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Activity of protein kinase CK2 uncouples Bid cleavage from caspase-8 activation

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Summary
In the present study, we quantitatively analysed the interface between apoptosis initiation and execution by determining caspase-8 activation, Bid cleavage and mitochondrial engagement (onset of mitochondrial depolarisation) in individual HeLa cervical cancer cells following exposure to tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). Employing resonance-energy-transfer probes containing either the caspase-8 recognition site IETD or full-length Bid, we observed a significant delay between the times of caspase-8 activation and Bid cleavage, suggesting the existence of control steps separating these two processes. Subsequent analyses suggested that the divergence of caspase-8 activation and Bid cleavage are critically controlled by kinase signalling: inhibiting protein kinase CK2 by using 5,6-dichloro-l-(β-D-ribofuranosyl-1)-benzimidazole (DRB) or by overexpression of a dominant-negative CK2α catalytic subunit largely eliminated the lag time between caspase-8 activation and Bid cleavage. We conclude that caspase-8 activation and Bid cleavage are temporally uncoupled events, providing transient tolerance to caspase-8 activities.

Key words: Apoptosis, Tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL), Protein kinase CK2, Bid, Caspase-8, Förster resonance energy transfer (FRET)

Introduction
Apoptosis is a central physiological cell-death process that occurs during the entire lifetime of metazoans and is executed by proteases of the caspase family. In response to intrinsic or extrinsic death signals, most human cells induce apoptosis via the mitochondrial (type II) pathway. Type-II apoptosis involves Bax-Bak-dependent mitochondrial-outer-membrane permeabilisation (MOMP), followed by co-release of mitochondrial-intermembrane-space proteins [cytochrome c, Smac (Diablo), Omi (HtrA2)] and concomitant mitochondrial depolarisation (Goldstein et al., 2000; Munoz-Pinedo et al., 2006; Rehm et al., 2003). Subsequent activation of caspase-9 and executioner caspase-3 leads to proteolysis of hundreds of substrates, nuclear condensation and fragmentation, and the dismantling of cells into apoptotic bodies. Once initiated, MOMP and effector-caspase activation proceed with rapid kinetics, reaching completion within minutes (Goldstein et al., 2000; Rehm et al., 2002). By contrast, the lag time between exposure to intrinsic or extrinsic death stimuli and MOMP or apoptosis execution is strongly stimulus- and dose-dependent, and is highly variable between cells from clonal populations (Rehm et al., 2002; Rehm et al., 2009).

Type-II apoptosis that is initiated via death ligands such as TRAIL requires the activation of caspase-8 upstream of mitochondrial engagement. TRAIL binds to its cognate death receptors (DR) 4 and 5, which, following receptor oligomerisation, recruit the cytosolic adaptor protein FADD and procaspase-8 (Bodmer et al., 2000; Sprick et al., 2000). Procaspase-8 is activated, and autokatallytically processed mature caspase-8 can be released into the cytosol (Chang et al., 2003; Donepudi et al., 2003; Martin et al., 1998), where it cleaves its prime proapoptotic substrate Bid. Truncated Bid (tBid) then accumulates at the mitochondria to induce MOMP via the formation of Bax-Bak pores (Li et al., 1998).

Förster resonance energy transfer (FRET) probes can be employed to measure intracellular protease activities that cleave peptides linking donor and acceptor fluorophores. In contrast to synthetic, proteolytically dequenchable fluorigenic substrates, ratiometric FRET readouts are highly sensitive because they are based on initial non-zero fluorescence signals and thus are not impaired by detection limits to low emission intensities. We and others developed CFP-YFP FRET probes containing an optimal caspase-8 recognition site (IETD) to measure caspase-8 signalling kinetics during TRAIL-induced initiation of apoptosis (Albeck et al., 2008; Hellwig et al., 2008). These studies highlighted high cell-to-cell variability and dose dependency of caspase-8 activation and activity upstream of mitochondrial engagement (Albeck et al., 2008; Hellwig et al., 2008). We could furthermore show that this variability is translated into a strict response of MOMP and apoptosis execution once a conserved amount of cleaved substrate has accumulated (Hellwig et al., 2008).

However, it remained unclear whether the cleavage of recombinant IETD substrates indeed reflects cleavage of physiological substrates of caspase-8, such as Bid. Although caspase-8 activity and large quantities of IETD-substrate cleavage can be detected several minutes and hours prior to MOMP (Albeck et al., 2008; Hellwig et al., 2008), biochemical studies suggested that very low tBid concentrations are already sufficient to induce MOMP (Li et al., 1998; Luo et al., 1998; Martinez-Caballero et al., 2009). Here, we therefore investigated the temporal relationship of caspase-8 activation and Bid cleavage in individual HeLa cervical cancer cells during TRAIL- and TNFα-induced apoptosis initiation by employing combinations of fluorescent reporter constructs and advanced time-lapse imaging.
Results and Discussion

Differential cleavage of IETD and Bid FRET substrates during TRAIL-induced apoptosis

To examine whether IETD-based probes reliably reflect the cleavage kinetics of Bid in individual cells, we compared FRET probes containing either an IETD peptide or full-length Bid (Hellwig et al., 2008; Onuki et al., 2002). We expressed both probes in HeLa cells and induced death-receptor-mediated apoptosis by the addition of TRAIL. Cycloheximide (CHX) was added in parallel to investigate the apoptosis signalling branch without strong interference from NFκB-mediated survival signalling (Falschlehner et al., 2007). CHX did not cause any detectable amount of cell death following 24 hours of treatment (not shown). HeLa cells require the mitochondrial pathway for the activation of effector caspases and apoptosis execution (Albeck et al., 2008; Engels et al., 2000; Hellwig et al., 2008), and are a widely used experimental cell system for the real-time photonic analysis of intracellular apoptosis signalling. For both FRET probes we optimised the acquisition procedure to minimise potential phototoxic damage while still achieving high signal-to-noise ratios. Confirming previous studies (Albeck et al., 2008; Hellwig et al., 2008), caspase-8 activation was detected before apoptotic mitochondrial engagement when using the IETD FRET probe (Fig. 1A), whereas, subsequent to MOMP, the activation of effector caspases contributed to a significant increase in the substrate cleavage rates (Fig. 1A). By contrast, the onset of Bid FRET disruption was closely correlated with mitochondrial engagement and then followed post-MOMP kinetics similar to the IETD FRET probe (Fig. 1B). Experimental baselines lacked slopes, ensuring that the measured signals were not a consequence of acceptor photobleaching/donor un-quenching during data acquisition (Fig. 1A,B). When defining the detection limit for probe cleavage as an irreversible deviation from the baseline noise, we achieved a sensitivity of 2.5% and 3.2% substrate cleavage for the IETD FRET and Bid FRET probes, respectively. Taken together, these results therefore suggest that Bid cleavage might not be initiated upon caspase-8 activation but instead might be subject to additional control steps.

The lag time between caspase-8 and Bid activation is sensitive to inhibition of protein kinase CK2

Previous in vitro and cell-population-based studies suggested that protein kinase CK2 might play an additional regulatory role at the interface between extrinsic apoptosis initiation and mitochondrial engagement (Desagher et al., 2001; Li et al., 2002; Shin et al., 2005). Human protein kinase CK2 represents two constitutively active serine/threonine kinase isoforms of broad substrate specificity, phosphorylating hundreds of known targets both in the cytosol and the nucleus (Litchfield, 2003; Sarno and Pinna, 2008). Abnormally elevated CK2 activities might be tumorigenic and indeed have been repeatedly detected in various cancers (Duncan and Litchfield, 2008).

In subsequent experiments, we therefore investigated whether CK2 activity contributes to the differential cleavage of IETD substrates and Bid. To investigate caspase-8 and Bid activation in parallel within individual cells, we combined the expression of the IETD FRET probe with a spectrally compatible red fluorescent Bid-Cherry fusion protein. As shown before for other fluorescently labelled Bid probes (Nechushtan et al., 2001), Cherry fluorescence redistributed into a punctate pattern during apoptosis, indicating tBid-Cherry accumulation at mitochondria (Fig. 2A). Likewise, the Bid-Cherry fusion protein was able to re-sensitize Bid-depleted HeLa cells to apoptosis induction by TRAIL and was cleaved in parallel with native Bid in immunoblotting experiments (supplementary material Fig. S1A,B).

To achieve rapid and specific CK2 inhibition in all cells observed, we employed a short (2 hour) pretreatment with the specific CK2 inhibitor 5,6-dichloro-1-(β-D-ribofuranosyl-1)-benzimidazole (DRB) (Meggio et al., 1990; Zandomeni and Weimann, 1984). Time-lapse imaging of control HeLa cells, as expected, showed that the IETD FRET probe was cleaved before tBid-Cherry translocation could be detected (Fig. 2B). By contrast, in DRB pretreated cells, the delay between the onset of IETD FRET cleavage and Bid activation was essentially abrogated (Fig. 2C,D). These results suggest that Bid processing is temporally uncoupled from caspase-8 activation in a DRB-sensitive manner. In additional control experiments, prolonged treatment of HeLa cells with 50 μM DRB did not result in any detectable apoptosis execution during the time course of the experiments (supplementary material Fig. S2A). Except for a mild reduction in cFlipL and slightly increased DR5 levels, the abundance of key proteins involved in death-inducing signalling complex (DISC) formation,
mitochondrial permeabilisation or apoptosis execution did not noticeably change in response to CK2 inhibition by DRB (supplementary material Fig. S2B-D). This suggests that the abrogation of the lag time between caspase-8 and Bid activation was not affected by perturbing the role of CK2 in transcriptional regulation (Litchfield, 2003) but is more likely a rather immediate response due to altered phosphorylation patterns of other CK2 substrates.

To investigate whether impaired CK2 activity affected kinetics other than the lag time between caspase-8 activation and Bid cleavage, we investigated parameters that can be obtained from individual cells by using the IETD FRET probe (Fig. 3A) (Hellwig et al., 2008): (i) the time from stimulus addition to caspase-8 activation, entailing the processes from death ligands binding to their receptors until caspase-8 activation; (ii) the time from caspase-8 activation until mitochondrial engagement; (iii) the amount of cleaved substrate at the time of mitochondrial engagement, which can serve to measure the decision threshold for subsequent apoptosis execution; and (iv) the short post-MOMP delay after which cleavage rates of the IETD substrate strongly increase owing to activation of effector caspase-3 and -7. We quantified these parameters in the absence or presence of CK2 inhibition (Fig. 3B-E). In addition to the pharmacological inhibition of CK2 by DRB, we also overexpressed a kinase-inactive mutant of the CK2α subunit (CK2α K68M), which competitively interferes with the functioning of endogenous CK2 (Vilk et al., 1999).

The lag time between TRAIL addition and onset of cleavage of the IETD FRET probe did not differ between the three groups (Fig. 3B), suggesting that the upstream signalling from ligand binding to caspase-8 activation was not affected by CK2 inhibition. As expected, we again noticed that the lag time between caspase-8 activation and mitochondrial engagement was completely abrogated upon DRB pretreatment, i.e. the onset of IETD-substrate cleavage coincided with mitochondrial engagement (Fig. 3C). Overexpression of CK2α K68M similarly reduced the lag time between caspase-8 activation and mitochondrial depolarisation (Fig. 3C). The time of mitochondrial engagement coincided with cleavage of approximately 10% of the IETD FRET substrate in control HeLa cells, whereas DRB treatment or CK2α-K68M overexpression abolished or reduced the amount of substrate cleaved at the time of mitochondrial engagement (Fig. 3D). Efficient effector-caspase activation, as indicated by a significant increase in the FRET-substrate cleavage rate, was detected within approximately 2-4 minutes following mitochondrial engagement, independent of whether CK2 was inhibited or not (Fig. 3E). Similar results were obtained in MCF-7 breast cancer cells treated with TRAIL and CHX in the presence or absence of DRB (n=9 or 15 cells analysed, respectively; not shown), or when inducing apoptosis in HeLa cells with TNFα and CHX (Fig. 3F). Furthermore, additional control experiments indicated that CK2 inhibition did not affect the activity of caspase-8 (supplementary material Fig. S2E). Overexpression of exogenous CK2α did not significantly alter the signalling kinetics (seven cells from n=3 experiments; not shown), suggesting that the activity of the native holoenzyme is not limited by availability of the α-subunit in HeLa cells. Taken together, these results indicate that CK2 activity does not affect either the time required for caspase-8 activation or the signalling kinetics subsequent to mitochondrial engagement. Instead, these data further suggest that CK2 activity is required to provide cells with transient tolerance to caspase-8 activity.

Our study demonstrated that, within living cells, caspase-8 activation and the proteolytic activation of caspase-8 substrate Bid are temporally separated events. In HeLa cells, the activity of protein kinase CK2 provided transient tolerance to caspase-8 activity by delaying Bid cleavage, a process that otherwise would induce MOMP immediately. Readings obtained from the use of recombinant IETD substrates therefore require careful interpretation (Albeck et al., 2008; Hellwig et al., 2008; Spencer et al., 2009). Likewise, system models that were designed to calculate a fast pore-formation process during MOMP so far assumed that the production of tBid could be deduced immediately from IETDase activities. Such models would benefit from including the biochemistry of an additional control step at the interface of caspase-8 and Bid activation to more authentically represent the biology of apoptotic signal transduction.

Short exposure to the CK2 inhibitor DRB was sufficient to eliminate the lag time between caspase-8 activation and Bid cleavage: this result is most likely caused by modulation of the phosphorylation status of CK2 substrates rather than by influencing CK2-dependent transcriptional processes. CK2 substrates whose anti- or proapoptotic potential was suggested to be modulated by...
phosphorylation include apoptosis repressor with caspase recruitment domain (ARC), caspase-2 and, probably most centrally, Bid itself (Desagher et al., 2001; Li et al., 2002; Shin et al., 2005). It was recently shown that the threonine within the caspase-8 cleavage site of Bid is a CK2 target site (Olsen et al., 2006), potentially explaining previous in vitro data that demonstrated reduced cleavage rates of Bid peptides that were phosphorylated at this position (Degli Esposti et al., 2003).

Besides CK2, other serine/threonine kinases such as CK1, mitogen-activated protein kinases and ataxia telangiectasia-mutated kinase might modify the functionality of Bid in cell death and survival signalling (Desagher et al., 2001; Holmstrom et al., 2000; Kamer et al., 2005; Vogel et al., 2006). Furthermore, several proposed TRAIL combination therapies build on the potential of TRAIL to synergize with clinically employed chemotherapeutic drugs. Among these are multiple kinase inhibitors, such as sorafenib and gefitinib (Johnstone et al., 2008). Unlike DRB and some of its derivatives, many of these inhibitors have been shown to be of rather modest target selectivity (Karaman et al., 2008; Meggio et al., 1990; Zandomeni and Weinmann, 1984). It is therefore conceivable that synergies observed in TRAIL co-treatments could in part be mediated by impaired CK2 activity or Bid phosphorylation.

It is currently unknown whether de-phosphorylation of Bid by protein phosphatases is required for apoptosis to proceed or whether uncatalysed hydrolysis of the phospho-group suffices. We identified that, in living cells, minimal quantities of tBid (estimated from the baseline noise of our FRET measurements as less than approximately 3% of the total Bid pool) are sufficient to trigger MOMP, a finding that agrees with in vitro biochemical and mitochondrial patch-clamping studies that found that tBid concentrations in the picomolar to low-nanomolar range are sufficient to permeabilise mitochondria (Li et al., 1998; Luo et al., 1998; Martinez-Caballero et al., 2009). An enzymatically catalysed process amassing significant amounts of de-phosphorylated Bid therefore might not be required during caspase-8-mediated apoptosis. However, glutamate-induced excitotoxic apoptosis and cell death by anoikis were shown to be mediated at least in part by translocation of large amounts of full-length Bid, which is far less efficient in inducing MOMP, to mitochondria (Li et al., 1998; Luo et al., 1998; Valentinj and Gilmore, 2004; Ward et al., 2006). Of note, PACS-2, a Bid-recruiting protein that functions also in controlling the formation of lipid-synthesizing centres in ER- and/or mitochondria-associated membranes, selectively recruits only non-phosphorylated Bid to the mitochondrial outer membrane (Simmen et al., 2005).
Ultimately, the interface between caspase-8 activation and tBid production (and, by extension, the decision to undergo apoptosis or not) might be of significant physiological importance, because caspase-8 activities can be functionally separated from cell-dead signalling. This has been demonstrated during macrophage and T-cell activation and differentiation, and during skin healing (Alam et al., 1999; Beisner et al., 2003; Kang et al., 2004; Kennedy et al., 1999; Lee et al., 2009; Rebe et al., 2007). More generally, it might provide cells with a safeguard mechanism for additional control steps or to reverse apoptosis initiation upon accidental caspase-8 activation.

Materials and Methods

Materials

Mineral oil and CHX were from Sigma (Tallaght, Ireland). TRAIL was from Leinco Technologies (St Louis, MO). Tetramethyl-rhodamine-methylster (TMRM) was from MobiTec (Göttingen, Germany). DRB was from Calbiochem (San Diego, CA). MitoTracker Green was from Invitrogen (Karlsruhe, Germany). TNFα was from Peprotech (London, UK).

Molecular cloning

For the generation of pBID-mCherry-N1, the coding sequence of Bid was PCR amplified from human cDNA and cloned in frame into the BglII/EcoRI sites of pEGFP-N1 (Clontech). To generate pBid-mCherry-N1, the mCherry gene was PCR amplified from pmCherry-C1 and sub-cloned in frame to BambHI-Ncol sites of pBID-EGFP following removal of EGFP by restriction. The verity of the construct was confirmed by sequencing. Plasmids for the expression of the IETD FRET probe, the YFP-Bid-CFP FRET probe, CK2αt and CK2αt K68M were described previously (Hellwig et al., 2008; Onuki et al., 2002; Vilk et al., 2003).

Cell culture

Cells were cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum at 37°C and 5% CO₂. For time-lapse imaging, cells were grown on 22-mm glass-bottom dishes (Willco Ireland (05/RFP/BIM056, 07/RFP/BICF601), the Health Research Board Ireland (RP/2006/258), the National Biophotonics and Imaging Platform (HEA PRTLI Cycle 4) and the EU Framework Programme 7 (APOSYS). A.H.L.-G. is a recipient of an NBPI Career Enhancement and Mobility Fellowship co-funded by Marie Curie Actions, the Irish Higher Education Authority Programme for Third Level Institutions Cycle 4 and the Italian National Research Council.

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