAC ratios. Sites shown in black were not detected in our study.
DNA-PK inhibitors, whereas inhibitors of ATM and/or ATR were without effect (Figure 3F). We also generated two Jurkat T cell lines with stable shRNA-mediated knockdowns of DNA-PK (Figure S2) and found that these showed substantially blunted [S/T]-Q phosphorylation following induction of apoptosis (Figure 3F). Finally, we performed a qP-PROTOMAP study of apoptotic Jurkat cells pretreated with NU-7441, which resulted in a two-fold or greater reduction in the majority of p[S/T]-Q events (~60%), with other non-p[S/T]-Q events being minimally affected (Figure S2). Interestingly, of the p[S/T]-Q events reduced by NU-7441 treatment, over 80% were apoptosis specific (Figure S2 and Table S4), which, when combined with our immunoblotting results (Figure 3F), indicate that DNA-PK is responsible for a large fraction of the p[S/T]-Q events observed in apoptotic cells.

We next investigated how DNA-PK might be activated during apoptosis. We found that, early in the apoptotic cascade, DNA-PK relocated from the nucleus to the cytoplasm, where, interestingly, the enzyme was cleaved to generate a stable ~150 kDa C-terminal fragment that contains the kinase domain (Figures 3G and 3H). The appearance of a cleaved form of DNA-PK in the cytoplasm directly correlated with the increased p[S/T]-Q immunoreactive proteins (Figure 3E) and the enhanced KinTAv signals for this kinase observed 2 hr after induction of apoptosis (Figure 3H, top). Pretreatment of cells with the caspase inhibitor Z-VD-FMK blocked STS-induced cleavage of DNA-PK and p[S/T]-Q events (Figure S2). Previous studies have also reported the caspase-mediated cleavage of DNA-PK in apoptotic cells (Casciola-Rosen et al., 1995), but have mostly interpreted this proteolytic event to inactivate DNA-PK. The assays used in such studies, however, typically measured DNA-dependent DNA-PK activity with a peptide substrate (Alalunis-Turner et al., 1995; Han et al., 1996; Song et al., 1996). Our data support an alternative model wherein caspase cleavage releases DNA-PK from genomic DNA to generate an active, truncated form of the enzyme that traverses into the cytoplasm to catalyze a large number of apoptosis-specific phosphorylation events.

**Caspase Cleavage Can Expose Phosphorylation Sites**

Previous studies that have examined the functional effects of phosphorylation on caspase cleavage with individual protein substrates in vitro have mostly uncovered instances where phosphorylation blocks caspase cleavage (Duncan et al., 2010; Kurokawa and Kornbluth, 2009; Tózsér et al., 2003), leading to a model where phosphorylation serves to “protect” proteins from proteolytic processing. Many of the apoptosis-specific phosphorylation events identified in our study, however, did not appear to conform to this scenario because they were located on half-tryptic peptides ending in C-terminal aspartates, the hallmark of caspase cleavage. One such example is SF3B2, which contains an apoptosis-specific phosphorylation event at Ser861 that is located at the P2 position adjacent to a site of caspase cleavage (Figures 4A and 4B). To ascertain whether this phosphorylation event occurs before or after caspase-mediated proteolysis, we used a targeted MS approach with isotopically labeled peptides to measure the four possible forms of the SF3B2 peptide: (1) uncleaved/unphosphorylated, (2) cleaved/unphosphorylated, (3) uncleaved/phosphorylated, and (4) cleaved/phosphorylated. These experiments provided two key lines of evidence supporting that phosphorylation of Ser861 occurs after caspase-mediated proteolysis. First, the cleaved/unphosphorylated peptide appeared at an earlier time point than the cleaved/phosphorylated peptide (Figure 4C). Second, the uncleaved/phosphorylated peptide was not detected at any time point, suggesting that the full-length (parental) form of SF3B2 is not phosphorylated at Ser861. These predictions were also supported by in vitro substrate assays, where we found that the unphosphorylated, but not phosphorylated peptide served as a substrate for caspases (Figure 4D and Figure S3).

A broader search of our qP-PROTOMAP data set identified several additional apoptosis-specific phosphorylation events that were found exclusively on half-tryptic, aspartate-terminating peptides (Table 1, Figure S3 and Table S5). One candidate was a previously unreported phosphorylation event found at the P4 position (S*QTD) on an N-terminal fragment of HCLS1 (Figure 4E). Similar to what was observed for pSer861 in SF3B2, phosphorylation of Ser112 completely blocked caspase-3 cleavage of the HCLS1 peptide (Figure 4F and Figure S3). Caspase-8 cleavage was also significantly reduced by phosphorylation of Ser112, although residual hydrolytic activity was detected (Figure 4F). These data, taken together, are consistent with phosphorylation of Ser112 occurring after caspase-mediated cleavage of HCLS1.

**Phosphorylation Can Promote Caspase Cleavage**

We next wondered whether phosphorylation might also, in certain instances, directly promote (rather than block) caspase-mediated proteolysis. We accordingly searched our qP-PROTOMAP data sets for apoptosis-specific phosphorylation events that were located on the parental forms of cleaved proteins in close proximity to sites of caspase-mediated proteolysis. A compelling example was found in the protein KHSRP (Figure 5A), where we observe an apoptosis-specific phosphorylation event at Thr100 at 2 hr post-STS treatment on the parental 74 kDa form of the protein (band 6/7), and at 4 hr on a half tryptic, aspartate (Asp103)-terminating peptide in an N-terminal ~15 kDa fragment (band 21/22) (Figures 5A and 5B, respectively). pThr100 was thus located at the P3 position relative to the Asp103 caspase cleavage site. We also detected the unphosphorylated version of the half-tryptic, Asp103-terminal peptide in the KHSRP fragment.

To determine the relative kinetics of Thr100 phosphorylation versus caspase-mediated proteolysis at Asp103 in KHSRP, we performed a targeted MS analysis using isotopically labeled
peptides following the protocol outlined above for the SF3B2 protein. In band 6, where the parental form of KHSRP migrates, we detected the uncleaved/phosphorylated form of the peptide, which was strongly increased over the first 2 hr following STS treatment and then decreased thereafter (Figure 5C). In contrast, the cleaved forms of the peptide in bands 21/22 did not appear until 2.5 hr and continued to accumulate throughout the remainder of the time course (Figure 5C). These data indicate that phosphorylation at Thr100 precedes proteolysis by a substantial time window during the apoptotic cascade. We should note that the vast majority of the cleaved peptide was found in the unphosphorylated form, with only trace levels of the cleaved/phosphorylated peptide being detected throughout the time course. Nonetheless, we were intrigued by the complementary time courses for phosphorylation versus proteolysis, as well as the similar stoichiometries of the uncleaved/phosphorylated and cleaved/unphosphorylated peptides, both of which peaked at ~10% of the total quantity of uncleaved/unphosphorylated peptide (Figure S4). These data correlate well with the low overall magnitude of cleavage for KHSRP (see peptide graphs in Figures 5A and 5B) and suggest further that phosphorylation and proteolysis may have a quantitative relationship wherein phosphorylation at Thr100 promotes caspase proteolysis at Asp103. In this model, the lack of accumulation of the cleaved/phosphorylated peptide could be explained by rapid dephosphorylation of pThr100 following caspase cleavage.

We tested whether phosphorylation at Thr100 directly affects caspase cleavage at Asp103 using in vitro peptide substrate assays. Caspase-3 hydrolyzed the phosphorylated and unphosphorylated KHSRP peptides at equivalent rates (Figure 5D and Figure S4); caspase-8, however, exhibited a dramatic increase in hydrolytic activity (>20-fold) for the phosphorylated form of the peptide (Figure 5D and Figure S4). The increased hydrolytic activity of caspase-8 could be completely blocked by preincubation with the inhibitor Z-VAD-fmk (Figure S4). These findings intrigued us because it is known that caspase-8, but not caspase-3, displays a strong preference for glutamic acid, which is an approximate isostere of phosphorylated serine/threonine residues (Pearman et al., 2011), at the P3 position (Chéreau et al., 2003; Fuentes-Prior and Salvesen, 2004). These data suggested that caspase-8 may have evolved a special capacity to accommodate and even prefer phosphorylated residues at the P3 position. To further explore this concept, we modeled the interaction of phosphorylated and unphosphorylated KHSRP peptides in the active sites of caspase-3 and caspase-8 (Figure 5E and Figure S4). These models predict a clear interaction between the pThr100 of the KHSRP substrate and an arginine residue (Arg177) in caspase-8 that is not found in caspase-3 (Figure 5E and Figure S4). Arg177 has also been found to interact with the P3 glutamic acid residue of inhibitors in caspase-8 co-crystal structures (Blanchard et al., 2000; Ekici et al., 2006). Inspired by this discovery, we searched our qP-PROTOMAP data for additional examples of apoptosis-specific Ser/Thr phosphorylation events occurring at the P3 position of known caspase cleavage sites (Table 1). We have already briefly discussed another such example - the apoptosis-specific pSer347 in RB1, which is located at the P3 position adjacent to the Asp350 cleavage site. Utilizing synthetic RB1 peptides, we again found that the phosphorylated peptide served as a much better caspase-8 substrate compared to the unphosphorylated variant (Figure 5F). In this case, caspase-3 also showed improved activity for the phosphorylated peptide, but exhibited a less dramatic increase than caspase-8 (Figure 5F). Finally, we noticed that caspase-3 itself possesses an apoptosis-specific phosphorylation event, pSer26 (detected at 2 hr post-STSS treatment), that is located at the P3 position relative to the known caspase-cleavage site Asp29 between the prodomain and the large catalytic subunit (Figure 5G). Cleavage at this site is thought to occur primarily by autocatalytic processing; however, there is some evidence that caspase-8 also proteolyses this site (Rank et al., 2001). As we found for KHSRP and RB1, caspase-8 displayed markedly greater hydrolytic activity for the phosphorylated versus unphosphorylated caspase-3 peptide (Figure 5H). These results, taken together, indicate that phosphorylation can promote the caspase cleavage of proteins during apoptosis primarily through a mechanism involving the P3 position of caspase proteolytic sites, which, upon phosphorylation, dramatically increases substrate hydrolysis by caspase-8.

**DISCUSSION**

The potential for crosstalk between phosphorylation and proteolytic pathways in apoptosis and other cell biological processes has long been recognized (Kurokawa and Kornbluth, 2009;...
López-Otin and Hunter, 2010); however, investigating such network interactions at a global level has proven technically challenging due to the lack of proteomic technologies that can coordinately profile protein phosphorylation and proteolysis in cells. qP-PROTOMAP addresses this problem by quantifying phosphorylation events in proteomes and incorporating these modifications into the topographical maps of proteins such that their relationship to proteolytic processing can be directly inferred. Using this approach, we have uncovered several ways that phosphorylation and proteolytic pathways intersect in apoptotic cells. This crosstalk is evident on a global level by the enrichment of phosphorylation events on proteolyzed proteins at locations that are in close proximity to caspase cleavage sites. From a functional perspective, we show that caspase cleavage can unveil new sites for phosphorylation on proteins and, conversely, apoptosis-specific phosphorylation events at the P3 position of caspase recognition sites can promote the cleavage of proteins (Figure 6). Caspase cleavage can also activate kinases, like

Table 1. Apoptosis-Specific Phosphorylation Sites Found within Six Residues (P6–P6) of Caspase Cleavage Sites

<table>
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See Table S1 for corresponding peptographs of the proteins.
Figure 4. Caspase Cleavage Exposes New Sites for Phosphorylation

(A and B) Quantitative peptographs showing SF3B2 at 2 hr (A) and 4 hr (B) post-STS treatment. A C-terminal apoptosis-specific phosphorylation event at Ser861 occurs at the P2 position relative to the caspase cleavage site at Asp862. An additional apoptosis-specific phosphorylation event is observed at Ser289 on the parental form of SF3B2 (band 3) at 2 hr, which is 10 residues from another site of caspase cleavage (Asp299, see band 13 in B).

(C) MS-based quantitation showing that the cleaved/unphosphorylated (Ser861) SF3B2 peptide is generated prior to the cleaved/phosphorylated (pSer861) peptide during apoptosis. Quantified peptides: uncleaved/unphosphorylated - EQQAQVEKEDFSDMVAEHAAK, uncleaved/phosphorylated - EQQAQVEKEDFS* DMVAEHAAK (endogenous form not detected), cleaved/unphosphorylated - EQQAQVEKEDFSD, cleaved/phosphorylated - EQQAQVEKEDFS*D.

(E) HCLS1

(F) Absolute product mass ion intensity (arbitrary units)
DNA-PK, that contribute to the creation of a network of phosphorylation events that are specific to apoptotic cells (Figure 6). This network is enriched in phosphorylation events that lack literature precedent, further supporting their potentially special relationship to the cell death process.

Although we do not yet understand precisely how caspase cleavage promotes the phosphorylation of proteins, it is possible that the kinases responsible for these phosphorylation events cannot gain access to their substrates due to steric hindrance. Caspase cleavage at a proximal location along the protein backbone could then relieve this steric blockade to expose sites for phosphorylation (Figure 6). Alternatively, there may be kinases that selectively phosphorylate proteins near their N or C termini, although we are not aware of any specific kinases that have been reported to show this substrate preference. Finally, it is possible that cleavage promotes the redistribution of kinases like DNA-PK to distinct subcellular compartments where they phosphorylate new sets of substrates.

Phosphorylation events that promote proteolysis were found to occur at the P3 position relative to caspase cleavage sites, where they dramatically enhanced substrate hydrolysis by caspase-8. This finding is unexpected and important because phosphorylation events within caspase consensus motifs (P4–P1 residues) have, in the past, been found to hinder caspase cleavage (Kurokawa and Kornbluth, 2009). Our results are, however, consistent with previous structural work on caspses, which have shown that caspase-8, as well as caspase-9, possess a unique arginine residue not found in other caspases that enhances binding to substrates with acidic residues in the P3 position (Blanchard et al., 2000; Chéreau et al., 2003; Fuentes-Prior and Salvesen, 2004). This feature has historically been interpreted to explain the preference that caspase-8 displays for substrates with a P3 glutamic acid residue (Fuentes-Prior and Salvesen, 2004), but our data suggest another level of biological significance, namely, that caspase-8 may have evolved to recognize a set of substrates selectively in their phosphorylated state. We should mention, however, that so far, we have only assessed the impact of P3-phosphorylation on a handful of caspase substrates, and it is therefore not yet clear whether P3-phosphorylation will serve as a general or substrate-selective mechanism to enhance proteolysis by caspase-8.

The intricate, systems-level interactions between kinase and caspase networks uncovered by qP-PROTOMAP analysis of apoptotic cells sets the stage for several important lines of future research. First, we have only examined one cell line (Jurkat T cells) and its response to a single apoptotic stimulus (STS). Although the rapid and near-complete apoptotic progression observed in STS-treated Jurkat cells has made it a preferred model for cell biological and proteomic investigations of programmed cell death (Dix et al., 2008; Mahrus et al., 2008; Short et al., 2007), and a recent study has shown that different apoptotic stimuli (STS versus TRAIL) cause similar overall patterns of proteolysis in cells (Agard et al., 2012), assessing the broader significance of our findings would certainly benefit from qP-PROTOMAP studies of additional cell types and with distinct apoptotic stimuli. Second, we do not yet fully understand which kinases are responsible for the phosphorylation events observed specifically in apoptotic cells. Although our results indicate that DNA-PK makes a substantial contribution to this apoptosis-specific phosphorylation network, many of its constituent phosphorylation events do not conform to the [S/T]-Q motif preferred by DNA-PK (Figure S2), pointing to the potential activation of other kinases (or inactivation of phosphatases) during apoptosis. Our functional proteomic data suggest candidates like AKT1 and 2, MAPK14, and BRAF for future investigation (Figure 3C). Disrupting such kinases could reveal the functional contribution that they (and their cognate substrates) make to apoptosis, as has been shown previously for DNA-PK (Bharti et al., 1998; Chakravarthy et al., 1999; Chen et al., 2005a, 2005b; Wang et al., 2000). Finally, there are other potential forms of crosstalk between phosphorylation and proteolytic pathways that may have eluded detection in our study. Phosphorylation events that, for instance, block caspase cleavage would not have been easily identified because the resulting phosphoprotein would not be detected as a cleaved product. Future studies that compare the apoptotic process under different cellular conditions may reveal context-dependent changes in protein cleavage that are due to such “protective” phosphorylation events. In fact, others have speculated, for instance, that cancer cells displaying resistance to apoptosis may possess specific kinase networks that mark proteins with phosphorylation events that protect against caspase cleavage (Ahmed et al., 2002). The qP-PROTOMAP method described herein represents a versatile proteomic platform for addressing such questions through its ability to generate global, quantitative, and integrated profiles of phosphorylation and proteolytic pathways in biological systems.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Induction of Apoptosis**

Jurkat cells were grown under standard conditions and seeded to a density of 1 × 10⁶ cells/ml prior to induction of apoptosis. Staurosporine (1 μM final) was added and the cells were incubated for 2 or 4 hr at 37°C prior to lysis. See Extended Experimental Procedures for more detail.

(D) In vitro peptide substrate assays (1 μM peptide substrate) demonstrating that phosphorylation of SF3B2 at Ser818 prevents cleavage by caspsases. Peptide substrates: EQQAQVEKEFDSVMAEHAAAK and EQQAQVEKEFDS’DVMVAEHAAAK. Peptide products: EQQAQVEKEFDS and EQQAQVEKEFDS’D.

(B) Quantitative peptograph of HCLS1 showing an apoptosis-specific phosphorylation event at Ser112 occurring at the P4 position of a caspase cleavage site at Asp115.

(F) In vitro peptide substrate assays (1 μM peptide substrate) demonstrating that phosphorylation of HCLS1 at Ser112 prevents proteolysis by caspase-3 and hinders proteolysis by caspase-8. Peptide substrates: SAVGHEYVAEVEKHSSTQQD and SAVGHEYVAEVEKHS’SQTD. Peptide products: SAVGHEYVAEVEKHSSTQQD and SAVGHEYVAEVEKHS’SQTD. See also Figure S3. For (D) and (F) error bars represent the SEM.
Figure 5. Phosphorylation at the P3 Position of Caspase Cleavage Sites Promotes Caspase-8-Mediated Proteolysis

(A and B) Quantitative peptographs showing an apoptosis-specific phosphorylation event at Thr100 on the parental form of KHSRP (A, band 6/7) at 2 hr, and on a half-tryptic, aspartate (Asp103)-terminating peptide of a stable fragment of this protein at 4 hr (B, band 21). Note that this half-tryptic peptide is shown in gray because it lacks an isotopically labeled amino acid.
Sample Preparation, SDS-PAGE, and Mass Spectrometry

400 µg of cytosolic protein (200 µg + 200 µg of light and heavy protein) was separated via a 10% SDS-PAGE gel and cut into 22 0.5 cm bands. Peptides were extracted via in-gel trypsin digestion and subjected to immobilized metal affinity chromatography (IMAC). IMAC eluate (enriched in phosphopeptides) or flow-through (for unphosphorylated peptides) was loaded onto MS-based quantitation showing a rapid increase in the uncleaved/phosphorylated (pThr100) KHSRP peptide (yellow line) from 0 to 2 hr post-STS treatment, which is ~1 hr prior to the appearance of the cleaved forms of this peptide. Note that the uncleaved/unphosphorylated KHSRP peptide was found at ten times higher levels and was therefore not shown in the figure for the sake of clarity (see Figure S4 for these data). Quantified peptides: uncleaved/unphosphorylated - IGGDAATTVNNSTPDFGFGGQK, uncleaved/phosphorylated - IGGDAATTVNNST*PDFGFGGQK, cleaved/unphosphorylated - IGGDAATTVNNSTPD, cleaved/phosphorylated - IGGDAATTVNNST*PD.

Caspase Activity Assays with Synthetic Peptide Substrates

Recombinant human caspases were diluted into buffer containing substrate peptide and an internal standard, and incubated for 1–2 hr at 37°C. Assays used for assessments of caspase-substrates. See Extended Experimental Procedures for more detail.

Data Analysis

MS2 data were searched using the ProLuCID algorithm (Xu et al., 2006) with a reverse-concatenated, nonredundant variant of the human IPI database. Peptides from each gel-band were grouped and filtered using DTASelect (Tabb et al., 2002), SILAC ratios were obtained with Cimage (Weerapana et al., 2010), and these data were assembled into quantitative peptographs using custom software. The “phosphorylation site data set” released on November 3, 2011 from the PhosphoSite database (Hornbeck et al., 2011) was used for assessments of phosphosites. The CASBAH database (Lüthi and Martin, 2007) was downloaded on December 19, 2011 and used for assessments of caspase-substrates. See Extended Experimental Procedures for more detail.

Several distinct forms of crosstalk between caspase and kinase pathways were uncovered by qP-PROTOMAP: (1) kinases (such as DNA-PK) can be cleaved and activated by caspases; (2) caspase cleavage can expose previously occluded residues that are then phosphorylated by kinases; (3) phosphorylation at the P3 position relative to scissile aspartates promotes proteolysis of proteins by caspase-8. This type of proteolysis-promoting P3 phosphorylation was also found on caspase-3 itself (4).

(C) MS-based quantitation showing a rapid increase in the uncleaved/phosphorylated (pThr100) KHSRP peptide (yellow line) from 0 to 2 hr post-STS treatment, which is ~1 hr prior to the appearance of the cleaved forms of this peptide. Note that the uncleaved/unphosphorylated KHSRP peptide was found at ten times higher levels and was therefore not shown in the figure for the sake of clarity (see Figure S4 for these data). Quantified peptides: uncleaved/unphosphorylated - IGGDAATTNNSTPDFGFGGQK, uncleaved/phosphorylated - IGGDAATTNNSTPDFGFGGQK, cleaved/unphosphorylated - IGGDAATTNNSTPD, cleaved/phosphorylated - IGGDAATTNNSTPD.

(D) In vitro peptide substrate assays (1 µM peptide substrate) demonstrating that phosphorylation at Thr100 of KHSRP enhances cleavage by caspase-8. Peptide substrates: IGGDAATTNNSTPDFGFGGQK and IGGDAATTNNSTPDFGFGGQK. Peptide products: IGGDAATTNNSTPD and IGGDAATTNNSTPD.

(E) Structure of caspase-8 (PDB: 1QTN) with the tetrapeptide ST*PD modeled into the active site. See the Extended Experimental Procedures and Figure S4 for additional details.

(F) In vitro peptide substrate assays (1 µM peptide substrate) demonstrating that phosphorylation at Ser882 of RB1 promotes cleavage by caspase-8 and, to a lesser extent, by caspase-3. Peptide substrates: IIHGSESMDGSLDNSYK and IIHGSESMDGSLDNSYK. Peptide products: IIHGSESMD and IIHGSESMD.

See also Figure S4. For (D), (F), and (H), error bars represent the SEM.
were quenched by acidification and subjected to ZipTip purification before MS analysis. Absolute quantities of product were calculated by comparison to synthetic standards. Assays were performed under nonsaturating substrate concentrations and resulted in less than 20% turnover of the substrate. See Extended Experimental Procedures for more detail.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.05.040.

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REFERENCES


Cell Culture and Induction of Apoptosis
Jurkat cells were grown at 37°C under 5% CO₂ in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. For metabolic labeling (SILAC), cells were maintained in RPMI media containing 2 mM glutamine and light or heavy arginine and lysine (Sigma) were supplemented at a concentration of 100 μg/ml. Cells were passaged six times in heavy media before testing for full incorporation of the heavy amino acids. Prior to induction of apoptosis, Jurkat cells were seeded to a density of 1 × 10⁶ cells/ml in RPMI 1640 media containing 10% FCS and 2 mM glutamine. DMSO or staurosporine (1 μM final concentration, Sigma) was added and the cells were incubated for 1, 2, or 4 hr at 37°C under 5% CO₂. For kinase inhibition experiments, 1 × 10⁶ cells/ml were pretreated with inhibitor for 1 hr prior to addition of staurosporine. Kinase inhibitors [CGK 733 (10 μM final), KU55933 (10 μM final), NU-7441 (1 μM final), and NU-7026 (10 μM final)] were purchased from Tocris, with the exception of CGK 733, which was purchased from Calbiochem. For caspase inhibition experiments, 1 × 10⁶ cells were pre-treated with 60 μM z-VAD-FMK (Roche) or DMSO for 1 hr prior to STS addition.

Preparation of Cell Lysates
Soluble and particulate fractions were prepared as previously described (Jessani et al., 2002). Briefly, cells were washed 3 times in cold PBS and resuspended in 200 μl of PBS containing protease inhibitors (complete EDTA-free protease inhibitor cocktail, Roche), phosphatase inhibitors (PHOSTOP, Roche), and z-VAD-FMK (Roche). Cells were then sonicated to lyse and centrifuged at 100,000 x g for 45 min. The supernatant was collected as the soluble fraction. For experiments containing cytosolic and nuclear fractions, samples were prepared according to manufacturers instructions (NE-PER Nuclear and Cytosolic Extraction Kit, Pierce).

Sample Preparation, SDS-PAGE, and IMAC Enrichment
For phosphopeptide enrichment, 200 μg of each control (light) and apoptotic (heavy) soluble fraction were combined and separated via a 10% SDS-PAGE gel for 850 V hr. The gel was washed in water and manually excised into twenty-two 0.5 cm bands. Bands corresponding to the migration of molecular-weight markers were noted and this information was used to estimate the molecular weights of proteins migrating in each band. Bands were subjected to in-gel trypsin digestion as previously described (Rosenfeld et al., 1992) with minor modifications. Briefly, bands were washed in 100 mM ammonium bicarbonate and proteins were reduced in 10 mM tris(2-carboxyethyl)phosphine (TCEP) at 37°C for 0.5 hr and then alkylation with 55 mM iodoacetamide in the dark for 0.5 hr. The bands were then dehydrated by washing in 50:50 acetonitrile:100 mM ammonium bicarbonate, followed by 100% acetonitrile. Gel bands were then dried and resuspended in 40 μl of trypsin at 10 ng/μl. Upon re-suspending of the gel bands, 25 mM ammonium bicarbonate was added to a final volume of 200 μl and the gel bands were placed at 37°C overnight. Supernatants containing peptides were removed, and the gel bands were further extracted with 5% formic acid and acetonitrile and dried down via speed vac. For phosphopeptide enrichment, the dried peptides were resuspended in 350 μl of IMAC binding buffer (250 mM acetic acid, 30% acetonitrile). 30 μl of equilibrated IMAC slurry (PHOS-Select, Sigma) was added to each band. Samples were then placed on a rotator at room temperature for 1.5 hr. The peptide/slurry mixture was then washed twice with 800 μl binding buffer followed by one wash with 300 μl water. Peptide elution was accomplished with 300 μl of 400 mM NH₄OH and then dried in a speed vac and stored at −80°C prior to use. The 4 hr data set was derived from five separate biological replicates and consisted of three IMAC flow-through (nonphosphopeptide) samples and six IMAC eluate (enriched for phosphopeptides) samples. The 2 hr data set was derived from two separate biological replicates and consisted of one IMAC flow-through sample and two IMAC eluate samples.

Mass Spectrometric Analysis
Phosphopeptides and unenriched peptides were analyzed separately via LC-MS/MS in the same way: peptides from each band were resuspended in 10 μl buffer A (95% H₂O, 5% acetonitrile, 0.1% formic acid) and loaded via autosampler onto a 100 μm (inner diameter) fused silica capillary column with a 5 μm tip that was packed with 10 cm of C18 resin (aqua 5μm, Phenomenex). LC-MS/MS analysis was performed on an LTQ-Velos Orbitrap mass spectrometer (ThermoFisher) coupled to an Agilent 1200 series HPLC. Peptides were eluted from the column using a 2 hr gradient of 5%-100% buffer B (5% H₂O, 95% acetonitrile, 0.1% formic acid). The flow rate through the column was 0.25 μl/min and the spray voltage was 1.7 kV. The mass spectrometer was operated in data-dependant scanning mode, with one full MS scan (400-1,800 m/z) occurring in the Orbitrap (60,000 resolution) followed by ten MS² scans of the nth most abundant ions with dynamic exclusion enabled (20 s duration).

Data Processing, Analysis, and Deposition
Raw mass spectrometry data were stored as RAW files generated by XCalibur version 2.1.0.1139 running on a Thermo Scientific LTQ-Velos Orbitrap mass spectrometer. RAW files were converted to MS2 format (McDonald et al., 2004) using RAW-Xtract version 1.8 and these MS/MS data were searched using ProLuCID (Xu et al., 2006). ProLuCID searches were performed using a reverse-concatenated nonredundant variant of the human IPI database version 3.33. Cysteine residues were required to be carboxymethylated (+57.02146 Da) and up to three differential phosphorylation marks (+79.9663 Da) were permitted on serine, threonine, or tyrosine residues in each peptide. Peptides were required to have at least one tryptic terminus. ProLuCID data from each gel band were quality-filtered and sorted with DTASelect version 2.0.25, which performs linear discriminant analyses within each charge- and
modification-state to achieve a peptide-false-positive rate below 1% (Tabb et al., 2002). Actual peptide false-positive rates (as defined in Elias and Gygi, 2007) were below 1% at this stage (0.9% and 0.7% in the 2- and 4 hr data sets, respectively). RAW data were also converted to mzXML format (Pedrioli et al., 2004) using ReAdW version 4.3.1 and SILAC quantitation was performed using an in-house software package called Cimage (described in Weerapana et al., 2010). Cimage was run using a 10 ppm mass-window and requiring an R² correlation value for light/heavy co-elution of 0.8 or greater. Peptides that were found exclusively in healthy or apoptotic cells (only light or heavy peak observed) were considered candidate singlet peptides and were subject to additional filters to remove singlet peaks with insufficient abundance or excessive background noise. At this stage the peptide false positive rates were 0.6% and 0.3% in the 2- and 4 hr data sets, respectively, indicating that incorporation of MS²-based SILAC information improves accuracy of peptide identifications. Peptides were then assembled into quantitative peptographs using in-house software. This process involved additional unbiased peptograph-level noise-filtering steps such as requirement that at least two distinct peptides be observed in a given band across all replicates. No reverse (decoy) peptides remained in either data set following application of these filters. All peptographs detected in both data sets can be viewed at: http://www.scripps.edu/chemphys/cravatt/protomap/. SILAC ratios and chromatographs from every peptide can also be found at this website. Additionally, a complete description including quality scores for peptide spectral matches (XCcorr and deltaCN values, Eng et al., 1994) of the 17,396 phosphopeptides detected in this study can be found in Table S2. Finally, all raw data generated in this study was deposited in the Proteome Commons repository (Smith et al., 2011), and can be accessed at https://proteomecommons.org using the following hashes: BFKUGbPTPJBlQIchJClUjSsF7nQVaMfs2beMkuBiKTs4J6SworZyNnU92D9R1JZHHHHoqg024CIAtAyI+ptQpieu/HkAAAAAAAO9A== and Bz6bOoFIAIロー7acYv8jXf6B6u2rvxcpUn8qdkxVYVkiCi3TNsGovshkSD0MxBH42G54G4g5j6/gQsXzp/Kvoq9f3UAAAAAACa1q== for the 2- and 4 hr data sets, respectively. Assessments of phosphosites were derived from the “phosphorylation site data set” released on November 3, 2011, from the PhosphoSitePlus database (Hornbeck et al., 2011), except for Figure 2C and Table S5, which were updated with the most-recent data available on the PhosphoSite website on April 18, 2012. The CASBAH database, which catalogs the known substrates of apoptotic proteolysis (Lüthi and Martin, 2007) was downloaded on December 19, 2011, and used for assessments of caspase-substrates.

Identification of Cleaved Proteins

Cleaved proteins were identified on the basis of the distribution of peptide-ratios in each band. Only proteins and fragments of sufficient abundance were considered: two spectral counts from at least two distinct peptide sequences were required in a given band and eight spectral counts from at least four distinct peptides were required for each protein. The distribution of peptide ratios in each band were organized into quartiles and, if the ratios in the upper three quartiles were more than 3-fold elevated in the control-cells, the band was flagged as control specific, indicative of a parental degradation event. If the ratios in the lower three quartiles were more than 3-fold elevated in the apoptotic cells, then the band was flagged as apoptosis specific, indicative of a cleaved fragment. Proteins that did not display apoptosis-specific fragments were further categorized as partially cleaved (where some bands were control specific and others were not) versus completely cleaved (where all bands showed control-specific ratios). Entries were flagged as candidate cleaved proteins if: (1) they displayed apoptosis-specific fragments at either time point, or (2) they showed complete parental cleavage at the 4 hr time point. Of the 2,867 proteins that met the abundance thresholds, 837 proteins were flagged as potential substrates of apoptotic proteolysis using this algorithm. This list was then manually pruned to remove ambiguous or mis-categorized protein entries resulting in a final high-confidence list of 744 proteins that are cleaved or degraded during apoptosis (Table S1).

Classification of Phosphorylation Sites and Motif-x Analysis

SILAC ratios for all peptides containing a given phosphorylated residue were extracted from the 2- and 4 hr data sets. Those sites with peptides displaying SILAC ratios that were all at least 2-fold enriched in apoptotic cells were deemed apoptosis specific. The remaining phosphosites had SILAC ratios that were either unchanged (“static,” either displaying less than 2-fold change in either direction or displaying both control-specific and apoptosis-specific ratios indicative of a static phosphorylation event on a cleaved protein) or control specific (at least 2-fold enriched in control-cells). For the DNA-PK inhibitor experiments, phosphorylation sites were categorized using the same algorithm described above, except that sites displaying two-fold or greater reduction upon treatment with NU-7441 were designated “suppressed by NU-7441” and all other sites were classified as “insensitive” (Table S4). For motif analysis, sequences surrounding each phosphosite (+/- 9 residues, referred to as “sequons”) were extracted from our 2- and 4 hr data sets and analyzed with the motif-x algorithm (Schwartz and Gygi, 2005) using either serine or threonine as the central residue. Apoptosis-specific sequons were submitted in one batch and control-specific and static sequons were submitted separately (Figure 3D and Table S4). The human proteome (ipi.HUMAN.fasta) was used as a background database and the significance and number-of-occurrences and significance thresholds were set at 20 and 0.000001, respectively.

Western Blotting

Either the soluble or the cytoplasmic and nuclear fractions of both control and staurosporine-treated Jurkat cells were analyzed via western blotting using standard methods. Blots were probed with antibodies for caspase-3, PARP1, DNA-PK, pS/T-Q, actin, lamin, and RB1 (Cell Signaling Technology numbers 9662, 9542, 4602, 2851, 4970, 2032, and 9313, respectively). The GRAP2 antibody was from R&D Systems (AF4640).
**Lentiviral Knockdown**

DNA-PK shRNA pLKO.1 lentiviral constructs were purchased from Open Biosystems. Short hairpin-plasmid DNA, along with envelope protein (psPAX2) and coat protein (CMV-VSVG) vectors were co-transfected into HEK293T cells. The virus-containing media was collected and filtered. Polybrene was added to the filtered media to a final concentration of 10 μg/ml. Varying amounts of virus-containing media were then used to infect Jurkat cells. Two days postinfection, Jurkat cells were resuspended in selection media containing 1 μg/ml puromycin. 7 days postselection, cells were collected and nuclear fractions were prepared. DNA-PK knockdown efficiency was measured by western blot (Figure S1). Sequences for the clones that gave the best knockdowns were CCAGTGAAGTCTGAATCATT and GCAGCTTTATTACAAAGACAT.

**Peptide Quantification by LC-MS**

1x10^6 cells/ml of Jurkat cells were treated with 1 μM staurosporine and collected every 30 min for 4 hr. The cells were then washed three times with PBS and resuspended in PBS containing protease inhibitors, phosphatase inhibitors, and z-VAD-FMK. The soluble fraction was isolated via high-speed centrifugation (see preparation of cell lysates) and then 200 μg was subjected to SDS-PAGE. Gel bands corresponding to relevant molecular weights were excised and digested in-gel with trypsin (as described above). Isotopically-labeled peptides (incorporating heavy lysine and arginine residues where present) were then added to tryptic digests prior to analysis via LC-MS/MS. When quantification of phosphopeptides was needed, an IMAC enrichment step was performed prior to LC-MS/MS analysis. LC-MS/MS analyses utilized targeted fragmentation by targeting the mass-to-charge ratios of relevant peptides for MS² fragmentation. Peptides masses were then extracted, and a diagnostic MS² ion was selected for quantitation via “pseudo-MRM.” This quantification method is referred to as pseudo-MRM because, unlike true MRM (multiple reaction monitoring, typically performed on a triple-quadrupole mass spectrometer) all of the fragment ion masses are measured in the ion-trap, rather than isolating a single daughter ion for quantification. Quantitation is then performed at the software level, after-the-fact, by measuring peaks comprised of a “transition” from parent ion to one of several diagnostic daughter ions (a similar approach is described in detail in Scheri et al., 2008).

**KiNativ Profiling of Active Kinases**

Jurkat cells were plated at 1 x 10⁶ cells/ml and treated with 1 μM STS for 1, 2, or 4 hr. Cells were then washed, pelleted, lysed in cell lysis buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% CHAPS, 1% Tergitol NP-40 type, 1% v/v phosphatase inhibitor cocktail II [EMD/Calbiochem, #524625]), and sonicated. Lysates were filtered and probe reactions were performed at room temperature with a final probe concentration of 5 μM. Samples were labeled with both Biotin-Hex-Acyl-ATP and Biotin-Hex-Acyl-ADP probes, as previously described (Patricelli et al., 2007). All reactions were performed in duplicate. Labeled lysates were denatured and reduced, alkylated, and digested with trypsin as described above. Desthiobiotinylated peptides were captured using high-capacity streptavidin resin (Thermo Scientific), and analyzed by mass spectrometry as previously described. The full results of these KiNativ profiling experiments can be found in Table S4.

**Substrate Assays**

Synthetic peptides were purchased from Thermo (HeavyPeptide AQUA standards) and diluted in assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT to a final reaction volume of 10 μl. Recombinant human caspase-8 or caspase-3 (carrier free, R&D Systems) was diluted to 100 nM into assay buffer containing substrate peptide as well as an internal standard peptide that does not serve as a caspase substrate. Samples were incubated for varying lengths of time (120 min for KHSRP substrate peptides and 60 min for SF3B2, Caspase 3, RB1, and HCLS1) at 37°C. Assays were then quenched by acidification with formic acid (0.5% final) and then subjected to ZipTip purification (Millipore) before mass spectrometric analysis on an LTQ-Velos Orbitrap mass spectrometer using a gradient that consisted of a 30 min loading phase followed by a 30 min gradient from 5% to 100% B (95% acetonitrile, 5% water, 0.1% formic acid). Ionization efficiencies of the product peptides relative to the internal standard were calculated using synthetic standards. These values were then used to calculate absolute turnover of the substrate. Assays were performed under nonsaturating substrate concentrations for a period of time that resulted in less than 20% turnover of the substrate (typically 1 μM substrate concentration for 60-120 min). Peptide substrates used for the assays are listed here, with the phosphorylated residue shown in bold. Samples were incubated with the phosphorylated residue shown in bold. SF3B2: substrate - EQAQVEKEDFSMDVAEHAAK, product - EQAQVEKEDFS. HCLS1: substrate - SAVGHEYAEEVKHSSQTDAAK, product - SAVGHEYAEEVKHSSQTD. RB1: substrate - TLQTDIDSFETGR, product - TLQTDIDS. KHSRP: substrate - IGGDAATVNNSTPFDGFQGGQK, product - IGGDAATVNNSTPD. Caspase-3: substrate - IILGSEMSMDGSLDNSYK, product – IILGSEMS. These peptide substrates were the only ones tested for the studies presented in this manuscript (i.e., no additional peptide substrate data were omitted from inclusion in the manuscript).

**Structural Modeling**

Models of caspase-substrate peptide interaction were generated using the flexible peptide docking program (Raveh et al., 2010) released in the Rosetta 3.0 software suite (Leaver-Fay et al., 2011). Crystal structures of caspase-8 (PDB: 1QTN) and caspase-3 (PDB: 1PAU) bound with tetrapeptide analogs were used as the starting template for modeling. First, the unphosphorylated substrate peptide sequences (STPD for KHSRP or ESMD for caspase-3) was grafted onto the peptide backbone in the crystal structure at the catalytic cysteine. Second, the rigid-body orientation between the catalytic cysteine in the caspase and the P1 aspartic acid in the...
peptide substrate was fixed to mimic the acyl-enzyme intermediate, as observed in the crystal structures. The rest of enzyme-peptide interaction across the binding interface was locally refined using Monte-Carlo energy minimization (MCM) by varying torsion angles in all protein sidechains as well as the peptide backbone and sidechains. Out of the 500 structural models generated, the one ranked by the best total energy was selected as the final model. Based on the models with unphosphorylated peptides, the phosphorylated peptides (STPD or ESMD) were generated by replacing the P3 serine or threonine residue with a phosphorylated variant that has an additional sidechain torsion angle allowing the position of the phosphate group to be energetically optimized. The same MCM-based flexible peptide docking method was applied to refine the local interaction across the caspase-peptide interface and after 50 structures were generated, the best-ranked model by total energy was subjected to a further round of gradient-based energy minimization to optimize the electrostatic interaction between the phosphorylated residue and its neighboring residues.

SUPPLEMENTAL REFERENCES


Figure S1. Exemplary Quantitative Peptograph, Related to Figure 1

(A) Western blot and peptograph for a representative cleaved and phosphorylated protein GRAP2, where apoptosis-specific (blue) and control-specific (red) phosphorylated peptides are marked by numbers 1 and 2, respectively. Control-specific (red) and apoptosis-specific (blue) unphosphorylated peptides are also marked on the parental (3) and fragment (4) bands of GRAP2, respectively. Note that the western blot confirmed reductions in the parental form of GRAP2 and the appearance of at least one of persistent fragment in apoptotic cells. An additional persistent fragment was detected by qP-PROTOMAP that was not observed by western blotting. As we have noted previously (Dix et al., 2008), PROTOMAP methods can detect fragments of proteins that are difficult to visualize by western blotting methods, which are reliant on antibody reagents that may recognize only a small number of epitopes on proteins.

(B) Representative MS1 chromatographs for peptides 1–4. Asterisks in the MS1 traces designate the times at which MS2 spectra were acquired. Asterisks in the listed peptide sequences designate phosphorylated residues.

(C) Description of the box-and-whisker plots used in the central panels of quantitative peptographs.
Figure S2. Crosstalk between Phosphorylation and Proteolysis during Apoptosis, Related to Figure 3

(A) Detection of new sites of phosphorylation occurred at a diminishing rate as additional data were acquired. The orange line represents the number of unique sites of phosphorylation identified for a particular number of phosphopeptides (green line).

(B) The diminishing rate of new phosphopeptide discovery with increasing replicate experiments was observed for both static/control-specific (red line) and apoptosis-specific (blue line) phosphorylation events.

(C) Apoptosis-specific (cyan) and static/control-specific (orange) phosphorylation events are both enriched in regions surrounding the scissile aspartate residues of known sites of caspase cleavage.

(D) Lentiviral knockdown of DNA-PK. See the Extended Experimental Procedures for details.

(E) Left: The increase in p\[S/T\]-Q phosphorylation observed upon treatment of Jurkat cells with STS is blocked by pre-treatment with the caspase inhibitor Z-VAD-fmk treatment also blocked DNA-PK cleavage (right).

(F) Treatment of Jurkat cells with the DNA-PK inhibitor NU-7441 prior to the induction of apoptosis resulted in a two-fold or greater reduction in the majority of p\[S/T\]-Q phosphorylation events. In contrast, all other non-p\[S/T\]-Q events were largely unaffected by NU-7441 treatment. The vast majority of NU-7441-sensitive p\[S/T\]-Q sites (>80%) were apoptosis specific (pie-chart).

(G) Representative examples of apoptosis-specific (i, iii, iv) and static (ii) phosphorylation events that are (i, iii) or are not (ii, iv) blocked by NU-7441-treatment. Note that examples i and ii represent S-Q and non-S-Q phosphorylation sites on the same protein, respectively, demonstrating the specificity of DNA-PK and the selectivity of the NU-7441 inhibitor. The chromatographs on the left represent the 2 hr apoptotic data set, with control-cells in red and apoptotic (STS) cells in blue. In the chromatographs on the right side, the blue trace again represents 2 hr apoptotic data, and the green line represents cells treated with both STS and NU-7441 for 2 hr.
Figure S3. In Vitro Substrate Assays Showing Linearity of Product Formation over the Tested Range of Substrate Concentrations, Related to Figure 4
(A) SF3B2 peptide substrates: EQQAQVEKDSDMVAEHAAK and EQQAQVEKDSDMVAEHAAK. SF3B2 product peptides: EQQAQVEKDSD and EQQAQVEKDSD. Note that the phosphorylated peptide substrate is not turned over at any concentration.
(B) HCLS1 peptide substrates: SAVGHEYVAEVEKHSSQTDAAK and SAVGHEYVAEVEKHSSQTDAAK. HCLS1 product peptides: SAVGHEYVAEVEKHSSQTD and SAVGHEYVAEVEKHSSQTD. Note that the phosphorylated peptide is not turned over at any concentration by caspase-3, although caspase-8 displays moderate activity with this substrate (see Figure 4).
(C) Phosphorylation events occurring within six amino acids of scissile aspartate residues in apoptotic proteomes are overrepresented in previously unreported phosphorylation sites.
See also Table S5.
Figure S4. Phosphorylation at the P3 Position Relative to the Scissile Aspartate Enhances Substrate Hydrolysis by Caspase-8, Related to Figure 5
(A and B) Phosphorylated and unphosphorylated tetrapeptide substrates representing the caspase-3 sequence containing pSer26 (ES*MD, (A) or the KHSRP sequence containing pThr100 (ST*PD, (B) were modeled into the active sites of caspase-8 or caspase-3 (PDB: 1QTN and 1PAU, respectively, see Extended Experimental Procedures for details). Hydrogen bonding interactions with the P3 residues are shown as dashed yellow lines. The lower panels in (A) show schematic representations of the interactions with the phosphorylated substrates. Hydrogen bonding interactions (<4Å) are shown as dashed lines. Notably, Arg177 in caspase-8 interacts with the phosphorylated, but not unphosphorylated substrates, and caspase-3 does not contain a homologous cationic residue. Note that the left panels of (B) are identical to the ones shown in Figure 5E, and are reproduced here for clarity.
(C–F) Representative in vitro substrate assays with a KHSRP peptide containing the Thr100 residue showing linear response of product formation by caspase-3 and caspase-8 over the tested range of substrate concentrations. Caspase-3 and caspase-8 proteolytic activity were completely blocked by pre-incubation with the caspase inhibitor z-VAD-FMK (D) and (F), 1 μM peptide substrate). Peptide substrates: IGGDAATTVNNSTPDFGFGGQK and IGGDAATTVNNST*PDFGFGGQK. Product peptides: IGGHAATTVNNSTPD and IGGHAATTVNNST*PD. Similar assays were conducted for RB1 and caspase-3 substrate peptides with both enzymes, but are not shown due to space constraints. (G) Quantitation of endogenous KHSRP peptides shows that the absolute amounts of uncleaved/uncleaved phosphorylated and cleaved/unphosphorylated peptide were similar at their respective peak accumulation values (2 and 4 hr time points, respectively). Peptide sequences are described in Figure 5.
Data in (D) and (F) are presented as means ± SEM. **p < 0.01, ***p < 0.001.