Point of VIEW

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Exploring the Intermembrane Space

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ABSTRACT Despite the importance that many components of the intermembrane space (IMS) of mitochondria play in cellular function, the targeting sequences that are responsible for transporting proteins to this subcellular compartment are not fully understood. A recent study has identified a minimized protein sequence that is sufficient for localizing the apoptogenic protein Smac/DIABLO in the IMS. This newly identified targeting sequence is capable of directing other proteins to this submitochondrial compartment as well.

Eukaryotic cells are divided into membrane-enclosed organelles that have distinct structures and functions. Many of these organelles are further partitioned into subcompartments that are capable of maintaining a distinct biochemical environment. The physical separation of cellular components allows for the creation of isolated local environments and for specialization in biological function. Knowledge of a protein's subcellular location can provide valuable information about its function and may also offer insight into its interactions with other cellular proteins and components. To this end, recent genome- and proteome-wide studies of protein localization have greatly increased our knowledge of the protein content of most organelles (*1, 2*). These biochemical studies have been complemented by live-cell imaging experiments that have further defined how organelles and their constituent proteins interact dynamically with the rest of the cell. An important tool for these imaging studies has been the use of genetically encoded fluorescent proteins and biochemical sensors that are targeted to specific subcellular compartments in the cell (*3, 4*). For example, the use of subcellularly localized fluorescent calcium sensors has increased our understanding of calcium flux in the nucleus (*5*), endoplasmic reticulum (*5–7*), mitochondrial matrix (*8, 9*), and the plasma membrane (*8, 10*). Integral to the success of these strategies is the ability to use the cell's intrinsic transport machinery to direct a desired protein to a specific part of the cell by appending a signal sequence to it. Although efficient targeting sequences are known for many subcellular compartments, there are still important cellular locations in which specific signaling sequences are either not known or poorly understood. In the March issue of *ACS Chemical Biology*, Umezawa and coworkers (*11*) use a genetic screen based on the covalent reassembly of a split enhanced GFP (EGFP) to identify a minimized sequence that is sufficient for the localization of the apoptogenic protein Smac/DIABLO to the intermembrane space (IMS) of mitochondria. The authors demonstrate that this minimized peptide sequence is able to efficiently localize three GFP variants and an intrabody to the IMS as well.

Mitochondria act as the cell's powerhouse by producing >90% of all cellular ATP through the oxidative phosphorylation of energy-rich molecules. Over the last decade, new evidence has emerged which shows that mitochondria are dynamic organelles that play diverse roles in other biological processes beyond energy production (*12, 13*). For example, this organelle is known to be central to the mechanism of apoptosis and is also the site of numerous metabolic functions. In addition, increasing evidence shows that mitochondria are integral components in a number of intracellular signaling cascades (*14, 15*). With this expanded view of mitochondrial function, research efforts have increased to understand this organelle's interrelationship with the rest of the cell and to develop new tools that will allow these interactions to be uncovered.

Mitochondria are double-membrane-enclosed organelles that contain two functionally distinct aqueous compartments that are separated by an inner membrane. The

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In healthy cells, Smac/DIABLO resides in the IMS, but upon permeabilization of the mitochondrial outer membrane during apoptosis, it is released into the cytosol.

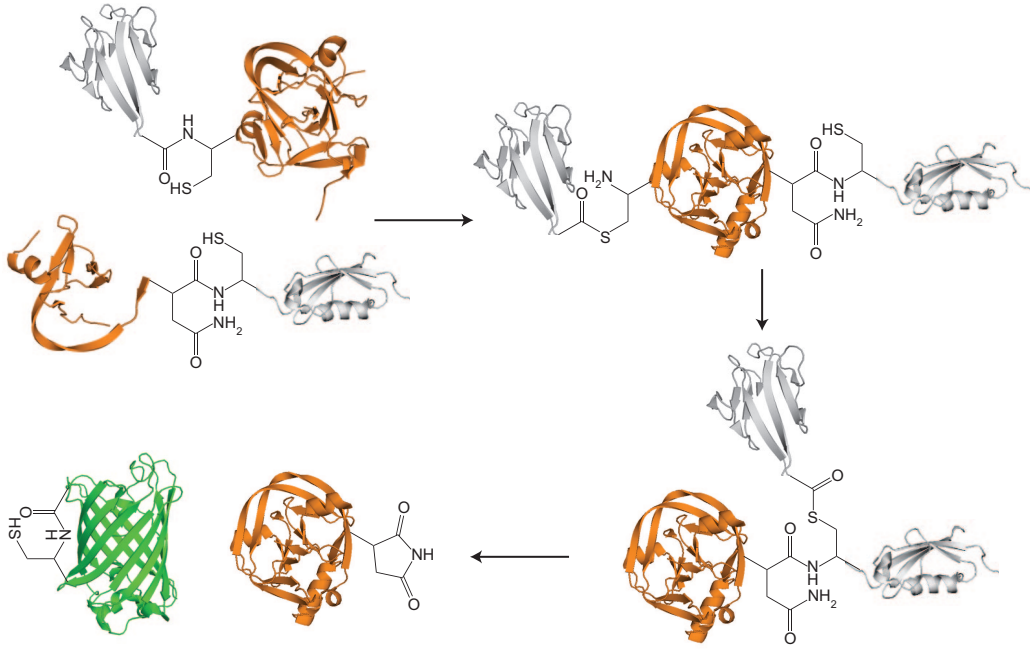

Figure 1. The split EGFP reconstitution assay used by Umezawa and coworkers to study the molecular determinants of Smac/DIABLO IMS localization. If both intein fragments are in the same subcellular compartment, the high-affinity interaction between DnaEn-EGFPn (top construct) and DnaEc-EGFPc (bottom construct) allows the efficient reassembly of EGFP through a protein trans-splicing mechanism. By restricting DnaEc-EGFPc localization to the IMS or mitochondrial matrix, the authors were able to rapidly determine the submitochondrial location of Smac/DIABLO mutants that are fused to DnaEn-EGFPn.

matrix is the subcellular compartment that is enclosed by the ion- and protein-impermeable inner membrane and contains a majority of the enzymes that are necessary for the citric acid cycle, as well as the oxidation of pyruvate and fatty acids. The IMS is the aqueous compartment between the outer and the inner membranes. Despite its small size, the IMS contains many proteins that are important for cellular function including a number of metabolic enzymes and components of the electron transport chain. In addition, various apoptotic factors are sequestered in this space until they are released during programmed cell death. Whereas mitochondria contain their own genomic DNA and ribosomes, most proteins localized in this organelle are transcribed in the nucleus and synthesized on cytosolic ribosomes. Because the outer and inner membranes of mitochondria are impermeable to large proteins, this organelle possesses a tightly regulated import machinery that allows for the specific localization of nuclearly encoded proteins to the correct mitochondrial space. Most proteins that are destined for the matrix contain a characteristic mitochondrial targeting presequence (MTS) that is cleaved by a protease after import. In contrast, proteins that are localized within the IMS are imported by a number of different mechanisms (*16, 17*). The diversity of transport mechanisms and the small size of the IMS have hindered studies of the exact molecular determinants that are responsible for the localization of proteins within this submitochondrial compartment.

Although many interesting IMS-resident proteins exist, the authors focused their analysis on the localization of the apoptogenic protein Smac/DIABLO. Murine Smac/DIABLO is translated as a 237-residue precursor protein that contains a 53-residue N-terminal MTS. Upon entering mitochondria, the MTS is cleaved by the inner membrane peptidase complex (*18*). This cleavage event produces a mature 184-residue protein that is soluble in the IMS. In healthy cells, Smac/DIABLO resides in the IMS, but upon permeabilization of the mitochondrial outer membrane during apoptosis, it is released into the cytosol with cytochrome *c* and other apoptotic factors (*19, 20*). Once released into the cytosol, Smac/DIABLO is believed to enhance apoptosis by binding to the BIR domains of members of the inhibitor of apoptosis protein family (IAPs) with its N-terminal four residues (Ala-Val-Pro-Ile) (*21, 22*). This binding event results in the direct displacement of caspases from IAPs, and this terminates the inhibition of these intracellular proteases and allows these enzymes to perform their role in programmed cell death.

The determination of the localization of a protein within the IMS is most often performed using immunoelectron microscopy or biochemical fractionation. Although these techniques have proven to be invaluable tools for studying the components of the IMS and other cellular subcompartments, they are not amenable to the throughput that is necessary for screening libraries of proteins. To circumvent this problem, Umezawa and coworkers (*23–25*) adapted a cell-based assay that they had previously developed that relies upon the naturally occurring trans-splicing intein from the DnaE gene of *Synechocystis* sp. PCC6803 (Ssp DnaE intein) (Figure 1) (*26*). The high affinities that the N-terminal (DnaEn) and C-terminal

(DnaEc) fragments of this intein have for each other allows for the efficient ligation of polypeptides that are appended to their termini. A fusion of a C-terminal segment of EGFP linked to DnaEc (DnaEc-EGFPc) and a fusion of an N-terminal segment of EGFP linked to DnaEn (DnaEn-EGFPn) are able to rapidly reconstitute EGFP if the two intein fragments are able to associate. These constructs are used for cell-based determination of protein localization by generating cell lines that stably express DnaEc-EGFPc that is localized exclusively within the IMS (BNL1MEims cells) or in the mitochondrial matrix (BML1MEmito cells) and screening libraries of proteins that are fused to DnaEn-EGFPn. If the protein that DnaEn-EGFPn is fused to directs it to the IMS in BL1MEims cells or to the mitochondrial matrix in BL1MEmito cells, then EGFP is reconstituted and the cells can be isolated by fluorescence activated cell sorting.

Using the split EGFP selection method described above, the authors performed a screen for mutations that cause Smac/DIABLO to be mislocalized to the mitochondrial matrix. Two libraries of Smac/DIABLO mutants were screened in BNL1MEmito cells, and two mutations were identified within the N-terminal MTS (M50K and C53R) that caused localization to the matrix. Various reasons exist that could account for the matrix localization of these mutants, including a decrease in the overall hydrophobicity of the targeting sequence or incomplete proteolytic processing, but the details of the mechanism were not explored further. To identify a minimized sequence that is sufficient for IMS localization, the authors screened a panel of Smac/DIABLO truncation mutants in BNL1MEims and BNL1MEmito cells. A truncation mutant consisting solely of the 53-residue N-terminal MTS of Smac/DIABLO was found to be localized exclusively in the matrix. Appending the next two amino acids (Ala54 and Val55) to the MTS gave the same submitochondrial distribution. Surprisingly, addition of only one more amino acid (Pro56) caused an almost complete switch from localization within the matrix to the IMS, with a construct consisting of residues 1–57 displaying an identical submitochondrial distribution as full-length Smac/DIABLO. This is an intriguing result because the four N-terminal residues (Ala-Val-Pro-Ile) that appear to be essential for proper IMS localization are the same four residues that are believed to be responsible for Smac/DIABLO's pro-apoptotic function. The N-terminal 57 residues of Smac/DIABLO appear to be a general targeting sequence, as demonstrated by their ability to direct an intrabody, a GFP-based pH indicator, a FRET-based calcium sensor, and a redox-sensitive GFP to the IMS.

The recent work of Umezawa and coworkers provides an exciting new tool for studying mitochondrial function by identifying a minimal sequence that is capable of directing a protein of interest to the IMS. In addition, these studies have provided valuable insight into the molecular determinants that are responsible for the submitochondrial localization of the pro-apoptotic protein Smac/DIABLO. Despite the importance of a number of IMS-localized proteins to overall cellular function, the mechanism of their transport is still poorly understood. Through the systematic analysis of other IMS-localized proteins with the split EGFP assay, a greater understanding of how the IMS interacts with the rest of the cell could be gained.

REFERENCES

1. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast, *Nature 425*, 686–691.
2. Foster, L. J., de Hoog, C. L., Zhang, Y. L., Zhang, Y., Xie, X. H., Mootha, V. K., and Mann, M. (2006) A mammalian organelle map by protein correlation profiling, *Cell 125*, 187–199.
3. Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) Creating new fluorescent probes for cell biology, *Nat. Rev. Mol. Cell Biol. 3*, 906–918.
4. Giepmans, B. N. G., Adams, S. R., Ellisman, M. H., and Tsien, R. Y (2006) Review—the fluorescent toolbox for assessing protein location and function, *Science 312*, 217–224.
5. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin, *Nature 388*, 882–887.
6. Palmer, A. E., Jin, C., Reed, J. C., and Tsien, R. Y. (2004) Bcl-2-mediated alterations in endoplasmic reticulum Ca2+ analyzed with an improved genetically encoded fluorescent sensor, *Proc. Natl. Acad. Sci. U.S.A. 101*, 17404–17409.
7. Ishii, K., Hirose, K., and Iino, M. (2006) Ca2+ shuttling between endoplasmic reticulum and mitochondria underlying Ca2+ oscillations, *EMBO Rep. 7*, 390–396.
8. Palmer, A. E., Giacomello, M., Kortemme, T., Hires, S. A., Lev-Ram, V., Baker, D., and Tsien, R. Y. (2006) Ca2+ indicators based on computationally redesigned calmodulin-peptide pairs, *Chem. Biol. 13*, 521–530.
9. Filippin, L., Magalhaes, P. J., Di Benedetto, G., Colella, M., and Pozzan, T. (2003) Stable interactions between mitochondria and endoplasmic reticulum allow rapid accumulation of calcium in a subpopulation of mitochondria, *J. Biol. Chem. 278*, 39224–39234.
10. Heim, N., and Griesbeck, O. (2004) Genetically encoded indicators of cellular calcium dynamics based on troponin C and green fluorescent protein, *J. Biol. Chem. 279*, 14280–14286.
11. Ozawa, T., Natori, Y., Sako, Y., Kuroiwa, H., Kuroiwa, T., and Umezawa, Y. (2007) A minimal peptide sequence that targets fluorescent and functional proteins into the mitochondrial intermembrane space, *ACS Chem. Biol. 2*, 176–186.
12. McBride, H. M., Neuspiel, M., and Wasiak, S. (2006) Mitochondria: more than just a powerhouse, *Curr. Biol. 16*, R551–R560.
13. Newmeyer, D. D., and Ferguson-Miller, S. (2003) Mitochondria: releasing power for life and unleashing the machineries of death, *Cell 112*, 481–490.
14. Pagliarini, D. J., and Dixon, J. E. (2006) Mitochondrial modulation: reversible phosphorylation takes center stage? *Trends Biochem. Sci. 31*, 26–34.
15. Horbinski, C., and Chu, C. T. (2005) Kinase signaling cascades in the mitochondrion: a matter of life or death, *Free Radical Biol. Med. 38*, 2–11.
16. Herrmann, J. M., and Hell, K. (2005) Chopped, trapped or tacked-protein translocation into the IMS of mitochondria, *Trends Biochem. Sci. 30*, 205–211.
17. Mesecke, N., Terziyska, N., Kozany, C., Baumann, F., Neupert, W., Hell, K., and Herrmann, J. M. (2005) A disulfide relay system in the intermembrane space of mitochondria that mediates protein import, *Cell 121*, 1059–1069.
18. Burri, L., Strahm, Y., Hawkins, C. J., Gentle, I. E., Puryer, M. A., Verhagen, A., Callus, B., Vaux, D., and Lithgow, T. (2005) Mature DIABLO/Smac is produced by the IMP protease complex on the mitochondrial inner membrane, *Mol. Biol. Cell 16*, 2926–2933.
19. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, *Cell 102*, 43–53.

20. Du, C. Y., Fang, M., Li, Y. C., Li, L., and Wang, X. D. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, *Cell 102*, 33–42.
21. Liu, Z. H., Sun, C. H., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain, *Nature 408*, 1004–1008.
22. Wu, G., Chai, J. J., Suber, T. L., Wu, J. W., Du, C. Y., Wang, X. D., and Shi, Y. G. (2000) Structural basis of IAP recognition by Smac/DIABLO, *Nature 408*, 1008–1012.
23. Ozawa, T., Sako, Y., Sato, M., Kitamura, T., and Umezawa, Y. (2003) A genetic approach to identifying mitochondrial proteins, *Nat. Biotechnol. 21*, 287–293.
24. Kim, S. B., Ozawa, T., Watanabe, S., and Umezawa, Y. (2004) High-throughput sensing and noninvasive imaging of protein nuclear transport by using reconstitution of split Renilla luciferase, *Proc. Natl. Acad. Sci. U.S.A. 101*, 11542–11547.
25. Kanno, A., Ozawa, T., and Umezawa, Y. (2006) Intein-mediated reporter gene assay for detecting protein-protein interactions in living mammalian cells, *Anal. Chem. 78*, 556–560.
26. Wu, H., Hu, Z. M., and Liu, X. Q. (1998) Protein trans-splicing by a split intein encoded in a split DnaE gene of Synechocystis sp. PCC6803, *Proc. Natl. Acad. Sci. U.S.A. 95*, 9226–9231.