cIAP-1, but not XIAP, is cleaved by caspases during the apoptosis induced by TGF-β in fetal rat hepatocytes

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Received 8 April 2002; revised 20 April 2002; accepted 23 April 2002

First published online 13 May 2002

Edited by Jesus Avila

Abstract We have studied the expression of XIAP, cIAP-1 and cIAP-2 in fetal rat hepatocytes and its possible regulation by pro-apoptotic stimuli (transforming growth factor-β (TGF-β)) and survival signals (epidermal growth factor (EGF)). The three forms of inhibitor of apoptosis proteins (IAPs) are expressed in fetal hepatocytes and only cIAP-1, but not XIAP or cIAP-2, is cleaved during TGF-β-induced apoptosis. The pan-caspase inhibitor Z-VAD.fmk blocked this effect, which indicates that cIAP-1 is a caspase substrate. EGF plays a dual role in the regulation of IAPs expression. On one hand, it increases cIAP-1 and cIAP-2 basal expression and, on the other hand, it blocks the cleavage of cIAP-1 by caspases induced by TGF-β. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Inhibitor of apoptosis protein; Hepatocyte; Transforming growth factor-β; Epidermal growth factor; Apoptosis

1. Introduction

Apoptosis has evolved as a physiological cell death in response to environmental and developmental signals, with the mitochondria playing an essential role. The key to understanding apoptosis is the activation and function of a set of proteinases, the caspases (cysteine proteases with specificity for aspartic residues) [1–3]. The functions of caspases are modulated by another set of proteins, the IAPs (inhibitor of apoptosis proteins) [2–5]. The IAP family of proteins are characterized by a novel domain of 70 amino acids termed the baculoviral IAP repeat, the name of which derives from the original discovery of these apoptosis suppressors in the baculoviruses [6,7]. Although the estructural requirements for IAP function may vary, numerous reports strongly suggest that at least some IAP family members play a conserved role in the regulation of cell death. Consistent with this, the human IAP family members XIAP, cIAP-1 and cIAP-2 can directly inhibit specific caspases [8,9]. XIAP appears to be widely expressed [10], whereas cIAP-1 and cIAP-2 expression is increased following activation of the NFκB transcription factor [11]. Although evidence for a direct oncogenic role for the IAPs has to be delineated, a number of evidences point towards this class of proteins playing a role in oncogenesis [12].

Apoptotic stimuli must impair the IAPs ability to suppress the caspase cascade. In this context, it has been described that, during the apoptosis induced by Fas, XIAP is cleaved into two fragments, which have a significantly reduced anti-apoptotic efficiency compared with full-length XIAP [13]. Thus, cleavage of XIAP may be one mechanism by which cell death programs circumvent the anti-apoptotic barrier posed by XIAP. In this same line of evidence, cIAP-1 is cleaved by caspases to produce a pro-apoptotic C-terminal fragment [14].

Numerous observations suggest that transforming growth factor-β (TGF-β), an extracellular polypeptide important in growth control, development and differentiation [15], plays an important biological role mediating hepatocyte death [16]. Previous work from our group showed that treatment of fetal rat hepatocytes with TGF-β1 is followed by apoptosis [17]. TGF-β1 mediates radical oxygen species (ROS) production [17,18], that precedes the loss of $\Delta\Psi_m$, and the release of cytochrome *c* [19]. TGF-β disruption of the mitochondrial transmembrane potential and release of cytochrome *c* are blocked by epidermal growth factor (EGF) [19]. TGF-β1 activates at least caspase-3, -8 and -9 in hepatocytes [20–22], which are not required for ROS production, glutathione depletion, Bcl-x_L down-regulation and initial cytochrome *c* release [22]. However, caspase activation mediates cleavage of Bid and Bcl-x_L that originate an amplification loop on the mitochondrial events [22].

The expression of IAPS in the liver and its possible regulation by apoptotic stimuli, such as TGF-β, or survival signals, such as EGF, have not been explored yet. Only a report from Shima et al. indicates that XIAP is expressed in human hepatoma cells (HuH7) and it is down-regulated by TGF-β [23]. According to this, the aim of this work was to study the expression of XIAP, cIAP-1 and cIAP-2 in fetal rat primary hepatocytes and its possible regulation by TGF-β and apoptosis inhibitors, such as EGF.

2. Materials and methods

2.1. Isolation of rat fetal hepatocytes and culture

Hepatocytes from 20-day-old fetal Wistar rats were isolated by collagenase disruption [24], plated in arginine free medium 199, supplemented with ornithine (200 μmol/l), fetal calf serum (10%), penicillin (120 μg/ml) and streptomycin (100 μg/ml) and incubated as previously described [19].

2.2. Western blot analysis

Supernatant cells and attached cells (after being scraped off in phosphate-buffered saline) were pelleted by centrifugation and resus-

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Abbreviations: EGF, epidermal growth factor; IAP, inhibitor of apoptosis proteins; TGF-β, transforming growth factor-β

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02774-6

pended in a lysis buffer (25 mmol/l HEPES, pH 7.5, 0.3 mol/l NaCl; 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l dithiothreitol (DTT), 0.1% Triton X-100; 20 mmol/l β-glycero-phosphate, 0.1 mmol/l Na_3VO_4, 2 μg/ml leupeptin and 1 mmol/l phenylmethylsulfonyl fluoride) during 10 min at 4°C. Then samples were clarified by centrifugation at 12 000×*g* for 10 min at 4°C. Anti-cIAP-1 and anti-cIAP-2 polyclonal antibodies were from R&D Systems, anti-XIAP monoclonal antibody was from Transduction Laboratories, anti-β-actin monoclonal antibody was from Sigma.

2.3. Analysis of nuclear DNA content by flow cytometry

The ploidy determination of hepatocytes was estimated by flow cytometry DNA analysis. Cells were detached by trypsinization, fixed in methanol (−20°C) for 1 min and treated with RNase (10 μg/ml) for 30 min at 37°C. The DNA content per cell was then evaluated in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) after staining cells with propidium iodide (0.05 mg/ml) for 15 min at room temperature in the dark.

2.4. Analysis of caspase-3 activity

Cells were lysed as previously described [22]. Protein concentration of cell lysates was determined by Bio-Rad protein assay kit. Reaction mixtures contained 20 mmol/l HEPES pH 7.0, 10% glycerol, 2 mmol/l DTT, 30 μg protein/condition and 20 μmol/l Ac-DEVD-AMC substrate (Pharmingen). After 2 h of incubation in the dark, enzymatic activity was measured in a Microplate Fluorescence Reader FL600 (Bio-Tek). A unit of caspase-3 activity is defined as the amount of active enzyme necessary to produce an increase of one arbitrary unit in the luminescence spectrophotometer after 2 h.

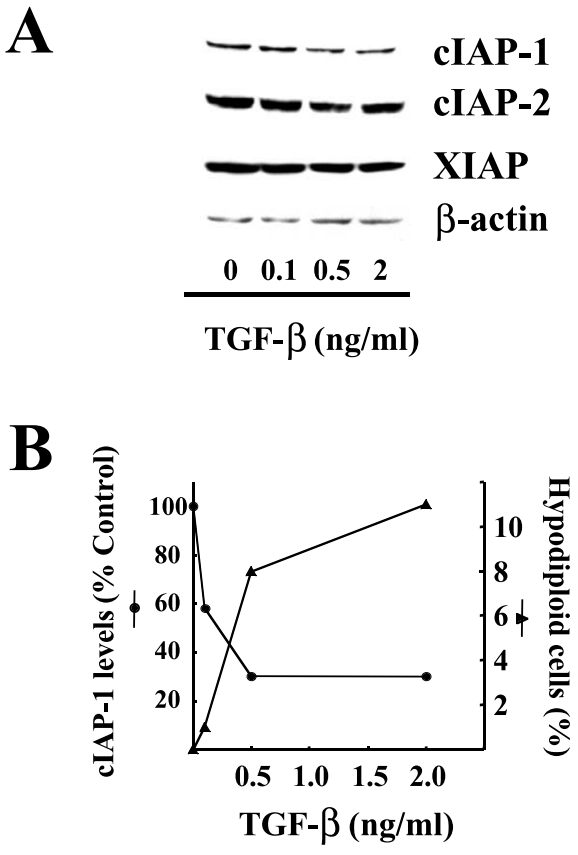

Fig. 1. Effect of TGF-β on IAPs family proteins in fetal rat hepatocytes. A: Dose response effect of TGF-β on cIAP-1, cIAP-2 and X-IAP protein levels (analyzed by Western blot), after incubation of cells for 15 h with the different concentrations of TGF-β. A representative experiment of three is shown. β-Actin is analyzed as loading control. B: Dose response effect of TGF-β on cIAP-1 protein levels as compared with the analysis of apoptosis, i.e. the appearance of hypodiploid cells. The densitometric analysis of cIAP-1 protein level (corrected by β-actin content and expressed as percentage of untreated cells) of a representative experiment ($n=3$) is shown. Parallel dishes in the same experiment were processed for analysis of nuclear DNA content by flow cytometry, to calculate the percentage of hypodiploid (apoptotic) cells. A representative experiment of three is shown.

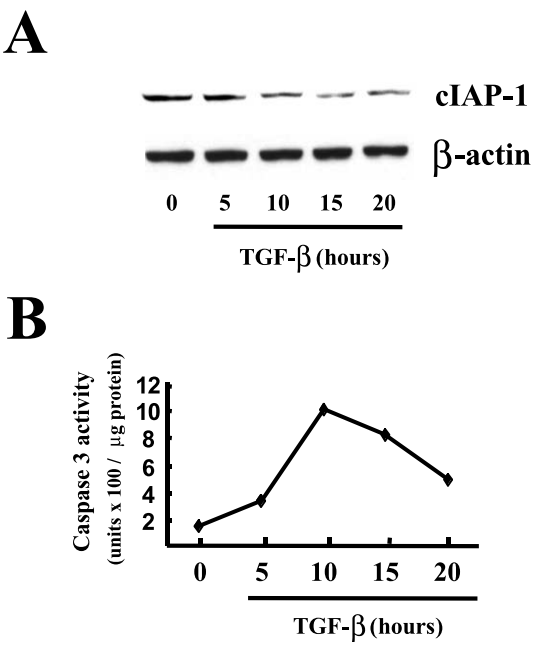

Fig. 2. Time course effect of TGF-β on cIAP-1 protein levels in fetal hepatocytes. A: Cells were incubated without (0) or with 2 ng/ml of TGF-β for different periods of time. Then, proteins were extracted and cIAP-1 protein levels were analyzed by Western blot. β-Actin is analyzed as loading control. A representative experiment of four is shown. B: Time course analysis of caspase-3 activation. Cells from parallel dishes in the experiment shown in A were lysed and caspase-3 was assayed. Results are expressed as units×100/μg protein. A representative experiment of three is shown.

3. Results and discussion

We have analyzed the presence of XIAP, cIAP-1 and cIAP-2 in fetal rat hepatocytes in primary culture. Under control conditions the three members of the IAP family were clearly expressed, cIAP-1 being the less abundant form (Fig. 1A). In response to TGF-β, and in a dose-dependent manner, cIAP-1 protein levels decreased, without change in the XIAP and cIAP-2 forms (Fig. 1A). Dose-dependence for cIAP-1 regulation was coincident with the appearance of hypodiploid cells, characteristic of an apoptotic process (Fig. 1B). A detailed timing analysis demonstrated that cIAP-1 levels decreased after 10 h of incubation in the presence of TGF-β. According to previous results of our group [22], at that time cytochrome *c* has been released from mitochondria and caspases must be activated. Thus, as it is shown in Fig. 2B, activity of caspase-3 reached a maximum at 10 h of TGF-β treatment.

Since cIAP-1 expression is under control of NFκB [11] and treatment of fetal hepatocytes with TGF-β produces NFκB inactivation [25], cIAP-1 levels could be under transcriptional control. However, taking into account that cIAP-1 is a caspase substrate [14], regulation of cIAP-1 protein levels could also be posttranslational. To solve this question, we incubated fetal rat hepatocytes in the presence of a pan-inhibitor of caspases: Z-VAD.fmk (Fig. 3). The result was very clear:

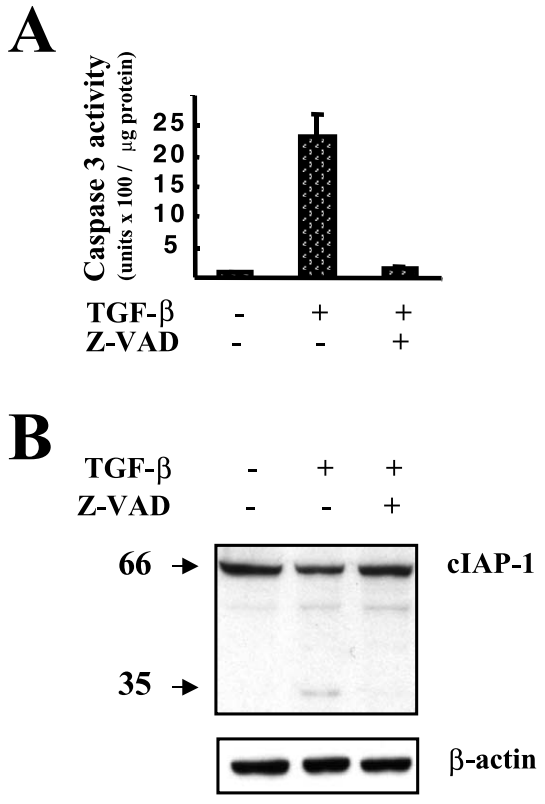

Fig. 3. cIAP-1 is cleaved by caspases during the apoptosis induced by TGF-β in fetal hepatocytes. A: Fetal hepatocytes were incubated in the absence or presence of TGF-β (2 ng/ml) or TGF-β (2 ng/ml)+Z-VAD.fmk (20 μmol/l, added 1 h before TGF-β) for 15 h. After this time, cells were lysed and caspase-3 activity was assayed. Results are expressed as units×100/μg protein and are mean ± S.E.M. of three independent experiments with duplicate dishes. B: Cells were incubated under the same conditions described in A. Then, proteins were extracted and cIAP-1 protein levels were analyzed by Western blot. β-Actin content is analyzed as loading control. A representative experiment of three is shown.

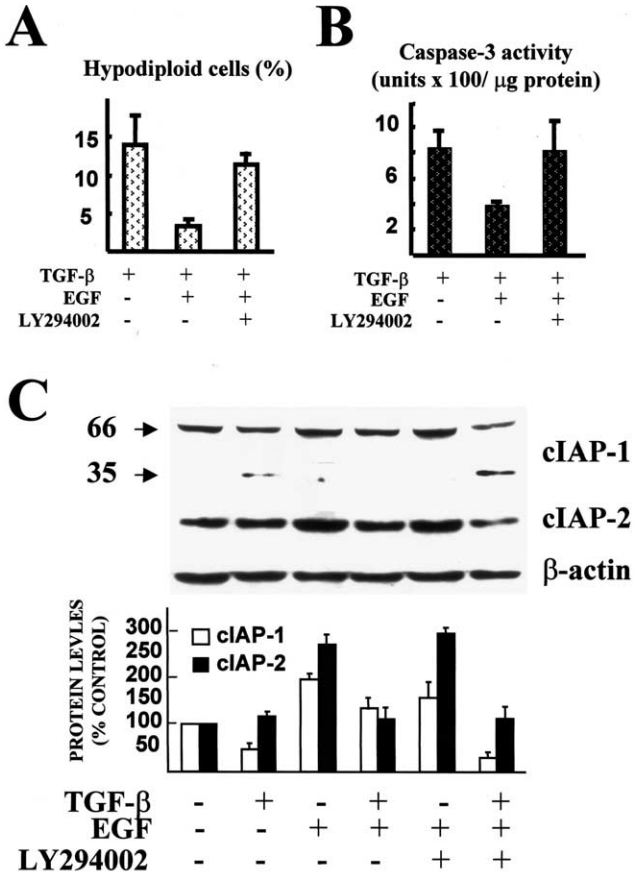

Fig. 4. Effect of EGF on IAPs expression. Role of PI 3-kinase. Fetal hepatocytes were incubated for 15 h in the absence (control cells) or presence of 2 ng/ml TGF-β and/or EGF (20 ng/ml) and/or LY294002 (a PI 3-kinase inhibitor used at 20 μmol/l and added 1 h before TGF-β and EGF), as indicated. A: Cells were detached by trypsinization and cell DNA content was analyzed by flow cytometry. Results are expressed as percentage of hypodiploid cells, and are mean ± S.E.M. of three independent experiments. B: Cells were lysed and caspase-3 assayed. Results are expressed as units×100/μg protein, and are mean ± S.E.M. of three independent experiments with duplicate dishes. C: Cells were lysed to analyze cIAP-1 and cIAP-2 protein levels by Western blot. In the case of cIAP-1, the proteolytic fragment is also shown. β-Actin content is analyzed as loading control. A representative experiment of three is shown. Densitometric analysis of Western blots was used to calculate the protein levels (corrected by β-actin content and expressed as percentage of untreated cells). Means ± S.E.M. of the three independent experiments are shown.

under conditions where Z-VAD.fmk completely blocked caspase-3 activity (Fig. 3A), the decrease in the cIAP-1 protein levels was also completely abolished (Fig. 3B). A detailed observation of the Western blot analysis revealed that under treatment with TGF-β, cIAP-1 appeared to be cleaved, showing a band of 35 kDa, which has been proposed to have pro-apoptotic activity [14]. In the presence of Z-VAD.fmk, this band completely disappeared (Fig. 3B). This is a clear demonstration that cIAP-1 is a substrate of caspases during the apoptosis induced by TGF-β in fetal hepatocytes. This event might contribute to a caspase-mediated amplification loop on the apoptotic events.

An intriguing result is that XIAP is not modulated by TGF-β in fetal rat hepatocytes, i.e. XIAP levels remain intact during the apoptosis induced by TGF-β. XIAP has been reported as a cofactor in the TGF-β superfamily signaling [26,27]. XIAP, but not the related proteins cIAP-1 and cIAP-2, associates with several members of the type I class of the TGF-β receptor and increases TGF-β-induced signaling [27]. This role for XIAP in TGF-β-mediated signaling appears to be distinct from and independent of its anti-apoptotic functions. Thus, XIAP might be necessary for the response of fetal hepatocytes to TGF-β.

The apoptosis induced by TGF-β in fetal hepatocytes is impaired by EGF [28], via a PI 3-kinase-dependent pathway [29]. Furthermore, EGF activates NFκB in fetal rat hepatocytes (Fabregat et al., unpublished observations). All these data pointed towards EGF as a possible regulator of cIAP-1 expression and/or cleavage. Results presented in Fig. 4 show that when fetal hepatocytes were incubated in the presence of both TGF-β and EGF apoptosis was impaired (Fig. 4A), and activation of caspase-3 notably attenuated (Fig. 4B). This impairment of apoptosis was completely blocked in the presence of LY294002, an inhibitor of PI 3-kinase activity. When we studied the protein levels of IAPs, we observed that EGF by itself was able to produce a significant increase in cIAP-1 and cIAP-2 (Fig. 4C). In contrast, it had no significant effect on XIAP protein levels (results not shown). The increase in both

cIAPs was not blocked by the presence of LY294002, which indicates that PI 3-kinase is not mediating this effect. Furthermore, in the case of cIAP-1, EGF was also able to abolish the loss of protein caused by TGF-β and the appearance of the proteolytic fragment of 35 kDa (Fig. 4C), this effect being completely dependent on PI 3-kinase. These results indicate that EGF plays a dual role on the regulation of IAPs. Firstly, this growth factor increases the basal levels of cIAP-1 and cIAP-2, the two members of the family that are under transcriptional control by NFκB [11]. Secondly, PI 3-kinase activation by EGF is responsible for the maintenance of cIAP-1 levels, even when TGF-β is present, probably through its ability to block caspase activation (Fig. 4B).

In conclusion, we describe that fetal rat hepatocytes express cIAP-1, cIAP-2 and XIAP and only cIAP-1, but not XIAP or cIAP-2, is substrate of caspases during the apoptosis induced by TGF-β. EGF plays a dual role in the regulation of these IAPs expression. On one hand, it blocks the cleavage of cIAP-1 by caspases and, on the other hand, it increases cIAP-1 and cIAP-2 basal expression. It is worthy to note that the less abundant form, cIAP-1, and one of the less effective in inhibiting caspases [30], is the form that is proteolysed by TGF-β. Probably this event can form part of an amplification loop initiated by caspases where cIAP-1 is transformed into a pro-apoptotic form [14]. In this same line of evidence, we have recently described that also Bcl-x_L and Bid are substrates of the caspases activated by TGF-β [22]. The absence of XIAP modulation could indicate that XIAP is essential for the TGF-β signaling, as has previously been suggested [26,27]. However, taking into account its high expression, other cellular events initiated by TGF-β must inactivate its anti-apoptotic activity. Future efforts must be done to clarify the possible appearance in the cytosol of some inhibitors of XIAP, such as DIABLO/Smac [31] or XAF1 [32], which can contribute to the activation of caspases by TGF-β.

Acknowledgements: The authors wish to thank Dr. Joan Gil (Universidad de Barcelona, Spain) for providing antibodies and setting up some of the techniques used in this work and Dr. A.M. Álvarez for expert assistance with the flow cytometer. This work was supported by grants from the Comunidad de Madrid, Spain (CAM0078/2000), and Ministerio de Sanidad y Consumo, Spain (FIS 01/0797). B.H. was recipient of a fellowship from the Ministerio de Educación y Cultura, Spain.

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