FEBS Letters 468 (2000) 134–136 FEBS 23356

Activation of a pro-apoptotic amplification loop through inhibition of NF-κB-dependent survival signals by caspase-mediated inactivation of RIP

Fabio Martinon, Nils Holler, Christelle Richard, Jürg Tschopp*

Institute of Biochemistry, University of Lausanne, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland

Received 6 January 2000

Edited by Marco Baggiolini

Abstract Death domain containing members of the tumor necrosis factor receptor (TNFR) superfamily can induce apoptosis or cell activation. However, the mechanisms by which these opposing programs are selected remain unclear. Frequently, NF-κB activation conveys protection against cell death. We show that the serine/threonine kinase RIP that is required for TNF-induced NF-κB activation is processed by caspase-8 into a dominant-negative (DN) fragment during death receptor-induced apoptosis, thereby leading to a blockade of NF-κB-mediated anti-apoptotic signals. Our results suggest that cleavage of RIP is part of an amplification loop which is triggered by Fas and most likely by other death receptors.

Key words: Apoptosis; Fas; Death receptor; Tumor necrosis factor; NF-κB; Survival; Kinase; RIP

1. Introduction

Tumor necrosis factor (TNF) binds two distinct cell surface receptors, TNFR1 and TNFR2 [1]. TNFR1 is thought to be the predominant receptor that signals both cytotoxic and inflammatory responses. The intracellular mediators of the TNF response include proteins characterized by 'death domains' such as TRADD and FADD/MORT1 [2]. These proteins bind a conserved 80 amino acid region found in the cytoplasmic domain of TNFR1 receptor family members and serve as adaptors to assemble a signaling complex that leads to apoptosis. FADD initiates apoptosis by mediating the activation of caspase-8 [2]. Another group of receptor-interacting molecules includes the TNF receptor-associated factors (TRAFs) and the serine/threonine kinase RIP. Upon TNFR1 trimerization, TNFR1 binds TRADD that recruits the RIP and TRAF2 [16]. RIP appears to mediate NF-κB activation [3,4]. Although RIP is an active serine/threonine kinase, the functional significance of its kinase activity is still unknown.

In response to TNF and Fas ligand treatment, NF-κB activation protects cells against TNF-induced apoptosis [5–9]. Thus, it has been proposed that the balance between life and death is regulated by NF-κB. Since RIP is a key effector in the pathway that transduces TNF signal to NF-κB activation, it was interesting to note that RIP is processed by receptors that signal apoptosis via the activation of caspase-8, resulting in the formation of a dominant-negative (DN) form impairing NF-κB signal transduction. Thus, the cleavage of RIP abolishes the induction of anti-apoptosis factors, leading to the amplification of death signals and rapid apoptosis.

2. Materials and methods

2.1. Antibodies/reagent/cell lines

Monoclonal antibodies used in Western blotting include: anti-Flag antibody (Kodak), anti-caspase-3 and RIP antibodies (Transduction Laboratories), anti-caspase-8 antibody (PharMingen). Human recombinant Fas ligand and caspase inhibitors were obtained from Alexis, Switzerland. Jurkat lymphoma cells were maintained in RPMI 1640 (Life Sciences, Basel, Switzerland) supplemented with 10% FCS and antibiotics.

2.2. Expression vectors

Expression vectors for RIP (559–671) and VSV-RIP were generated by PCR amplification/PCR-based mutagenesis, and subcloned into pCR3-derived vectors (Invitrogen) conferring an N-terminal and/or C-terminal Flag-tag. v-FLIP expression vectors have been described [10].

2.3. Transfection

293T Cells were seeded at 4×10^5 in a 3.5 cm plate and transfected by the calcium phosphate precipitation method as previously described [11]. The luciferase assays have been described [11].

3. Results and discussion

3.1. RIP is rapidly cleaved by caspases during Fas-induced apoptosis

As RIP is one of the molecules whose deficiency was demonstrated to completely abrogate TNF-induced NF-κB activation [3,4], we considered RIP as an ideal target. Indeed, when BJAB, Raji and Jurkat T cells were killed by FasL, complete conversion of the 74 kDa protein into a 38 kDa fragment was detected by Western blot analysis (Fig. 1A and data not shown). Since the epitope recognized by the monoclonal anti-RIP antibody lies within the death domain, cleavage had to occur between the kinase domain and the intermediary domain (Fig. 1B). When Asp-324 was mutated to Glu, cleavage of RIP was abolished, whereas the mutation of two other putative caspase cleavage sites (D300E and D418E) had no effect (Fig. 2A). The preceding sequence of this cleavage site is (321)LQLD(324) which correlates with the preferred cleavage motif of caspase-8 (LXXD). RIP cleavage was indeed inhibited by the caspase-8 inhibitors FLIP (Fig. 2B) and zVAD, IETD-fmk but to a lesser extent by DEVD-fmk and YVAD-fmk (Fig. 2C), suggesting that RIP processing is caused by caspase-8.

*Corresponding author. Fax: (41)-21-692 5705.
E-mail: jurg.tschopp@ib.unil.ch

PII: S0014-5793(00)01212-6

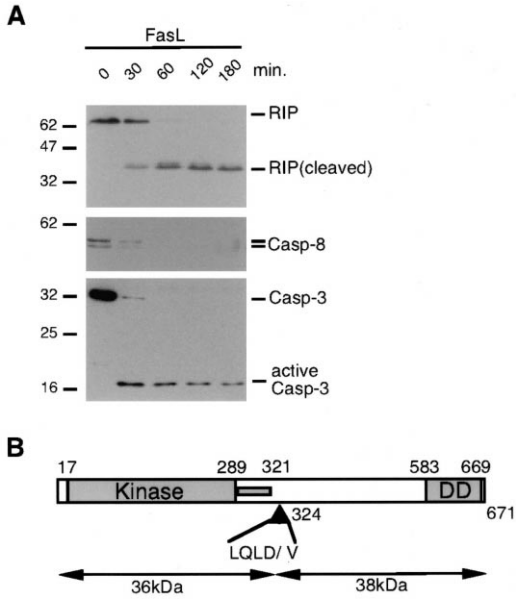

Fig. 1. RIP is processed during apoptosis. (A) Jurkat T cells were treated with cross-linked sFasL (300 ng/ml) for the indicated times. Processing of RIP, caspase-8 and caspase-3 was assessed by Western blot analysis of whole cell lysates using the appropriate antibodies. (B) Schematic representation of the structure of RIP. The N-terminal kinase domain is followed by a short coiled-coil region (stippled box) which is followed by a death domain (DD). The caspase cleavage site is indicated.

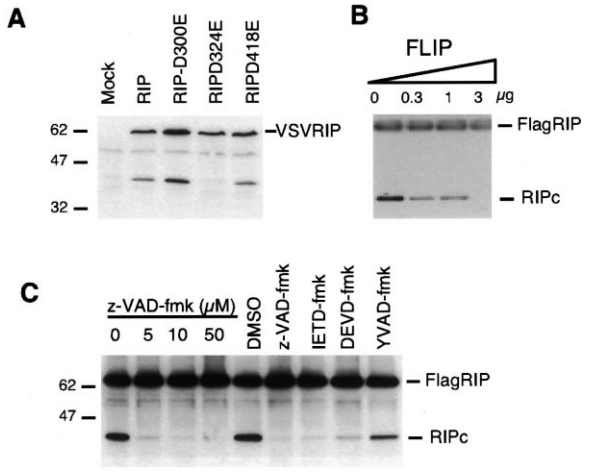

Fig. 2. Cleavage of FLIP occurs at Asp-324 most likely by caspase-8. (A) FlagRIP and the indicated mutants were overexpressed (2 μg plasmid) in 293T cells and processing was analyzed by immunoblotting using an antibody detecting an epitope within the DD of RIP. (B) Inhibition of FlagRIP processing by v-FLIP. Increasing amounts of v-FLIP were co-transfected with FlagRIP expression plasmid (2 μg) into 293T cells and processing was analyzed by Western blot using an anti-Flag antibody. (C) FlagRIP-transfected 293T cells were treated with cross-linked FasL for 12 h in the presence of various caspase inhibitors (50 μM unless indicated). Whole cell lysates were analyzed by immunoblotting using an anti-RIP antibody.

Similar to processed TRAF1 (see accompanying paper), the C-terminal fragment of RIP generated during apoptosis acted as a DN inhibitor of NF-κB activation (Fig. 3). Compared to the death domain of RIP (RIP 559–671), RIP 324–671 was slightly less potent, but was still capable of reducing TNFα-induced NF-κB activation by more than 70%.

3.2. *Activation of a pro-apoptotic amplification loop during death receptor-mediated apoptosis*

While the exact role of RIP in TNF-induced NF-κB activation is still unknown, it is clear that its absence blocks NF-κB signaling as cell lines or primary cells lacking RIP are unable to activate NF-κB in response to TNF [3,4]. We found rapid cleavage of RIP into two major fragments when apoptosis was induced by FasL. Cleavage occurred after Asp-324, in agreement with a very recent publication [12].

As NF-κB activation is part of the response of several death receptors (TNFR1, TRAIL-R1/R2, TRAMP/DR3 and possibly Fas) [2], conflicting signals can be triggered that on one hand activate gene transcription resulting in an anti-apoptotic response and cell survival, and on the other hand induce cell death (Fig. 4). The pathway which is initially chosen may depend on the presence or absence of low constitutive NF-κB activation, caused by signals emanating from growth factor receptors, adhesion receptors or others. We observed that low constitutive activation of NF-κB was sufficient to protect HT1080 cells against TNF, but not FasL and TRAIL-induced cell death (see accompanying paper). Massive NF-κB activation was necessary to render these cells resistant to the latter two death ligands. Once the NF-κB pathway is selected in response to TNF, a self-amplifying loop is initiated which further augments NF-κB signals, thus further deflecting the pro-apoptotic signal toward survival (Fig. 4).

A different scenario may be envisaged if the pro-apoptotic response is chosen in response to death ligands, probably under conditions of low NF-κB activity. A hallmark in the induction of apoptosis is the activation of the caspase cascade. Some of the caspase-mediated cleavage products are pro-apoptotic proteins, resulting in self-amplification and acceleration of cell death. Examples are the cleavage of BID [13,14], MEKK1 [15], ICAD [16] and PKC delta [17]. Acceleration of cell death, however, is also achieved by blocking the activity of inhibitors of apoptosis. Caspase-3-dependent Bcl-2 cleav-

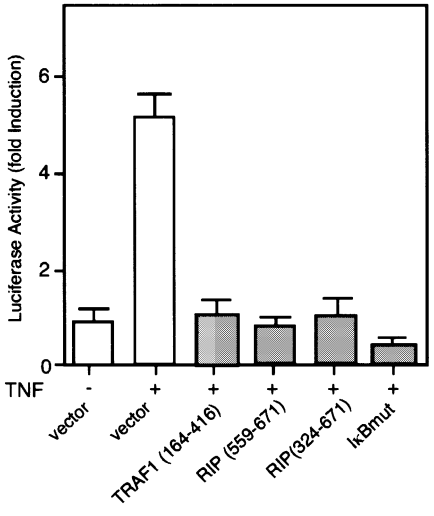

Fig. 3. Transcription factor NF-κB is blocked by the C-terminal RIP cleavage product. 293T Cells were transfected with VSV-RIP (324–671) or DN forms of other signaling molecules. 24 h after transfection, cells were treated with TNF (10 ng/ml) for 6 h and NF-κB activity was measured.

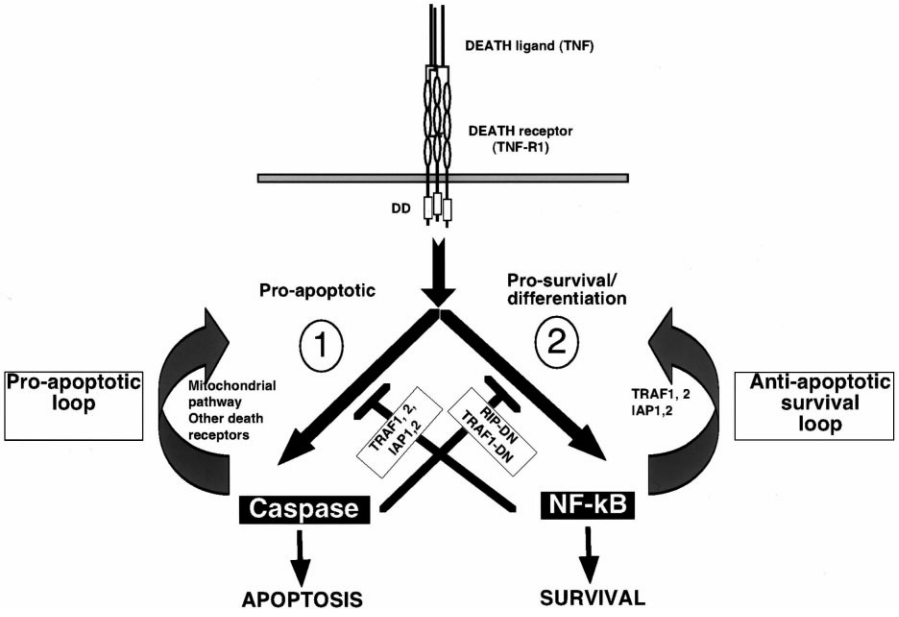

Fig. 4. Model of caspase-induced inactivation of NF-κB-mediated protection from apoptosis. TNF- and to a lesser extend FasL-induced death signals (1) are antagonized by proteins such as IAP-1, IAP-2, TRAF1, TRAF2, which, if present at the time of signal initiation, deviate the pro-apoptotic signal into NF-κB activation signals (2). The concentration of these anti-apoptotic proteins is gradually increased as their expression is under the control of the NF-κB transcription factor (anti-apoptotic amplification loop). When these NF-κB-dependent anti-apoptotic proteins are absent or at low concentration during signal initiation, death signals propagate, resulting in the activation of caspases and the subsequent cleavage of anti-apoptotic proteins (TRAF1, RIP). This allows other pro-apoptotic pathways (other death receptors, mitochondrial pathway) to become more active, leading to an enormous acceleration of cell death (pro-apoptotic amplification loop).

age is observed during apoptosis, converting Bcl-2 into an inducer of apoptosis [18]. Interestingly, both RIP and TRAF1 (see accompanying paper) conversion do not lead to their inactivation but rather to the formation of DN versions. We found that the C-terminal fragments of TRAF1 and RIP which are generated during apoptosis both interfere with TNF-induced activation of NF-κB. Thus, cleavage of only a fraction of TRAF1 and RIP can result in a complete blockade of the NF-κB-dependent anti-apoptotic pathway (Fig. 4).

Recent results show that NF-κB inactivation is also used to amplify pro-apoptotic signals that are triggered upon growth factor withdrawal [7]. In this case, inactivation occurs further downstream and is achieved by the cleavage of the NF-κB/Rel A subunit of NF-κB by caspase-3 and/or -6. NF-κB/Rel A processing may be complementary to the TRAF1/RIP inactivation pathway.

There is substantial cross-talk between pro-apoptotic and survival pathways. Our results suggest that cleavage of TRAF1 and RIP is part of an amplification loop which is triggered by Fas and most likely by other death receptors which trigger caspase-8 (or -10) activation. As a result, cross-sensitization between different pro-apoptotic signaling pathways occurs. For example, weak Fas signals will sensitize cells to signals by TNF and TRAIL. Considering that the NF-κB response leads to the protection against apoptosis by many different apoptotic stimuli, cross-sensitization to other stimuli such as those triggered by mitochondria and vice versa is likely to occur. Interestingly, we found that detachment of adherent cells leads to a highly increased sensitivity to FasL-induced cell death (unpublished data). Thus, the simultaneous inactivation of anti-apoptotic pathways controlled by NF-κB will ultimately result in a rapid cell death, thus avoiding the situation that a cell is half dead and half alive.

Acknowledgements: This work was supported by grants of the Swiss National Science Foundation (to J.T.).

References

[1] Wallach, D. (1997) Trends Biochem. Sci. 22, 107–109.
[2] Ashkenazi, A. and Dixit, V.M. (1998) Science 281, 1305–1308.
[3] Kelliher, M.A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B.Z. and Leder, P. (1998) Immunity 8, 297–303.
[4] Ting, A.T., Pimentel-Muinos, F.X. and Seed, B. (1996) EMBO J. 15, 6189–6196.
[5] Beg, A.A. and Baltimore, D. (1996) Science 274, 782–784.
[6] Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V. and Baldwin Jr., A.S. (1998) Science 281, 1680–1683.
[7] Levkau, B., Scatena, M. and Raines, E. (1999) Nat. Cell Biol. 1, 227–233.
[8] Peter, M.E., Kischkel, F.C., Scheuerpflug, C.G., Medema, J.P., Debatin, K.M. and Krammer, P.H. (1997) Eur. J. Immunol. 27, 1207–1212.
[9] Dudley, E., Hornung, F., Zheng, L., Scherer, D., Ballard, D. and Lenardo, M. (1999) Eur. J. Immunol. 29, 878–886.
[10] Thome, M. et al. (1997) Nature 386, 517–521.
[11] Burns, K. et al. (1998) J. Biol. Chem. 273, 12203–12209.
[12] Lin, Y., Devin, A., Rodriguez, Y. and Liu, Z. (1999) Genes Dev. 13, 2514–2526.
[13] Li, H., Zhu, H., Xu, C.J. and Yuan, J. (1998) Cell 94, 491–501.
[14] Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Cell 94, 481–490.
[15] Deak, J.C., Cross, J.V., Lewis, M., Qian, Y., Parrott, L.A., Distelhorst, C.W. and Templeton, D.J. (1998) Proc. Natl. Acad. Sci. USA 95, 5595–5600.
[16] Sakahira, H., Enari, M. and Nagata, S. (1998) Nature 391, 96–99.
[17] Ghayur, T. et al. (1996) J. Exp. Med. 184, 2399–2404.
[18] Kirsch, D.G. et al. (1999) J. Biol. Chem. 274, 21155–21161.