Resource

Cell

Activation of Specific Apoptotic Caspases with an Engineered Small-Molecule-Activated Protease

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SUMMARY

Apoptosis is a conserved cellular pathway that results in the activation of *c*ysteine-*asp*artyl proteases, or caspases. To dissect the nonredundant roles of the executioner caspase-3, -6, and -7 in orchestrating apoptosis, we have developed an orthogonal protease to selectively activate each isoform in human cells. Our approach uses a split-tobacco etch virus (TEV) protease under small-molecule control, which we call the SNIPer, with caspase alleles containing genetically encoded TEV cleavage sites. These studies reveal that all three caspases are transiently activated but only activation of caspase-3 or -7 is sufficient to induce apoptosis. Proteomic analysis shown here and from others reveals that 20 of the 33 subunits of the 26S proteasome can be cut by caspases, and we demonstrate synergy between proteasome inhibition and dose-dependent caspase activation. We propose a model of proteolytic reciprocal negative regulation with mechanistic implications for the combined clinical use of proteasome inhibitors and proapoptotic drugs.

INTRODUCTION

Apoptosis, or programmed cell death, is a ubiquitous and conserved cellular pathway required for development, immune cell maturation, and to prevent oncogenesis (Taylor et al., 2008). Apoptosis is triggered by signaling events involving a diverse array of protein networks, organelles, and macromolecular complexes that converge upon the activation of caspases (Fuentes-Prior and Salvesen, 2004). The executioner caspases are the final proteases to be activated in apoptosis. This leads to cleavage of upwards of 1000 proteins (Dix et al., 2008; Mahrus et al., 2008) to produce the characteristic apoptotic phenotypes of membrane blebbing, nuclear condensation, DNA fragmentation, and ultimately phagocytosis by immune cells. Executioner caspases have been recently linked to nonapoptotic phenotypes such as axonal pruning, stem cell differentiation, and red blood cell enucleation (Yi and Yuan, 2009). The cellular processes that stop caspases short of inducing apoptosis in these diverse biological settings remain a mystery.

There are 12 caspase isoforms in humans, but their precise and nonredundant roles for mediating apoptosis, nonapoptotic phenotypes, and innate immunity are only beginning to be fully elucidated. Extensive biochemical and structural analyses indicate that the inflammatory and initiator caspases are recruited to larger complexes that induce caspase dimerization, autoproteolysis, and protease activation (Shi, 2004). Upon activation, the apoptotic initiator caspase-8 and -9 cleave the executioner procaspase-3, -6, and -7. The executioner caspases are translated as inactive dimers; there are at least two sites of processing in the executioner caspases; and the specific role of each proteolytic event in regulating executioner caspase activity is still unclear, both in vitro and in cells, as is the role of the prodomain in executioner caspase function.

Gene knockout studies in murine embryonic fibroblasts demonstrate that cells deficient in both caspase-3 and -7 are more resistant to a variety of apoptotic stimuli as compared to single knockouts, suggesting functional redundancy in these two executioner isoforms (Lakhani et al., 2006). Furthermore, single-cell studies reveal that the induction of executioner caspase activity is highly stochastic (Albeck et al., 2008b; Rehm et al., 2002). To further probe the function of each caspase with sharp temporal resolution, we needed to activate an individual isoform selectively and synchronously. Recent progress was reported toward building small-molecule activators of the procaspases (Wolan et al., 2009), but these compounds are not yet selective enough to trigger each one of the caspases individually.

To address the goal of activating a single executioner caspase isoform in a site-selective manner, we describe the optimization of a previously split NIa *t*obacco *e*tch *v*irus (TEV) protease (Wehr et al., 2006). We demonstrate that an engineered variant of split-TEV can be robustly activated in cells to specifically cleave each of the executioner caspase isoforms. These studies reveal that activation of caspase-3 or -7, but not caspase-6, is sufficient to induce apoptosis. Remarkably, the activation is transient for

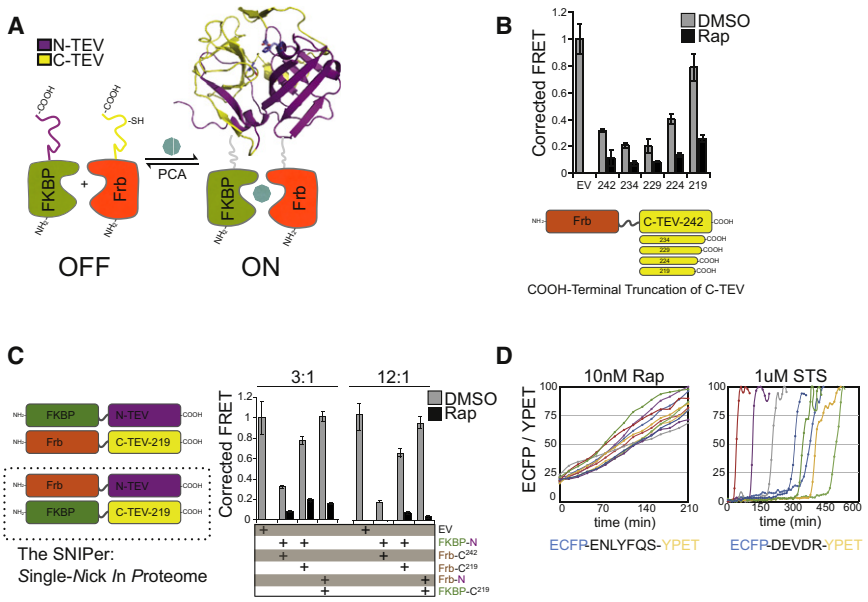

Figure 1. Engineering the SNIPer for Conditional Proteolysis

(A) Generalized scheme for a ligand-inducible orthogonal protease based on the protein complementation system.

(B) Plasmids with deletions at the C terminus of Frb-C-TEV or empty vector (EV) were cotransfected with FKBP-N-TEV and ECFP-TevS-YPET in 293T cells. Ten nanomolar Rap was added for 12 hr and FRET measurements were recorded using a fluorescent microplate reader. Data are presented as corrected averages of FRET/ECFP from an experiment performed in quadruplicate (error bars ± standard deviation [SD]).

(C) N-TEV and C-TEV-219 were fused to either FKBP or Frb and assayed by FRET in quadruplicate as above. Transfections were performed with 3× or 12× molar excess of Tev constructs to reporter plasmid (error bars ± SD).

(D) The kinetics and cell-to-cell heterogeneity of SNIPer-mediated protease activity were compared to endogenous caspase activation by live-cell fluorescent imaging. HEK293 cells were transfected with the SNIPer and ECFP-TevS-YPET or ECFP-DEVDR-YPET, a reporter for caspase-3/-7 activity in cells, and treated with 10 nM Rap or 1 μM staurosporine. Live-cell FRET measurements were recorded every 15 min.

See also Figure S1.

each isoform. These data support a model of antagonistic proteolysis between the executioner caspases and the proteasome.

RESULTS

The SNIPer: Design, Optimization, and Characterization in Mammalian Cells

Our initial split-TEV construct was based on a previous design developed for protein-complementation assays (Wehr et al., 2006) (Figure 1A). The full-length TEV variant S219V was split into two component fragments: N-TEV (residues 1–118) and C-TEV (residues 119–242). This design inactivates TEV protease by separating the catalytic triad residues Asp44 and His81 from the Cys151 nucleophile. N-TEV was fused to the C terminus of FKBP with a 10 amino acid $(GGGGS)_{2x}$ linker between the two domains, and C-TEV was fused to the C terminus of Frb (the rapamycin-binding domain of mTOR kinase). Therefore, protease activity is conditionally restored by the addition of rapamycin, which promotes split-TEV fragment association and ligand-dependent protein folding.

We assayed for reconstituted protease activity in 293T cells by cotransfecting both N- and C-TEV halves and a FRET-based ECFP-YPET reporter that is cleaved by TEV. In the presence of rapamycin (Rap), the split-TEV construct extending to residue 242 cleaved the protease reporter as compared to an empty vector control (Figure 1B). However, significant processing also occurred in the absence of Rap, suggesting that the two halves can associate and reconstitute activity in the absence of a small-molecule dimerizer. Another group recently implemented the published N-TEV and C-TEV design in mammalian cells and similarly observed leaky TEV protease activity in the absence of Rap (Williams et al., 2009). They reduced Rap-independent activation by lowering the amount of split-TEV DNA that was transiently transfected or by targeting one fragment to the cell membrane via a genetically encoded myristoylation tag. However, our goal was to engineer cell death pathways using stable cell lines, which requires a split-TEV design with no background activity. We hypothesized that the C terminus of the TEV was causing spontaneous refolding of the fragments in the absence of Rap (Figure S1A, available online, for a model). A systematic set of deletion mutants was created from the $C\text{-}TEV^{119–242}$ fragment to generate four truncated C-TEV constructs. Coexpression of FKBP-N-TEV and Frb-C-TEV-219 yielded inducible proteolysis upon addition of Rap with reduced leaky proteolytic activity in mock-treated cells as confirmed by the FRET assay (Figure 1B) and immunoblot analysis (Figure S1B, top panel).

Further optimization was required due to detectable TEV activity for FKBP-N-TEV and Frb-C-TEV-219 in the absence of Rap (Figure 1C and Figure S2B, bottom panel). We tested the opposite FKBP and Frb protein fusions to N-TEV and to the enhanced C-TEV-219 fragment, and we observed that Frb-N-TEV(1–118) and FKBP-C-TEV(119–219) maintained robust TEV activity in the presence of Rap with little background. As an initial test of split-TEV stability, we found that proteolysis of the FRET reporter was not enhanced after treatment with Rap and 500 nM PS-341 (Bortezomib), a clinically available proteasome inhibitor (Figure S1C), indicating that a proteasome inhibition was not sufficient to promote increased split-TEV activity in cells. Each split-TEV protein fragment was also detected by immunoblotting after blocking protein translation with cyclohexamide for 8 hr, suggesting both were stable in cells (Figure S1D). Both removal