

## A Sting in the Tail: Ubiquitin Ligase Function of Inhibitor of Apoptosis Proteins

Peter Mace, Sarah Shirley and Catherine Day\*

Biochemistry Department, University of Otago, Dunedin 9054, New Zealand

\*Corresponding author: [catherine.day@otago.ac.nz](mailto:catherine.day@otago.ac.nz)

Programmed cell death (apoptosis) is required during development and to maintain homeostasis of multicellular organisms. The apoptotic pathway is tightly regulated, with the balance between pro-apoptotic and pro-survival molecules finely tuned, so that cell death only occurs when appropriate. The inhibitor of apoptosis (IAP) proteins, such as XIAP, cIAP1 and cIAP2, are key negative regulators of apoptosis that function by inhibiting the executioners of cell death (caspases) or by blocking pathways that activate them. The IAP proteins possess ubiquitin ligase activity, and this ability to promote the addition of ubiquitin to themselves and interacting proteins is critical to their function. Ubiquitin was initially thought of as a tag that targets proteins for degradation by the proteasome. However, in recent years, many subtleties of ubiquitin signalling have been revealed, and it is now recognised that rather than just throwing out the garbage, many different ubiquitin modifications exist and these can regulate signalling cascades, such as apoptosis.

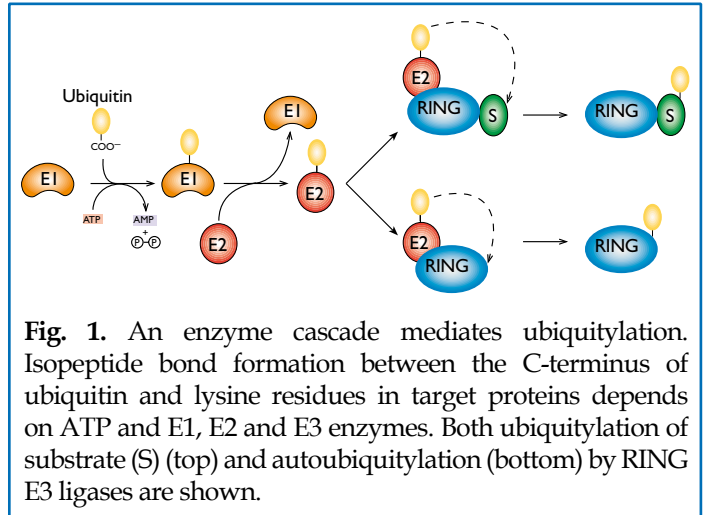
### Ubiquitylation

Ubiquitin can be attached to proteins as a single moiety or as chains. Ubiquitin has seven lysine residues and variation in the lysine residue used to build chains, together with the extent of polymer formation, results in distinct conformations of ubiquitin that mediate a diverse range of molecular signals. For example, lysine 48 (K48) linked chains of four or more ubiquitin moieties will target substrate proteins for degradation by the 26S proteasome. In contrast, ubiquitin chains built via linkage of lysine 63 (K63) typically lead to non-proteolytic events.

Attachment of ubiquitin to proteins requires the sequential action of three enzymes: an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating (Ubc) enzyme, and a ubiquitin E3 ligase (**Fig. 1**) (1). Following activation of ubiquitin by the E1 and transfer to the E2, the E3 ligase facilitates the transfer of ubiquitin from the E2 to a lysine sidechain in the substrate protein. If the E3 contains a HECT (homologous to E6-Ap C-terminus) domain, ubiquitin is transferred to the E3 before it is transferred to the substrate. In contrast, RING E3 ligases, such as IAPs, facilitate direct transfer of ubiquitin from the E2 to the target protein by providing a scaffold that brings the E2:ubiquitin conjugate and substrate into close proximity (**Fig. 1**). The substrate may be either an interacting protein or, in the case of autoubiquitylation, the E3 itself (**Fig. 1**).

More than 500 RING domains have been identified in mammalian genomes. At the core of the RING domain are two zinc ions coordinated by conserved cysteine and histidine residues. However, aside from the zinc-binding residues, sequences are variable and it is likely that this underpins the distinct properties of each domain. For example, each RING domain interacts with a subset of

the available (about 30) E2 proteins. The various E2:E3 combinations can mediate distinct ubiquitin modifications, thus determining a range of substrate protein fates (2).



**Fig. 1.** An enzyme cascade mediates ubiquitylation. Isopeptide bond formation between the C-terminus of ubiquitin and lysine residues in target proteins depends on ATP and E1, E2 and E3 enzymes. Both ubiquitylation of substrate (S) (top) and autoubiquitylation (bottom) by RING E3 ligases are shown.

### IAP Function and Interactions

IAP proteins are characterised by the presence of at least one baculoviral IAP repeat (BIR) domain, with the mammalian IAPs (XIAP, cIAP1 and cIAP2) each containing three BIR domains at their N-terminus (**Fig. 2A**). XIAP is the most extensively studied IAP and has been shown to be capable of directly inhibiting the protease activity of caspases (3). This occurs via two mechanisms: either the BIR3 domain interacts with caspase-9 to trap it in an inactive monomeric form, or the BIR2 domain and preceding linker interact with caspases-3 and -7 to block the active site. The IAPs therefore inhibit apoptosis by sequestering latent levels of caspases. When an apoptotic stimulus is received, restraint by the IAPs must be relieved. This occurs when the mitochondrial membrane is disrupted and pro-apoptotic proteins, such as Smac/Diablo, are released. These proteins have four conserved residues at their N-terminus that constitute the IAP binding motif (IBM). The IBM binds to a conserved groove on the BIR2 and BIR3 domains of IAPs, displacing caspases that can then go on to cleave their substrates (4).

In contrast to XIAP, cIAP1 and cIAP2 bind to caspases, but do not inhibit caspase activity directly. These two IAPs modulate tumour necrosis factor (TNF) signalling by binding to TNF-receptor associated factor (TRAF) proteins. The BIR1 domain of cIAP1 and cIAP2 binds to TRAFs, and is essential for recruitment of IAPs to death receptor complexes, allowing them to influence NF- $\kappa$ B signalling. Both cIAP1 and cIAP2 also contain a caspase recruitment domain (CARD).

Following the BIR3 domain in all IAPs is a region that has recently been shown to have homology with ubiquitin-associated (UBA) domains that bind ubiquitin (5,6). In IAPs,

this domain binds K63-linked ubiquitin chains that are at least four ubiquitin moieties in length (6), although some variation in binding specificity has been reported (5). The UBA domain does not appear to be required for dimerisation, E3-ligase activity (6), or for engagement in TNF signalling complexes (5). However, the UBA domain is required for efficient activation of the NF- $\kappa$ B pathway, suggesting that interaction of IAPs with proteins modified by K63-linked polyubiquitin chains is important for signalling.

Lastly, IAPs have a C-terminal RING domain that, in conjunction with a short stretch of 13 residues at the extreme C-terminus, is required for both dimerisation of IAPs and E3 ligase activity (7,8). While IAPs have been reported to ubiquitylate proteins associated with apoptosis, the importance of this has been uncertain. However, recent studies in *Drosophila* and using IAP antagonists in mammalian cells (9-11) suggest that the E3 ligase activity of IAPs is central to their role in signalling.

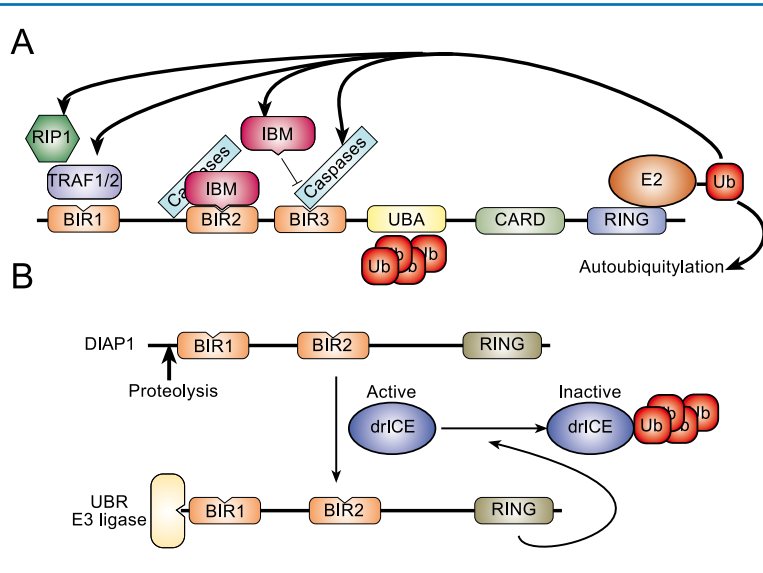
**IAP RING Domains - Linking Ubiquitylation and Apoptosis**

Activation of apoptosis by therapeutic molecules is a major goal of pharmaceutical companies. Given the role of IAPs as inhibitors of caspases, significant effort has been devoted to developing compounds that allow their activity to be manipulated. Early studies suggested that mimicking the function of Smac/Diablo to activate caspases had potential for promoting apoptosis, and several groups have developed small molecule mimics of the IBM motif (12). When these Smac-mimetic IAP antagonist compounds proved effective at inducing apoptosis, XIAP was the presumed target because it is the only IAP capable of inhibiting caspases (12). Surprisingly, however, IAP antagonist action is independent of caspase-9 and instead depends on caspase-8. This indicates that antagonists trigger apoptosis through the extrinsic apoptotic pathway controlled by death receptors, such as the TNF receptor (10,11). Extensive analysis by several groups has shown that binding of antagonists to cIAP1 and cIAP2 triggers their rapid degradation by the proteasome. This degradation ultimately results in NF- $\kappa$ B activation, TNF $\alpha$  secretion, caspase-8 activation and cell death. Mutation of the RING

domain in cIAP1 and cIAP2 abrogates E3 ligase activity and blocks degradation in response to antagonists, pointing to a crucial role for the RING domains of cIAP1 and cIAP2.

Like many RING E3-ligases, IAPs are able to target themselves and substrate proteins for ubiquitylation (Fig. 2). The balance between autoubiquitylation and substrate ubiquitylation is important. For example, promotion of IAP autoubiquitylation by addition of IAP antagonists results in diminished ubiquitylation, and stabilisation, of NF- $\kappa$ B inducing kinase (NIK) (10). Recent studies also suggest that the abundance of the cIAPs, and ubiquitylation of receptor-interacting protein 1 (RIP1), might be intimately linked, and that this is crucial for the function of IAP antagonist compounds (13). Notably, Bertrand *et al.* (2008) have shown that cIAP1 and cIAP2 can mediate the formation of both K63 and K48 linked ubiquitin chains on RIP1 (13). Attachment of K63-linked chains on RIP1 promotes the assembly of pro-survival complexes. However, RIP1 ubiquitylation is diminished upon addition of IAP antagonists that promote autoubiquitylation and degradation of IAPs. As a result, RIP1 fails to form a pro-survival complex and instead interacts with caspase-8, which ultimately leads to cell death (13).

Not only can the E3 ligase activity of IAPs indirectly regulate caspases, but a new study now suggests a direct link (9). Using *Drosophila* DIAP1, an IAP protein that, like XIAP, binds caspases via its BIR domains and flanking sequences, Ditzel *et al.* (2008) have unravelled a complex negative feedback mechanism that regulates the levels of active effector caspases in cells. In its unmodified form, DIAP1 ubiquitylates caspases poorly. However, following cleavage by drICE, DIAP1 is able to bind the UBR-domain-containing E3 ligase and polyubiquitylate effector caspases (DCP-1 and drICE) (Fig. 2B). Although a proportion of this polyubiquitylation is K48-linked, drICE is not targeted for degradation, but rather has reduced catalytic potential. Thus, following activation of DIAP1 by caspases, DIAP1 in turn inactivates caspases - an elegant autoregulatory loop. In mammals, cleavage of XIAP and cIAPs by caspases following apoptotic stimuli has been reported (14), as has ubiquitylation of caspases by IAPs (15). It is now important to reconcile these separate observations to determine if a similar regulatory loop exists in mammals.



**Fig. 2.**  
**A.** Interactions of the domains in IAPs. The BIR domains of IAPs bind to a number of proteins. Molecules discussed in the text are highlighted and the interactions described reflect those of XIAP and cIAP1/2. The recently identified UBA domain is shown and interaction with ubiquitin chains is indicated. The CARD domain and interaction with TRAF2 are specific to cIAP1 and cIAP2.  
**B.** The DIAP1 autoregulatory feedback loop in *Drosophila*. Proteolytic cleavage by the caspase drICE activates DIAP1, allowing binding of UBR E3 ligase and resulting in polyubiquitylation and inactivation of effector caspases.

In recent years, a picture of IAP function at the TNF receptor has begun to emerge and now new clues about a direct role in regulating caspases have been suggested. However, considerable uncertainty remains and the role of IAPs in normal and cancerous cells requires further investigation. In particular, it is unclear how binding of IAP antagonists to the BIR domain modulates ubiquitin E3 ligase activity.

### Regulation of IAP E3 Ligase Activity

IAPs form dimers that depend on the presence of the RING domain, with extensive contacts at the RING:RING interface (8). However, mutation of residues at the C-terminus, which are remote from the E2-binding site, abolish dimer formation and significantly diminish E3 ligase activity (7). This suggests that, as in other RING domains, dimerisation is intimately associated with IAP E3 ligase activity. Consistent with this, analysis of the RING domains from MDMs, which are very similar to those of IAPs, has shown that the functional unit extends across the dimer interface (16,17).

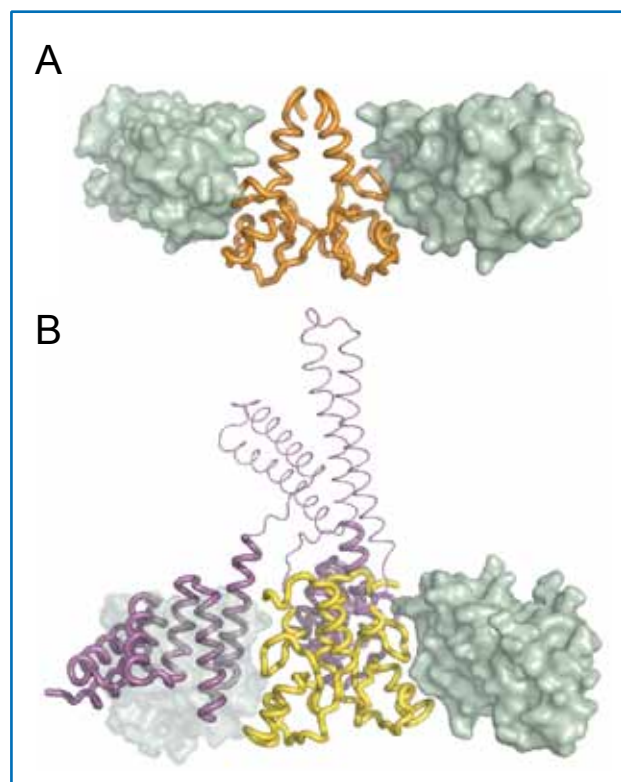
Crosstalk between domains within IAPs must also play a role in regulating E3 ligase activity because autoubiquitylation of purified IAPs is increased by IAP antagonists that bind to BIR domains (10). Clues as to how this occurs may lie with the E3 ligase CHIP (C-terminal HSP70 interacting protein), which contains a TPR domain and a U-box domain that is responsible for CHIP's E3 ligase activity. Although the U-box forms a dimer like the RING domains, in the crystal structure only one U-box has an exposed E2 binding site, with the E2 binding site on the other monomer occluded by the TPR domain (Fig. 3) (18). Furthermore, the C2 domain of the HECT E3 ligase Smurf2 performs an autoinhibitory function and prevents cycles of futile autoubiquitylation (19). Given these examples, it is tempting to invoke a similar mechanism for IAPs, where antagonist binding to a BIR domain modulates E3 ligase activity of the remote RING domain.

### Future Directions

IAPs are positively correlated with tumour survival and recent studies have revealed a number of surprises that have widened the scope of their influence on signalling pathways. While these studies have emphasised the potential value of targeting IAPs therapeutically, the increasing complexity may pose new challenges and it is apparent that the role of the E3 ligase activity of IAPs needs to be more clearly defined. The factors governing the balance between substrate and autoubiquitylation are key. It is possible that the oligomeric state of IAPs is one of these factors. Alternatively, the BIR domains, although remote in primary structure, may interact with the RING domain and influence E3 ubiquitin ligase activity. A better understanding of the relationship between the distinct domains of IAPs is central to establishing how these proteins are able to regulate the apoptotic pathway. The recent discovery of the UBA domain adds a further piece to this three-dimensional jigsaw, and deducing its binding partners and their fates upon IAP recruitment will no doubt enrich our understanding of IAP function.

### References

1. Pickart, C.M. (2001) *Annu. Rev. Biochem.* **70**, 503-533
2. Christensen, D.E., Brzovic, P.S., and Klevit, R.E. (2007) *Nat. Struct. Mol. Biol.* **14**, 941-948
3. Eckelman, B.P., Salvesen, G.S., and Scott, F.L. (2006) *EMBO Rep.* **7**, 988-994
4. Wu, G., Chai, J., Suber, T.L., Wu, J.W., Du, C., Wang, X., and Shi, Y. (2000) *Nature* **408**, 1008-1012
5. Blankenship, J.W., Varfolomeev, E., Goncharov, T., *et al.* (2009) *Biochem. J.* **417**, 149-160
6. Gyrð-Hansen, M., Darding, M., Miasari, M., Santoro, M., Zender, L., Xue, W., Tenev, T., da Fonseca, P.C., Zvelebil, M., Bujnicki, J., Lowe, S., Silke, J., and Meier, P. (2008) *Nat. Cell Biol.* **10**, 1309-1317
7. Mace, P.D., Linke, K., Feltham, R., Schumacher, F.R., Smith, C.A., Vaux, D.L., Silke, J., and Day, C.L. (2008) *J. Biol. Chem.* **283**, 31633-31640
8. Silke, J., Kratina, T., Chu, D., Ekert, P.G., Day, C.L., Pakusch, M., Huang, D.C., and Vaux, D.L. (2005) *Proc. Natl. Acad. Sci. USA* **102**, 16182-16187
9. Ditzel, M., Broemer, M., Tenev, T., Bolduc, C., Lee, T.V., Rigbolt, K.T., Elliott, R., Zvelebil, M., Blagev, B., Bergmann, A., and Meier, P. (2008) *Mol. Cell* **32**, 540-553
10. Varfolomeev, E., Blankenship, J.W., Wayson, S.M., Fedorova, A.V., Kayagaki, N., Garg, P., Zobel, K., Dynek, J.N., Elliott, L.O., Wallweber, H.J., Flygare, J.A., Fairbrother, W.J., Deshayes, K., Dixit, V.M., and Vucic, D. (2007) *Cell* **131**, 669-681



**Fig. 3. Comparison of the CHIP and IAP RING domains.**

- A.** Structure of the cIAP2 RING domain dimer (orange) bound to E2 (green).
- B.** A model that summarises the available structures of CHIP. The two structures solved were (i) the U-box (yellow) bound to E2 (green), and (ii) the full CHIP protein including U-box and TPR domains (purple). In full-length CHIP, the TPR domain of one monomer in the dimer blocks its own E2 binding site, leaving a single binding site exposed on the opposite monomer.

11. Vince, J.E., Wong, W.W., Khan, N., Feltham, R., Chau, D., Ahmed, A.U., Benetatos, C.A., Chunduru, S.K., Condon, S.M., McKinlay, M., Brink, R., Leverkus, M., Tergaonkar, V., Schneider, P., Callus, B.A., Koentgen, F., Vaux, D.L., and Silke, J. (2007) *Cell* **131**, 682-693
  12. Vucic, D., and Fairbrother, W.J. (2007) *Clin. Cancer Res.* **13**, 5995-6000
  13. Bertrand, M.J., Milutinovic, S., Dickson, K.M., Ho, W.C., Boudreault, A., Durkin, J., Gillard, J.W., Jaquith, J.B., Morris, S.J., and Barker, P.A. (2008) *Mol. Cell* **30**, 689-700
  14. Clem, R.J., Sheu, T.T., Richter, B.W., He, W.W., Thornberry, N.A., Duckett, C.S., and Hardwick, J.M. (2001) *J. Biol. Chem.* **276**, 7602-7608
  15. Huang, H., Joazeiro, C.A., Bonfoco, E., Kamada, S., Levenson, J.D., and Hunter, T. (2000) *J. Biol. Chem.* **275**, 26661-26664
  16. Linke, K., Mace, P.D., Smith, C.A., Vaux, D.L., Silke, J., and Day, C.L. (2008) *Cell Death Differ.* **15**, 841-848
  17. Uldrijan, S., Pannekoek, W.J., and Vousden, K.H. (2007) *EMBO J.* **26**, 102-112
  18. Zhang, M., Windheim, M., Roe, S.M., Peggie, M., Cohen, P., Prodromou, C., and Pearl, L.H. (2005) *Mol. Cell* **20**, 525-538
  19. Wiesner, S., Ogunjimi, A.A., Wang, H.R., Rotin, D., Sicheri, F., Wrana, J.L., and Forman-Kay, J.D. (2007) *Cell* **130**, 651-662
- 

