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Letter to the Editor

The active caspase-8 heterotetramer is formed at the CD95 DISC

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Dear Editor,

Caspase-8 plays an important role in the CD95 (APO-1/Fas) signalling pathway.[1–3] It is activated at the CD95 DISC. The mechanism of caspase-8 activation at the DISC is in the focus of intensive studies. It is well established that binding of procaspases-8/a and -8/b to the DISC results in autocatalytic cleavage via a two-step mechanism[4,5] (Figure 1a). The initial cleavage at Asp^{374} generates two cleavage intermediates p43/p41 and p12. Subsequently, p12 is rapidly converted to p10. Afterwards, an additional cleavage occurs at Asp^{216}, producing the active enzyme subunit p18 and the inactive p26/p24 prodomain. The DED-containing cleavage products p43/p41 and p26/p24 were reported to be present in the DISC in previous biochemical studies.[5,6]

We analysed in more detail all cleavage products of procaspase-8 in the DISC formed upon stimulation of the B-lymphoblastoid cell line SKW6.4. Cells were treated with LZ-CD95L for different time points, followed by immunoprecipitation of the DISC components with the agonistic anti-APO-1 and by immunoblotting with a panel of mAbs. The epitopes recognised by these antibodies are shown in Figure 1a. In addition to the established components of the DISC, including full-length procaspase-8 (p55/p53) and its cleavage product p43/p41, we clearly detected the p18 and p10 subunits of procaspase-8 in the DISC (Figure 1b). The presence of p10 and p18 at the DISC was also observed using other T-cell lines Hut78, CEM, H9 as well as primary T cells (data not shown). The detection of these products became possible because we used different conditions for electrophoresis, focusing on lower molecular weight products in comparison to previous studies. In addition, the presence of the p10 subunit was detected using the C5 antibody that had not been used in the analysis of the lower molecular weight procaspase-8 subunits in the CD95 DISC before.

The kinetic analysis in Figure 1b demonstrated that p10 and p18 are detectable at the CD95 DISC within 30 s after stimulation. The amount of both p10 and p18 at the DISC reached its maximum 1–5 min after receptor engagement. After 10 min, the intensity of the bands corresponding to p10 and p18 started to decrease and after 20 min, the intensity of bands was markedly reduced. The kinetic analysis performed with lysates of SKW6.4 cells (Figure 1b) showed the same main features as reported earlier.[5] P10 and p18 were detected in the cytosol 10 min after the receptor stimulation, and their presence reached a maximum after 20 min, exactly at the time when their amount in the DISC was found to be decreased.

To ensure that the p10 and p18 subunits are components of the DISC under different stimulation conditions, SKW6.4 were stimulated in parallel with LZ-CD95L and anti-APO-1 antibody. We observed the presence of p10 and p18 in the DISC in both cases (data not shown). To exclude the possibility that p10 and p18 subunits are generated during the washing steps, DISC formation and consecutive immunoprecipitation experiments were performed in the presence of the caspase inhibitor zVAD-fmk. Under these conditions, we could also detect active caspase-8 subunits at the DISC (data not shown).

Additionally, we addressed the question of whether the caspase-8 subunits generated at the DISC were functionally active and did not require additional cytosolic processing and can cleave caspase-8 substrates. Thus, *in vitro* translated, [^{35}S]-labelled procaspase-3 was added to protein-A sepharose containing CD95 immunoprecipitates from unstimulated and stimulated cells. Incubation of protein-A sepharose beads with caspase-3 resulted in the cleavage of procaspase-3 to p20, p19, p17 and p12 subunits only in the stimulated case (Figure 1c, lane 3). Cleavage was blocked in the presence of the specific inhibitor of caspase-8 IETD-fmk (Figure 1c, lane 4).

To ensure that autocatalytic processing of procaspase-3 does not blur our results in these experiments, we performed a similar assay with procaspase-3 carrying a mutation in the catalytic site (C163S). This mutant was supposed to be a substrate of caspase-8, but it excludes the formation of any cleavage product as a result of genuine caspase-3 proteolytic activity. In the presence of immunoprecipitates from the CD95 DISC, we observed only the first cleavage step with the formation of the p20 subunit (Figure 1c, lane 7). No further processing to the p17 subunit was detected. This observation is in accordance with published data indicating that the generation of the mature p17–p12 enzyme of caspase-3 only occurs autocatalytically.[7] Thus, the caspase-3 mutant C163S is catalytically inactive in our *in vitro* system. Cleavage was not observed in the presence of IETD-fmk (Figure 1c, lane 8), as well as in the case of immunoprecipitates from unstimulated cells (Figure 1c, lane 6). These data point out that the cleavage of caspase-3 results from the catalytic activity of the caspase-8 heterotetramer formed at the DISC.

In previous studies, it has been suggested that formation of the tetramer takes place in the cytosol.[5] In our view, this is a less likely event than the interaction between subunits remaining bound to the DISC. Thus, the DISC not only brings the molecules of procaspase-8 into close proximity to each other, but it also places them in a spatial orientation favourable for their activation. A number of biochemical studies provide evidence that the activation of procaspase-8 occurs via its dimerisation or oligomerisation.[8–10] The conditions for the dimer formation and the following cleavage steps must be provided by the spatial structure of the DISC. However, further

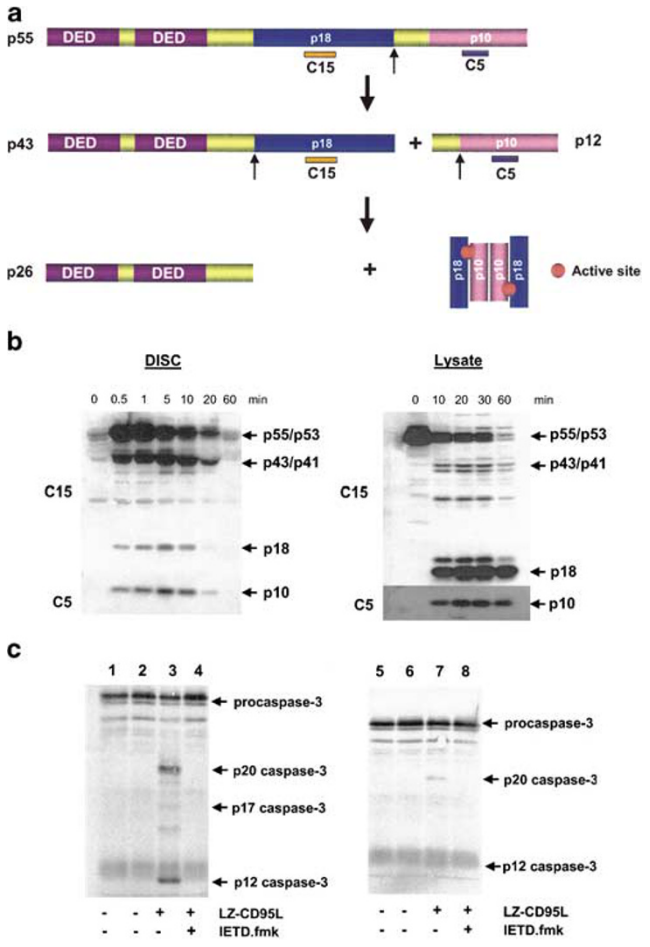

Figure 1 Mature subunits p10 and p18 of caspase-8 are detectable in the CD95 DISC. (**a**) Two-step mechanism of procaspase-8a cleavage. The epitopes for the monoclonal anti-caspase-8 antibodies C15 and C5 are indicated. (**b**) Time course of p10 and p18 presence in the DISC. SKW6.4 cells were treated with LZ-CD95L for the indicated periods of time. CD95 was immunoprecipitated from either 5×10^7 SKW6.4 cells untreated (−) or treated (+) with LZ-CD95L. Immunoprecipitated proteins (IP) and cytosolic extracts (L) were separated by 12% SDS-PAGE and immunoblotted with anti-caspase-8 mAb C15 and anti-caspase-8 mAb C5. Migration positions of full-length procaspase-8 (p55/p53), which exists in two isoforms, and its further processed products p43/p41, p18 and p10 are indicated. (**c**) CD95 was immunoprecipitated from 3×10^7 SKW 6.4 cells untreated (−) or treated (+) with LZ-CD95L. The beads containing immunoprecipitated CD95 were incubated with *in vitro* translated, [^{35}S]-labelled procaspase-3 (lanes1–4) or [^{35}S]-labelled procaspase-3 (C163S) (lanes 5–8) for 24 h at 4°C in buffer conditions mimicking cytosolic composition as described.[5] The analysis of cytosolic products is also shown in the presence of 50 μM IETD-fmk (lanes 4, 8). In lanes 1 and 5, *in vitro* translated procaspase-3 and its mutant, respectively, were incubated without beads

biochemical studies are necessary in order to reveal all the molecular details of events occurring at the CD95 DISC.

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