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Transcriptional and translational control of Mcl-1 during apoptosis

Daniel Iglesias-Serret, Maria Piqué,[1] Joan Gil, Gabriel Pons, and José M. López*,[1]

Unitat de Bioquímica, Departament de Ciències Fisiològiques II, Universitat de Barcelona, Campus de Bellvitge, E-08907 L'Hospitalet, Spain

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Abstract

Mcl-1 is an antiapoptotic member of the Bcl-2 family whose protein and mRNA have a short half-life. In this report, we studied the changes in Mcl-1 protein and mRNA expression induced by staurosporine and aspirin. Both drugs induced apoptosis in Jurkat cells and reduced the levels of Mcl-1 protein. The caspase inhibitor Z-VAD.fmk and the proteasome inhibitor MG132 partially protected Mcl-1 from decay, indicating that both caspase-dependent and proteasome pathways are involved during apoptosis. Staurosporine also reduced Mcl-1 mRNA levels and this reduction was mostly caspase-dependent. In addition, staurosporine reduced the transcriptional activity of the Mcl-1 promoter fused to a luciferase gene reporter more than actinomycin D, a general inhibitor of transcription. Thus, we conclude that staurosporine down-regulates Mcl-1 mRNA levels by inhibiting transcription in a caspase-dependent manner and reduces Mcl-1 protein levels by a caspase-independent post-transcriptional mechanism. In contrast aspirin, at doses and times that induced loss of viability and decay of Mcl-1 protein, had no effect on Mcl-1 mRNA levels. Aspirin rapidly inhibited de novo protein synthesis before caspase activation. Moreover, the translational factor eIF2α was transiently phosphorylated and therefore inhibited very soon after aspirin treatment. Aspirin also inhibited the luciferase reporter activity of several attached promoter constructs, but it did not affect the luciferase activity of a construct containing an internal ribosome entry site (IRES) in its mRNA 5′UTR. We conclude that staurosporine inhibits transcription and translation, whereas aspirin only inhibits cap-dependent translation. Treatment with cycloheximide, at doses that inhibit protein synthesis without affecting cell viability, also induced Mcl-1 protein decay. Mcl-1 disappearance might be necessary but not sufficient for the induction of apoptosis by staurosporine and aspirin. A model for the control of Mcl-1 during drug-induced apoptosis is presented.

Keywords: Mcl-1; Apoptosis; Translational control; Transcription; Aspirin

Mcl-1 is an early-induction gene that was originally isolated from the ML-1 human myeloid leukemia cell line during phorbol ester-induced differentiation along the monocyte/macrophage pathway [1]. The mRNA and protein levels of Mcl-1 increase in response to a variety of cell growth, differentiation, and activation factors but it is also rapidly turned over within the cell [2–5]. Mcl-1 mRNA and protein levels are rapidly and transiently increased during the early stages of TPA-induced ML-1 differentiation [1–3] and are also up-regulated by the granulocyte–macrophage colony-stimulating factor (GM-CSF), by interleukin 3 (IL-3) [6], and by gonadotropins in the rat ovary [7]. An increase in the Mcl-1 protein levels usually correlates with an increase in the mRNA levels, mostly due to enhanced transcription [3]. Some regulatory sequences have been identified in the Mcl-1 promoter: an upstream sequence between −197 and −69 is responsible for cytokine activation of the Mcl-1 mouse gene [6], and a 162-bp segment of the human Mcl-1 promoter mediates transcriptional induction by TPA [8]. IL-3 stimulation of the Mcl-1 mouse promoter is mediated through modulation of at least two transcription factors, the CRE-2 and SIE binding proteins [9]. The Mcl-1 gene knock out in mice results in the failure of embryos to implant, indicating that it plays a key role in mammalian development [10]. Overexpression of Mcl-1 prolongs the survival of cells exposed to a variety of apoptosis-inducing stimuli, including cytokine withdrawal, c-myc overexpression, staurosporine, etoposide, calcium ionophore, and UV irradiation [6,11–15], and depletion of Mcl-1 by

* Corresponding author. Fax: +34-93-224-0899.
E-mail address: jose.lopez@crg.es (J.M. López).
[1] Present address: Centre de Regulació Genòmica, Programa d'Expressió Gènica, Pg. Marítim 37-49, 08003, Barcelona, Spain.

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antisense oligonucleotides facilitates entry into apoptosis [6,15–21]. As expected for a short-term survival regulator, Mcl-1 protein expression decreases when cells undergo apoptosis with various stimuli [6,22–28]. Most studies of the down-regulation of Mcl-1 during apoptosis analyse the protein but not the mRNA levels. Therefore, it is difficult to assess whether down-regulation of Mcl-1 is due to transcriptional or translational repression. It is also unclear whether the mechanisms involved are dependent on caspase activation. In order to further clarify and investigate this subject, we have studied the effect of two apoptotic agents, staurosporine and aspirin, on Mcl-1 protein and mRNA levels, as well as the role of caspase activation in both conditions. Aspirin induces apoptosis in many cell types, including colon cancer cells [29] and leukemia cells [26,30,31]. Although the target of aspirin that mediates its apoptotic effect is unknown, aspirin induces apoptosis through mitochondrial cytochrome *c* release [30]. Staurosporine is a protein kinase inhibitor that induces apoptosis in most cell types. Both aspirin [26] and staurosporine [27] have been reported to induce a decrease in the levels of Mcl-1. In this report, we show that staurosporine decreases both Mcl-1 mRNA and protein levels, whereas aspirin mediates a reduction in the Mcl-1 protein without any apparent change in the amount of mRNA. Surprisingly, aspirin rapidly inhibits protein synthesis and induces transient phosphorylation, and thus inhibition, of the translation factor eIF2α[2] before caspase activation. eIF2α is a subunit of the eIF2 protein complex, which is essential for recruiting $tRNA_i^{Met}$ to the 40S ribosomal subunit and initiating mRNA translation. These results point to the importance of translational control of Mcl-1 during apoptosis.

Materials and methods

Reagents

Aspirin (acetylsalicylic acid), staurosporine, actinomycin D, cycloheximide, propidium iodide (PI), and MG-132 (Z–Leu–Leu–Leu–al) were from Sigma Chemicals (St. Louis, MO). Z-VAD.fmk (benzyloxycarbonyl–Val–Ala–Asp–fluoromethyl ketone) was from Bachem AG (Bubendorf, Switzerland). L-[4,5-^{3}H]Leucine was from Perkin–Elmer Life Sciences (Boston, MA). Staurosporine (1 μM) was dissolved in DMSO. Aspirin (10 mM) was dissolved in ethanol and neutralized to pH 7 with 4 M NaOH. The concentrations used have been described to induce apoptosis in Jurkat cells [30,32].

Cell culture

The human leukemia T cell line Jurkat was from the European Collection of Cell Cultures. Cells were grown in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Paisley, UK), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere at 5% carbon dioxide.

Analysis of viability by phosphatidylserine exposure and PI uptake

Cell viability was determined by analysing phosphatidylserine exposure and membrane integrity, by double staining with Annexin V-FITC (Bender MedSystems, Vienna, Austria) and PI (Sigma Chemicals), prior to flow cytometric analysis (FACSCalibur and the CellQuest software, Becton Dickinson, Mountain View, CA). Cells (150,000–300,000) were washed with PBS, resuspended in 150 μl of annexin binding buffer, and incubated with 0.4 μl of Annexin V-FITC. After 20 min of incubation in the dark at room temperature, 150 μl of annexin binding buffer with 3 μl of propidium iodine (PI) (50 μg/ml) was added just before flow cytometric analysis. Cell viability was measured as the percentage of annexin V and PI negative cell population.

Plasmids

The human XIAP 5′UTR (−3999 to −5) was obtained from a genomic clone (RPCI1, "dJ"315G1, The Sanger Center) by PCR amplification with the primers: forward (CGGAGCTCGTTGAATGATGGTTTTAT CTAA), reverse (CGAAGCTTTCTTGAAAATAGGA CTTGTCCA), and was cloned into pGL2-Basic plasmid (Promega). The rat fatty acid synthase (FAS) promoter (−1594 to +65) was obtained as previously described [33]. The mouse Mcl-1 promoter–luciferase construct (−2400 to +10) was kindly supplied by Dr. H.-F. Yang-Yen [6]. The cytomegalovirus (CMV) luciferase construct was supplied by Dr. Lee A. Witters.

Transient transfection and luciferase assay

Jurkat cells were transiently transfected by electroporation. Approximately 10×10^6 cells were collected per electroporation, washed with RPMI, and resuspended in 0.65 ml of complete media without antibiotics. The cell suspension was mixed with 2 μg of the green fluorescent protein reporter plasmid pEGFP-C1

[2] *Abbreviations used:* eIFs, eukaryotic initiation factors; IRES, internal ribosome entry site; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; XIAP, X-linked inhibitor of apoptosis protein; Z-VAD.fmk, benzyloxycarbonyl–Val–Ala–Asp–fluoromethyl ketone.

(Clontech, Palo Alto, CA), together with 10 μg of the luciferase reporter plasmid of interest. Cells were electroporated in 0.4 cm gap cuvettes at 250 V, 975 μF, and 72 Ω by using an Electro cell manipulator 600 (BTX, San Diego, CA) and returned to 10 ml of complete media. After 24 h of incubation, transfection efficiency was determined measuring the green fluorescence mean of each sample by flow cytometry. At the same time, the remaining cells were treated with various factors, as indicated, and luciferase activity was assessed by lysing cells in Cell Culture Lysis Reagent (Promega) (2.5×10^6 cells in 150 μl) and mixing 40 μl of cellular lysates with 100 μl of Luciferase Assay Reagent (Promega). Light emission was recorded with the TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) at 10 s integration time. The protein concentration of each cellular lysate was also determined with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Unless otherwise indicated, the luciferase activity data correspond to the luciferase units measured and normalized to the transfection efficiency and the protein concentration of each sample.

Northern blot analysis

For each condition, between 10 and 15×10^6 cells were treated with the indicated drug of study. Total RNA was isolated from cultured cells with the Ultraspec RNA isolation system kit (Biotecx, Houston, TX). RNA was denatured in formamide and formaldehyde and electrophoresed through formaldehyde-containing agarose gels. RNA was blotted to nylon transfer membranes (Hybond-N^+, Amersham), fixed 2 h at 80 °C, and hybridized overnight with ^{32}P-labelled probes at 42 °C. Blots were washed in 0.1% SSC, 0.1% SDS, at 50–60 °C. To increase the transfer of the high size XIAP mRNA, an alkaline treatment (0.05 M NaOH and 1.5 M NaCl, for 15 min) and neutralization (0.5 M Tris–HCl, pH 7.5, 1.5 M NaCl) of the gel was performed before blotting to nylon membranes. cDNA probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol) by the random-priming method (Amersham) to a specific activity of at least 10^9 cpm/μg.

Western blot analysis

Whole-cell protein extracts were obtained by lysing cells with Laemmli sample buffer. Protein was measured by the Micro BCA assay reagent and 50 μg of protein was loaded in each lane. The protein concentration slightly decreases when cell viability decreases and for that reason we loaded equal amounts of protein in each condition. Bcl-2 is a good control for protein loading because it has a very long half-life [34,35]. Fifty micrograms of the protein extract was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h with 5% dried skimmed milk in TBST (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) and were incubated with the following antibodies: polyclonal anti-Mcl-1 (sc-819), polyclonal anti-Id1 (c-20, sc-488), polyclonal anti-eIF2α (sc-7629) (Santa Cruz Biotechnology), polyclonal anti-caspase-3 (Transduction Laboratories, Lexington, KY), polyclonal anti-caspase-9 (New England Biolabs), monoclonal anti-Bcl-2 (Dako A/S, Glostrup, Denmark), polyclonal anti-PARP (Boehringer–Mannheim), monoclonal anti-XIAP (Transduction Laboratories), and polyclonal anti-Phospho-eIF2α (Ser 51) (Cell Signaling Technology, USA). Antibody binding was detected with horseradish peroxidase-coupled secondary antibody and the enhanced chemiluminescence (ECL) detection kit (Amersham, Bucks, UK).

[^{3}H]Leucine incorporation

Jurkat cells (2×10^6) were isolated by centrifugation and incubated in RPMI 1640 medium containing [^{3}H]leucine (42.50 Ci/mol) in the presence or absence of aspirin (10 mM) for 6 h. Cells were collected at different times by centrifugation and washed in PBS with cold leucine (0.5 mg/ml). Cells were lysed with Igepal buffer (20 mM Tris–HCl, pH 7.6, 10 mM potassium acetate, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, and 0.25% Igepal), spun 30 min at 12,000*g* at 4 °C, and the supernatant was separated. One aliquot was used to measure total protein content and the rest were precipitated with TCA (10% final concentration). After centrifugation (30 min at 12,000*g* at 4 °C) the supernatant (soluble fraction) was collected. The pellet was resuspended in 100 μl of 0.2 M NaOH (TCA fraction). Biogree 103 scintillation liquid was added in both the soluble and the TCA fraction and cpm counted in a Wallac 1409 liquid scintillation counter. Results were expressed as cpm TCA fraction/(cpm soluble fraction × mg protein).

Results

Down-regulation of Mcl-1 protein levels during apoptosis

We analysed by Western blot the expression of Mcl-1 and Bcl-2 in Jurkat cells treated with staurosporine (1 μM) or aspirin (10 mM). As shown, staurosporine (Fig. 1A) and aspirin (Fig. 1B) significantly reduced the protein levels of Mcl-1 at 0.5 and 3 h, respectively. However, changes in the levels of Bcl-2 were negligible. The viability of the cells was determined during the time-course assay showing a progressive decrease, which was more evident for staurosporine treated cells. We assayed the effect of the proteasome inhibitor MG132 in Mcl-1 protein levels during aspirin and staurosporine-induced

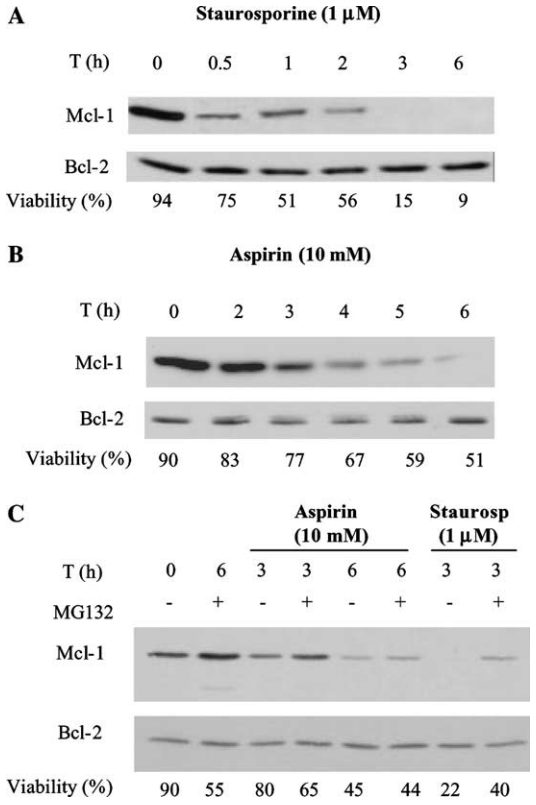

Fig. 1. Mcl-1 protein is down-regulated during apoptosis. Jurkat cells (0.5×10^6 cells/ml) were treated with staurosporine (1 μM) (A) or with aspirin (10 mM) (B) for different times and collected (2×10^6 cells) at the same moment for Western blot analysis as described in Materials and methods. (C) The proteasome inhibitor MG132 partially blocks the decrease of Mcl-1 during apoptosis. Jurkat cells were incubated with staurosporine (1 μM) or with aspirin (10 mM) alone or in the presence of MG132 (2.5 μg/ml) for different times and assayed as before. Fifty micrograms of protein was assayed for the presence of Mcl-1 and Bcl-2 by using specific antibodies. Viability, expressed as the percentage of annexin V and PI negative cells, is shown for each time at the bottom of the figure.

apoptosis. MG132 partially blocked Mcl-1 protein decay (Fig. 1C). However, MG132 also induced a significant decrease of cell viability (lane 2, bottom line), which was not incremented by the drug treatment. These results indicate that Mcl-1 is degraded, at least in part, by the proteasome pathway (see Discussion).

Down-regulation of Mcl-1 protein during apoptosis is only partially protected by Z-VAD.fmk

To address the contribution of caspases to the mechanism of Mcl-1 down-regulation during apoptosis, we used the broad caspase inhibitor Z-VAD.fmk. We analysed by Western blot the expression of Mcl-1 in cells treated with staurosporine or aspirin in the presence or absence of Z-VAD.fmk (200 μM). We analysed caspase-3 disappearance as a parameter of caspase activation. For aspirin-treated cells, we also measured the activation of caspase-9 since caspase-3 disappearance was not clear at short times of incubation. The decrease of Mcl-1 protein during apoptosis was partially protected by Z-VAD.fmk in both staurosporine (Fig. 2A) and aspirin (Fig. 2B) treated cells. Bcl-2 did not change in any condition assayed and was thus used as a control for protein loading. The efficiency of Z-VAD.fmk was evident since caspase-3 activation, determined by the disappearance of the precursor form, was largely blocked in the presence of the inhibitor. Caspase-9 proteolysis, determined by the appearance of the intermediate cleavage product of 37 kDa, was observed 3 h after aspirin treatment and efficiently protected by Z-VAD.fmk (Fig. 2B). Z-VAD.fmk also protected from the decrease of viability, measured by the exposure of phosphatidylserine, during apoptosis. The Mcl-1 protein was quantified by densitometry from Figs. 2A and B and corrected by the Bcl-2 protein levels (Fig. 2C). Altogether, these results demonstrate that the decay of Mcl-1 is not merely due to caspase activation, but instead both caspase-independent and caspase-dependent pathways are responsible for Mcl-1 down-regulation during apoptosis.

Expression of Mcl-1 mRNA during staurosporine-induced apoptosis

The reduction of Mcl-1 protein levels during apoptosis can be explained either by transcriptional or translational inhibition, since both the protein and mRNA have very short half-life [2,3]. We measured the Mcl-1 mRNA levels by Northern blot. A major band of 3.8 kb and a minor band of 2.5 kb have been described previously for human samples [1]. The 2.5 kb transcript was difficult to detect in Jurkat cells and its regulation followed the same pattern as that of the 3.8 kb transcript (data not shown). As shown in Fig. 3A, staurosporine treatment reduced mRNA levels in parallel to Mcl-1 protein decay, suggesting that staurosporine inhibits transcription of Mcl-1 gene in Jurkat cells. However, these results do not rule out the possibility that Mcl-1 mRNA levels may be influenced by processing or stability changes during staurosporine-induced apoptosis. Next, we examined whether down-regulation of Mcl-1 mRNA by staurosporine was caspase-dependent. As shown in Fig. 3B, Z-VAD.fmk inhibited staurosporine-induced mRNA decay. In contrast, the degradation of Mcl-1 protein was only partially protected by Z-VAD.fmk indicating that a caspase-independent post-transcriptional mechanism/s might be involved in Mcl-1 down-regulation. To provide further insight into the transcriptional regulation of Mcl-1 during apoptosis induced by staurosporine, we transfected Jurkat cells with the Mcl-1 mouse promoter (−2400 to +10) fused to the luciferase gene reporter and we compared the time-course decay of luciferase activity after incubation with staurosporine or actinomycin D, a known inhibitor of

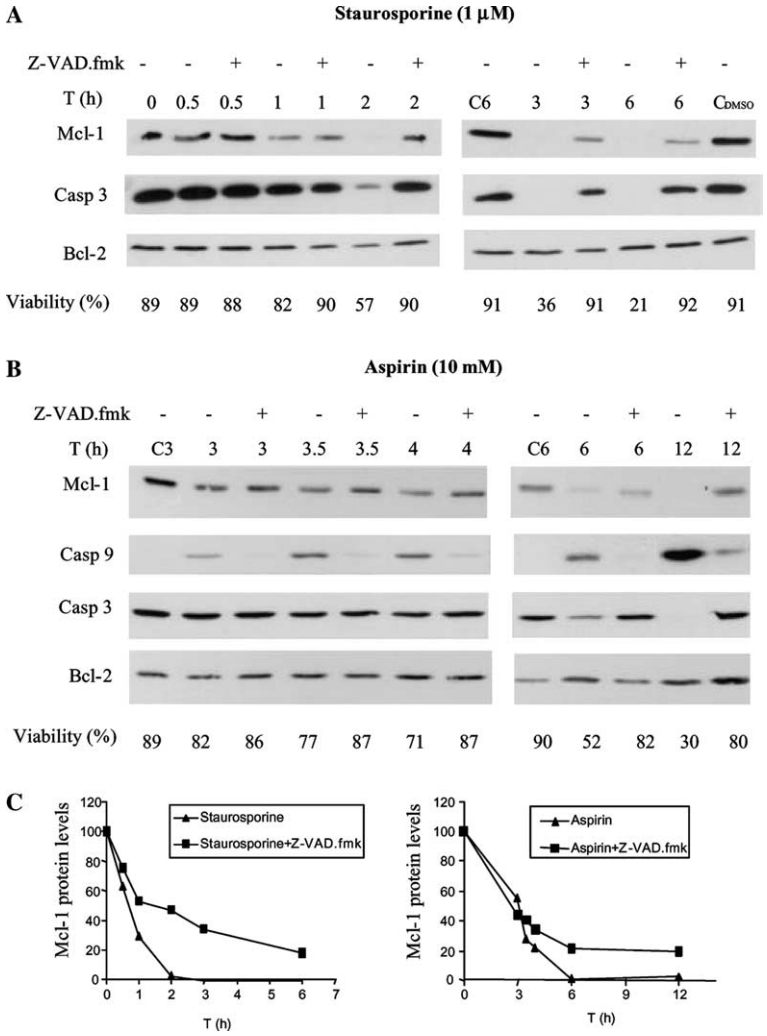

Fig. 2. Z-VAD.fmk partially protects Mcl-1 protein decay during apoptosis. Jurkat cells were treated with staurosporine (1 μM) (A) or with aspirin (10 mM) (B) alone or in the presence of Z-VAD.fmk (200 μM) for different times and collected at the same moment for Western blot analysis. Fifty micrograms of protein was assayed for the presence of Mcl-1, caspase-3, caspase-9, and Bcl-2 with specific antibodies. Cells incubated for 3 or 6 h without any drug (C3, C6) or with DMSO, the solvent of staurosporine, (C_{DMSO}) were used as additional controls. Viability, expressed as the percentage of annexin V and PI negative cells, is shown for each time at the bottom of the figure. (C) Mcl-1 protein levels were quantified by densitometry from (A) and (B) and corrected by the Bcl-2 protein levels.

transcription. As shown in Fig. 3C, the luciferase activity decreased with both treatments, but it was more marked with staurosporine. The combination of staurosporine plus actinomycin D had the same effect as the incubation with staurosporine alone (data not shown). The concentration of actinomycin D used in this experiment (5 μg/ml) has been reported to inhibit transcription in Jurkat cells [36] without affecting cell viability for short times of incubation [37]. Accordingly, the viability of Jurkat cells was not reduced significantly during two hours of incubation with actinomycin D, whereas staurosporine reduced it (Fig. 3C). However, longer times of incubation with actinomycin D significantly reduced viability of Jurkat cells (data not shown). These data, together with the previous results, suggest that staurosporine inhibits transcription of the Mcl-1 promoter construct, inducing down-regulation of luciferase activity. Moreover, a post-transcriptional effect might also be present since the effect of staurosporine is more marked than that of actinomycin D.

Aspirin inhibits protein synthesis in Jurkat cells and induces eIF2α phosphorylation

Inhibition of protein synthesis can be important in the control of cell death if we consider anti-apoptotic proteins with short half-life, like Mcl-1. Aspirin had no effect on Mcl-1 mRNA levels over the 8 h period of incubation, whereas the protein decayed soon (Fig. 4A). This result suggests a translational inhibition of Mcl-1 mRNA during aspirin-induced apoptosis. Alternatively, the stability of Mcl-1 protein might

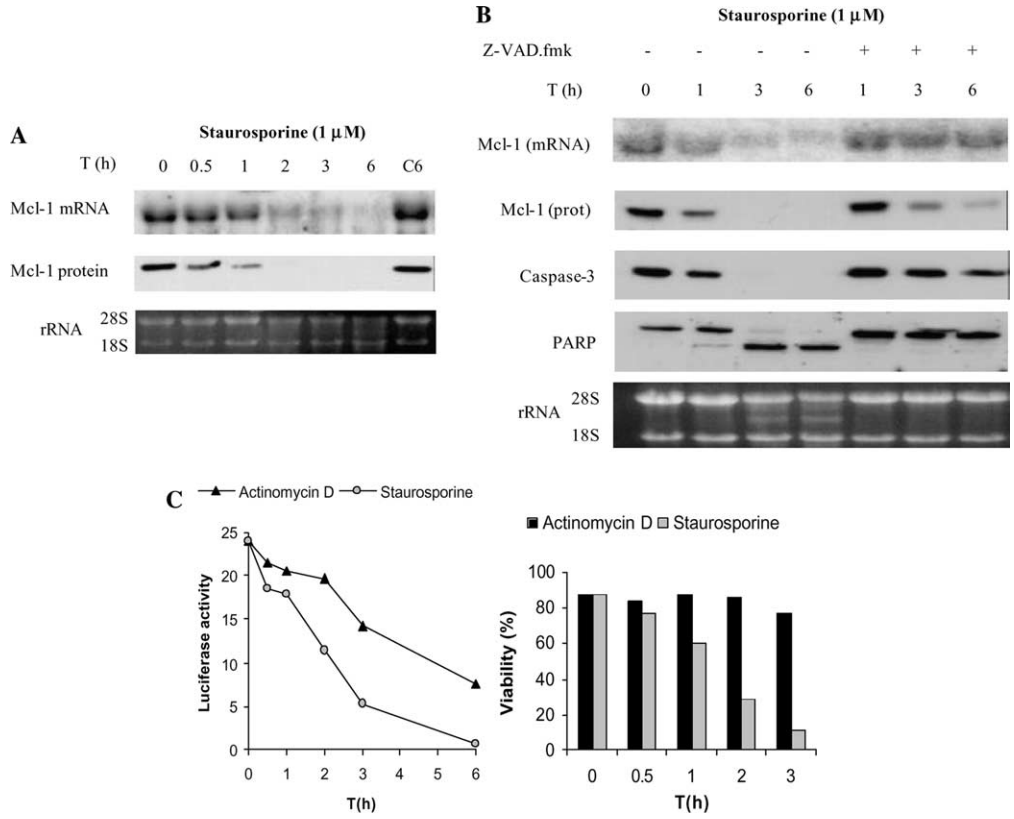

Fig. 3. Expression of Mcl-1 mRNA during staurosporine-induced apoptosis. (A) Jurkat cells were treated with staurosporine for different times and collected at the same moment (10×10^6cells per condition). Total RNA was isolated as described in Materials and methods and 10 μg per lane was assayed by Northern blot with a Mcl-1 ^{32}P-labelled cDNA probe. Ethidium bromide-stained 28 and 18S rRNAs are shown as a loading control. Fifty micrograms of protein was assayed by Western blot for the presence of Mcl-1. One representative experiment of three is shown. C6: Jurkat cells incubated for 6 h without staurosporine. (B) Jurkat cells were treated with staurosporine (1 μM) with or without Z-VAD.fmk (200 μM) for different times and collected at the same moment. Ten micrograms of total RNA was loaded in each lane and Mcl-1 mRNA was determined by Northern blot using a specific ^{32}P-labelled cDNA probe. Ribosomal RNAs are shown as a loading control. Samples were also collected for Western blot analysis and 50 μg of protein was loaded in each lane and assayed for the presence of Mcl-1, caspase-3, and PARP with specific antibodies. (C) Jurkat cells were electroporated with 10 μg of mouse Mcl-1 promoter construct and 24 h after transfection cells were treated with staurosporine (1 μM) or actinomycin D (5 μg/ml) for different times. Cells were collected at the same moment and luciferase activity was determined. Values are expressed as relative luciferase units/mg protein. Viability was also measured by analysis of phosphatidylserine exposure and PI uptake as described in Materials and methods. Data are shown as mean values of two experiments.

decrease during aspirin-induced apoptosis. To address whether aspirin inhibits de novo protein synthesis, we determined the incorporation of [^{3}H]leucine in the TCA precipitated fraction of Jurkat cells incubated with aspirin. We expressed the results as TCA fraction/soluble fraction to avoid any mistake due to differential [^{3}H]leucine uptake. A decrease of protein synthesis was observed as soon as 1 h after treatment (Fig. 4B), when viability was unaffected (data not shown). It should be pointed that cytochrome *c* release and subsequent caspase activation does not occur until 2–3 h after aspirin addition, as we described in a previous work [30]. One of the factors necessary for ribosomal recruitment and protein translation is the protein complex eIF2 (reviewed in [38]). Phosphorylation of a small amount of a subunit of eIF2 (eIF2α) can virtually shut down protein synthesis initiation [39]. We measured the levels of both the phosphorylated eIF2α and total eIF2α by Western blot with specific antibodies and expressed their relation after densitometric quantification. As seen in Fig. 4C, a little amount of phosphorylated eIF2α was present in Jurkat cells in normal conditions, and after aspirin addition a transient increase in phosphorylation was observed between 15 and 90 min of treatment. Another phosphorylation peak was also evident at 4–5 h after aspirin incubation, when caspases are activated [30, this manuscript]. These results indicate that aspirin inhibits protein synthesis in Jurkat cells, at least in part, by inducing eIF2α phosphorylation. Importantly, this early and transient phosphorylation event must be caspase-independent. Additional effects of aspirin in the translational machinery and/or Mcl-1 protein turnover rate cannot be ruled out. These data also indicate that transcriptional inhibition is not a general effect during apoptosis.

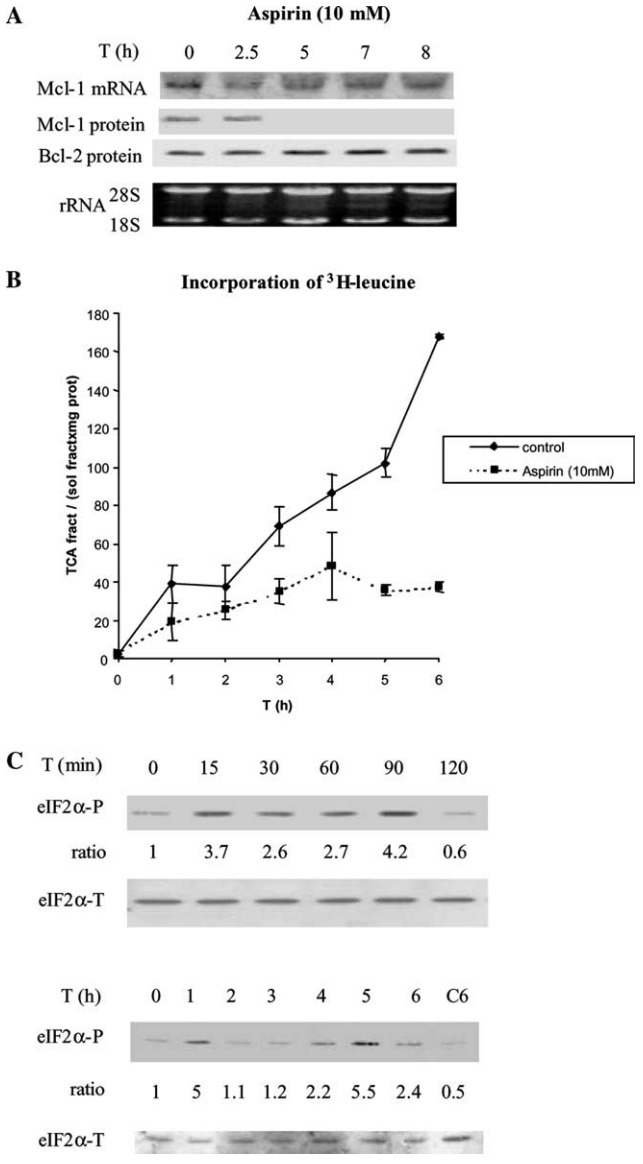

Fig. 4. Aspirin inhibits [^{3}H]leucine incorporation in Jurkat cells and induces eIF2α phosphorylation. (A) Jurkat cells were treated with aspirin for different times and collected at the same moment. Total RNA was isolated as described in Materials and methods and 15 μg per lane was assayed by Northern blot with a Mcl-1 ^{32}P-labelled cDNA probe. Ribosomal RNAs are shown as a loading control. Fifty micrograms of protein was assayed by Western blot for the presence of Mcl-1 and Bcl-2 with specific antibodies. One representative experiment of three is shown. (B) Jurkat cells, at a concentration of 0.5×10^6 cells/ml, were incubated with [^{3}H]leucine (42.5 Ci/mol) in the absence or presence of aspirin (10 mM) for 6 h. The cells (1×10^6) were collected at different times and incorporation of [^{3}H]leucine into protein was determined by TCA precipitation. [^{3}H]Leucine in the soluble fraction was also measured and the results were expressed as cpm TCA fraction/(cpm soluble fraction × mg protein). One representative experiment of three is presented and the values are the average ± SEM of three determinations. (C) Jurkat cells were treated with aspirin (10 mM) for different times and collected at the same moment. Cell lysates were analysed by Western blot (50 μg per lane) with antibodies specific for Ser^{51}-phosphorylated eIF2α (eIF2α-P) and total eIF2α (eIF2α-T). The immunoreactive signal of intact eIF2α was quantified densitometrically and the ratio eIF2α-P/eIF2α-T, normalized for 0 h, was determined. C6: cells were incubated in medium without aspirin for 6 h.

The XIAP promoter luciferase construct, which contains an IRES, is down-regulated by staurosporine but not by aspirin

The results presented above indicate that aspirin affects protein synthesis at the level of initiation by transiently increasing the level of eIF2α phosphorylation. It is known that during apoptosis, the translation of mRNAs containing IRES is less affected than in other mRNAs with cap-dependent translation [40]. We addressed the question of what could happen with luciferase promoter constructs codifying for mRNAs with cap-dependent or independent translation during staurosporine or aspirin-induced apoptosis. The gene XIAP contains a functional internal ribosome entry site (IRES) in the 5′UTR of its mRNA that mediates cap-independent translation [41]. It has been reported that in some stress conditions, like serum withdrawal or γ-irradiation, the expression of a CAT reporter gene under the control of the XIAP 5′UTR is unchanged or slightly increased, whereas the same reporter gene under cap-dependent translation is down-regulated [41,42]. We performed transfection experiments in Jurkat cells with the 5′UTR of the XIAP gene (nucleotides −3999 to −5) fused to the luciferase gene reporter. This construct expressed significant luciferase activity indicating that it contains a functional promoter. When Jurkat cells were transfected with the XIAP construct and treated with the apoptotic agents, a marked reduction of luciferase activity was observed with staurosporine but not with aspirin (Fig. 5A). However, the mRNAs whose translation is cap-dependent (Mcl-1, FAS, and CMV promoter constructs) showed a significant reduction of luciferase activity by both staurosporine and aspirin treatment. To further investigate this point, we studied the effect of aspirin on the endogenous XIAP mRNA and protein levels. As expected, neither the XIAP protein nor the mRNA were affected by aspirin treatment (Figs. 5B and C). This result is in agreement with the maintenance of Mcl-1 mRNA levels, indicating that transcriptional inhibition during apoptosis is not a general and unspecific phenomenon. The data from Fig. 5A also suggest that aspirin down-regulates the levels of short-lived proteins (luciferase) by inhibiting protein synthesis rather than by altering protein-rate turnover. XIAP mRNA, whose translation is cap-independent, can escape from this translational repression. Down-regulation of XIAP is important in several models of apoptosis [43], however XIAP does not change during aspirin treatment.

Down-regulation of Mcl-1 protein levels by cycloheximide does not affect cell survival

As Mcl-1 is a critical antiapoptotic short-lived protein, we examined the effect of Mcl-1 protein decay on cell viability. Thus, we treated Jurkat cells with

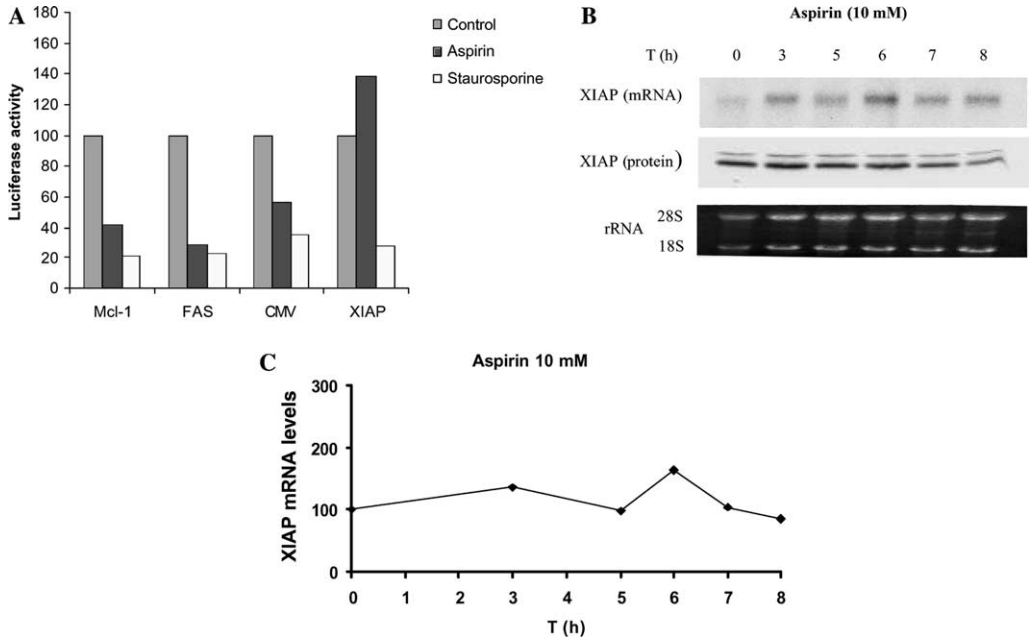

Fig. 5. XIAP 5′UTR-luciferase construct is down-regulated by staurosporine but not by aspirin. (A) Jurkat cells were transfected by electroporation with 2 μg of the green fluorescent protein reporter plasmid pEGFP-C1 together with 10 μg of one of the following promoters fused to the luciferase reporter: CMV, FAS, Mcl-1, and XIAP. Twenty-four hours after transfection staurosporine (1 μM) or aspirin (10 mM) was added to the culture medium for 3 or 6 h, respectively, and the luciferase activity was determined. The values were expressed as relative luciferase units normalized with the transfection efficiency and protein concentration. Data are shown as mean values of two experiments and normalized for each construct relative to the basal activity in the absence of the drug. (B) XIAP mRNA and protein levels were analysed by Western and Northern blot in Jurkat cells after aspirin (10 mM) treatment. Cell were treated for different times and collected at the same moment and 50 μg of protein assayed with a specific antibody. The upper and less marked band is unspecific. Total RNA was isolated and 15 μg per lane were assayed by Northern blot with a XIAP ^{32}P-labelled cDNA probe. Ribosomal RNAs are shown as a loading control. (C) XIAP mRNA was analysed by Northern blot in Jurkat cells after aspirin treatment. Jurkat cells were incubated with aspirin (10 mM) for different times and cells were collected at the same moment. Total RNA was isolated and blotted as described in Materials and methods and 15 μg per lane was assayed by Northern blot with a XIAP ^{32}P-labelled cDNA probe. XIAP mRNA levels were quantified by densitometry from (B) and the values corrected by the 18S ribosomal RNA.

cycloheximide, a general inhibitor of translation, at doses that did not affect cell viability. Cycloheximide reduced Mcl-1 protein levels (Fig. 6A) in a similar way to aspirin (Fig. 6B). Id1, a short-lived protein [44] not related with apoptosis, also decayed following aspirin and cycloheximide treatment. Bcl-2, in contrast, was not affected by either cycloheximide or aspirin. Further, preincubation of cells with Z-VAD.fmk did not protect Mcl-1 from decay induced by cycloheximide (Fig. 6C). These results rule out a non-specific effect of Z-VAD.fmk on the translational or protein degradation machinery.

Taken together, these results demonstrate that aspirin down-regulates short-lived proteins such as Mcl-1, Id1 or even luciferase by inhibiting the translation machinery. However, as viability is not lost when Mcl-1 is down-regulated, Mcl-1 disappearance might be necessary but not sufficient to trigger apoptosis induced by aspirin or staurosporine in Jurkat cells.

Discussion

The present study shows that Mcl-1 protein is rapidly depleted after treatment of Jurkat cells by two well known apoptotic stimuli, staurosporine and aspirin. On the contrary, Bcl-2 remains practically constant over the cell death process. Bcl-2 is a long-lived protein, having a half-life of 10–14 h [34,35], whereas Mcl-1 has a very short half-life ranging from 20 min to 3 h, depending of the cell type considered [2,45]. Mcl-1 is probably degraded by the proteasome pathway, since proteasome inhibitors (MG115, MG132, and lactacystin) that efficiently block the degradation of Mcl-1 also block actinomycin D-induced apoptosis [20], UV-induced apoptosis [46], and lipopolysaccharide-induced apoptosis [47]. Moreover, very recently it has been reported that Mcl-1 is polyubiquitinated and subsequently degraded by the proteasome [46]. However, in several cell lines the proteasome inhibitors only partially protect from staurosporine-induced apoptosis [47,48]. In our model, the proteasome inhibitor MG132 partially blocked Mcl-1 protein decay but also induced apoptosis (Fig. 1C). This could explain the partial protection of Mcl-1 decay. It has been reported that inhibitors of the proteasome can induce apoptosis in Jurkat cells [49,50]. The down-regulation of Mcl-1 protein during apoptosis agrees with other studies [6,22–28] and suggests that Mcl-1 might be important for survival. However, we

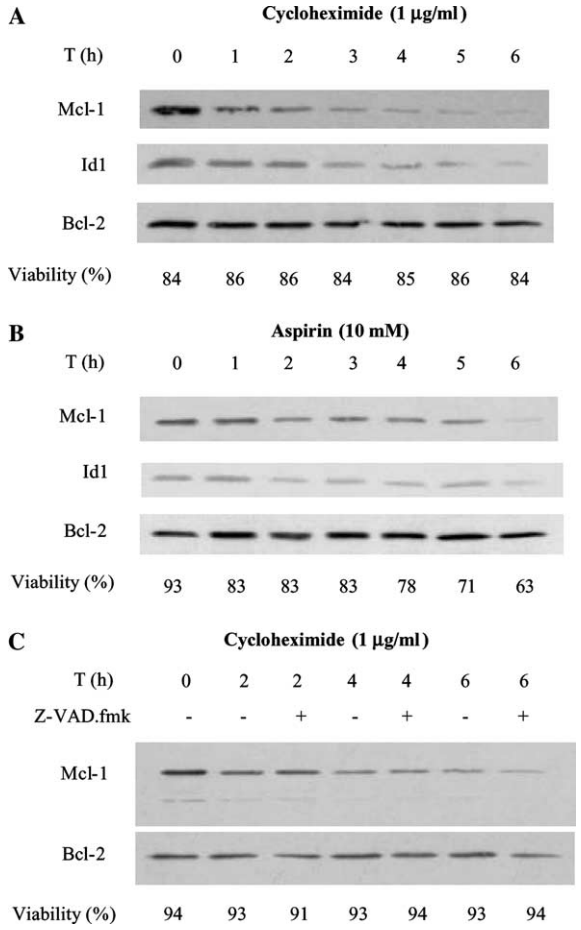

Fig. 6. Down-regulation of Mcl-1 protein by cycloheximide does not affect cell survival. Jurkat cells (1×10^6 cells/ml) were treated with cycloheximide (1 μg/ml) (A) or with aspirin (10 mM) (B) for different times and collected at the same moment for Western blot analysis as described in Materials and methods. (C) Z-VAD.fmk (200 μM) does not protect from cycloheximide-induced Mcl-1 protein decay. Jurkat cells were treated with cycloheximide (1 μg/ml) alone or in the presence of Z-VAD.fmk (200 μM) for different times and collected at the same moment for Western blot analysis. Fifty micrograms of protein was assayed for the presence of Mcl-1 and Id1 by using specific antibodies. Viability, expressed as the percentage of annexin V and PI negative cells, is shown for each time at the bottom of the figure.

also observed down-regulation of other proteins of short half-life, which are not involved in apoptosis (luciferase, Id1). Moreover, down-regulation of Mcl-1 by cycloheximide is not correlated with a decrease in survival of Jurkat cells. Therefore, the role of Mcl-1 in mediating apoptosis of Jurkat cells by aspirin and staurosporine is uncertain. Further experiments should clarify this issue. Presently, we can only assure that Mcl-1 down-regulation is not sufficient to induce apoptosis in Jurkat cells. During the revision of this manuscript, it has been reported that Mcl-1 is a sensor of protein synthesis inhibition and that the decrease of Mcl-1 protein is necessary to induce apoptosis [46]. These authors found that Mcl-1 elimination during UV-induced apoptosis is caused by a lack of protein synthesis and suggest that another UV-induced event in addition to the elimination of Mcl-1 must be required to promote apoptosis.

The Mcl-1 mRNA levels in cells treated with staurosporine also showed a rapid decrease, which was correlated with the protein decay. It is reported that Mcl-1 mRNA has a very short half-life of less than 2 h [3]. Mcl-1 has a long 3′ untranslated region (2.8 kb) and contains multiple mRNA destabilization signals [1]. In this study, we have not addressed the destabilization of Mcl-1 mRNA during apoptosis. Surprisingly, Mcl-1 mRNA levels did not change in aspirin-induced apoptosis, but the protein and cell viability were strongly reduced after treatment, suggesting a rapid translational inhibition. We have studied the caspase-dependent and independent mechanisms involved in the Mcl-1 protein and mRNA decay. Both pathways seem to be present in staurosporine and aspirin-induced apoptosis. The broad inhibitor of caspases Z-VAD.fmk largely blocked caspase-3 processing and viability decrease. However, Z-VAD.fmk only partially protected from Mcl-1 protein decay after staurosporine or aspirin treatment, indicating that Mcl-1 down-regulation is not merely due to caspase activation. Very recently, it has been described that Mcl-1 is substrate for caspases during Fas-mediated apoptosis [51]. Here, we demonstrate a caspase-independent pathway for Mcl-1 down-regulation during apoptosis. Interestingly, the decrease in Mcl-1 mRNA levels after staurosporine-induced apoptosis was blocked by Z-VAD.fmk, pointing to a caspase-dependent mechanism for the shut-down of Mcl-1 transcription. Staurosporine is a potent inhibitor of multiple protein kinases and inhibits transcription of different genes [52–54]. However, we cannot rule out that the stability of the Mcl-1 mRNA could be affected during staurosporine-induced apoptosis. Although Z-VAD.fmk reverted the down-regulation of the Mcl-1 mRNA by staurosporine, the protein levels still decrease (Fig. 3B), suggesting a caspase-independent translational control. It is reported that staurosporine inhibits protein synthesis in several cell lines [55,56], leads to pronounced cleavage of eIF4G (which acts as a bridge between the cap-binding protein eIF4E and eIF3 complex allowing the mRNA molecule to associate with the 40S ribosomal subunit) performed by caspase 3 [57], and induces eIF2α phosphorylation after caspase activation [58]. It is also reported that several apoptotic stimuli induce caspase-dependent degradation of the polypeptide initiation factor eIF4G and eIF4B in Jurkat cells [59,60]. Our results suggest an additional caspase-independent mechanism for translational inhibition. It has been reported that inhibition of protein kinase C (PKC) by staurosporine decreases lipoprotein lipase synthesis by inhibition of translation [61]. Recently, it has also been described that staurosporine inhibits phosphorylation of translational regulators linked to mTOR by a caspase-independent mechanism [62]. It is thus possible

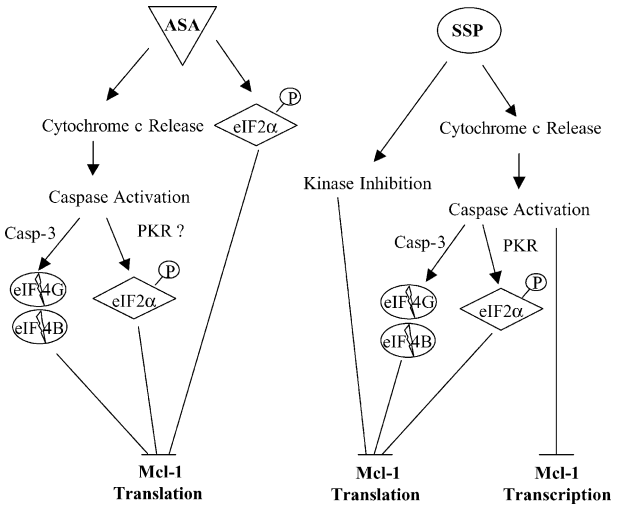

Fig. 7. Model for translational and transcriptional control of Mcl-1 by aspirin and staurosporine-induced apoptosis. Mcl-1 is depleted after aspirin (ASA) addition by caspase-dependent and independent mechanisms. The caspase-independent pathway would mediate rapid phosphorylation of eIF2α (15 min) and subsequent impairment of translation. The caspase-dependent pathway, engaged after cytochrome c release from the mitochondria (2–3 h), would induce proteolysis of the translation initiation factors eIF4G and eIF4B, and phosphorylation of eIF2α (probably by PKR proteolysis). Staurosporine (Ssp) also induces translational inhibition by caspase-dependent and independent pathways. The caspase-dependent pathway is identical to that of aspirin, involving cytochrome *c* release. The caspase-independent pathway can be mediated by kinase inhibition. In addition, staurosporine, but not aspirin, induces transcriptional inhibition of Mcl-1 by a caspase-dependent mechanism.

that staurosporine mediates this caspase-independent translational control by kinase inhibition (see diagram in Fig. 7).

In this study, we demonstrate a translational control of Mcl-1 during aspirin-induced apoptosis. In staurosporine-induced apoptosis, the transcriptional and translational effects may be overlapped making it more difficult to interpret the results. Therefore, we focused our research on the caspase-independent translational control by aspirin because it is well known that after caspase-3 activation, with other apoptotic stimuli, the translational machinery is shutdown [63]. There is only one report that describes the inhibition of protein synthesis by aspirin, but a detailed time-course study and the molecular mechanisms were not addressed [64].

We confirmed that aspirin inhibited protein synthesis in Jurkat cells using different approaches. Transfection experiments in Jurkat cells with gene promoter constructs fused to the luciferase gene reporter showed that staurosporine and aspirin treatment decreased the luciferase activity. The decay of luciferase could be due to transcriptional or translational inhibition. However, when we considered the XIAP construct, which contains an IRES element in its 5′UTR that confers resistance to translational inhibition [40], there was no decrease in the luciferase activity after aspirin treatment. In contrast, staurosporine produced a down-regulation of all promoter constructs, including XIAP. Leucine incorporation, a reliable measure of de novo protein synthesis, was inhibited in Jurkat cells as soon as 1 h after aspirin treatment, when caspases are still inactive and the viability of the cells is unaffected. In a previous report, we described that caspases-8, -9, and -3 were activated 3 h after aspirin treatment simultaneously with cytochrome *c* release from the mitochondria [30]. In the present study, we observed a transient phosphorylation of eIF2α between 15 and 90 min after aspirin treatment, when caspases are not active. Phosphorylation of only a fraction of the cellular eIF2α is sufficient to severely impair translation initiation [39]. Interestingly, it has been described that induction of apoptosis in Jurkat cells by activation of the Fas/CD95 receptor promotes an early transient increase in the level of eIF2α phosphorylation, correlated with the inhibition of translation [65]. In that report, the enhancement of eIF2α phosphorylation does not require caspase-8 activity and occurs prior to the cleavage of eIF4G. The mechanism for eIF2α phosphorylation independent of caspase activation is presently unknown and deserves further investigation. We also observed eIF2α phosphorylation at 4–5 h after treatment, when caspases are active. Recently, Saelens et al. [58] have described a caspase-dependent activation of the protein kinase PKR during apoptosis that induces eIF2α phosphorylation. A similar mechanism could be responsible for the late phosphorylation of eIF2α induced by aspirin.

In conclusion, different patterns of transcriptional and translational control during apoptosis emerge depending on the stimulus although translational shutdown seems to be a common point during apoptosis (Fig. 7). Short-lived antiapoptotic proteins, such as Mcl-1, are important mediators of apoptosis, since they are expected to be rapidly lost by translational inhibition. Caspase-dependent and independent mechanisms are responsible for this translational inhibition, whereas cap-independent mRNA translation seems to escape that inhibition. As previously discussed [40–42], that might be a survival or stress strategy to keep some proteins as XIAP alive during these threatening situations. An apparent paradox of this model is that eIF2α should be necessary for cap-dependent and independent translation, since it is essential for $tRNA_i^{Met}$ and ribosome recruitment. However, it is reported that other apoptotic regulators, like Fas and Bax, increase their expression after eIF2α phosphorylation [66]. It seems clear that different mRNAs have a heterogeneous affinity for the remaining functional ribosomal machinery, although the mechanism/s involved are still unknown [38]. Recently, Gerlitz et al. [67] described that eIF2α phosphorylation is required for activation of cellular IRES elements. Our data also suggest that

transcriptional inhibition during apoptosis is not a general and unspecific phenomenon, since Mcl-1 and XIAP mRNA levels are maintained under aspirin treatment when viability is falling down and caspases are activated. It is becoming clear that control of translation plays a major role in the programmed cell death. This should be kept in mind when designing new and more effective drugs to induce apoptosis in cancer cells.

Acknowledgments

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