

Rapid Induction of the Intrinsic Apoptotic Pathway by L-Glutamine Starvation

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While the amino acid L-glutamine is known to play a role in the survival of several cell types, the underlying molecular mechanisms are still poorly defined. We show in this report that L-glutamine starvation rapidly triggered apoptosis in Sp2/0-Ag14 hybridoma cells. This process involved the activation of both caspases-9 and -3, suggesting that L-glutamine deprivation initiated an intrinsic apoptotic pathway in Sp2/0-Ag14 cells. Supporting this idea, the cytosolic release of the mitochondrial proteins SMAC/DIABLO and cytochrome c (Cyt c) was observed, with an initial limited leakage occurring during the first 30 min of L-glutamine deprivation, followed by a greater release after 60 min. The latter occurred simultaneously with the translocation of the pro-apoptotic protein Bax to the mitochondria. Finally, a decline in XIAP levels and the activation of caspases-3 and -9 were observed. Thus, L-glutamine deprivation of Sp2/0-Ag14 cells rapidly triggers intracellular events, which target the mitochondria, leading to the cytosolic release of apoptogenic factors, the activation of caspases-9 and -3, and the commitment to the death program. This work introduces the Sp2/0-Ag14 hybridoma as a unique model for the study of the molecular events underlying the pro-survival function of L-glutamine. *J. Cell. Physiol.* 202: 912–921, 2005. © 2004 Wiley-Liss, Inc.

L-glutamine (Gln) is the most abundant free amino acid in the bloodstream and plays an important role the modulation of cellular physiology. In addition to being the main inter-organ carrier of nitrogen, Gln provides carbon atoms for intermediary metabolism, participates in the regulation of cellular pH and volume, contributes to the production of glutathione (GSH), polyamines, and amino sugars, and is essential to the synthesis of NAD(P) co-factors (Young and Ajami, 2001; Newsholme et al., 2003). Gln has been shown to influence several cellular signaling pathways including the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Blikslager et al., 1999; Rhoads et al., 2000; Ko et al., 2001), the cAMP-activated protein kinase (PKA) (Xia et al., 2003) and the mammalian target of rapamycin (mTOR) pathways (Xia et al., 2003). Gln is also involved in the control of gene expression (Abcouwer et al., 1999; Huang et al., 1999; Wischmeyer, 2002) and it has been shown to modulate the activity of the ubiquitin-proteasome protein degradation pathway (Zellner et al., 2003). Consequently, it is not surprising that several cellular processes, including proliferation (Ko et al., 1993; Turowski et al., 1994; Blikslager et al., 1999; Chang et al., 1999), motility (Fu et al., 2003), and differentiation (Turowski et al., 1994; Spittler et al., 1997), are influenced by the availability of Gln.

Accumulating evidence also suggest that Gln is involved in the control of cell survival. Gln supplementation increases the tolerance threshold of several cell lines to the toxic effect of doxorubicin (Tavares et al., 1998), ionizing radiation (Klimberg et al., 1990; Winters et al., 1994), heat shock (Vidair and Dewey, 1987; Wischmeyer et al., 1997; Exner et al., 2002), serum starvation (Xu et al., 1997), ischemia (Blikslager et al., 1999), oxidative stress (Wischmeyer et al., 1997), PMA, and ionomycin (Chang et al., 2002), and the cytokines FAS ligand and TNF- α (Ko et al., 2001; Exner et al., 2002). Moreover, Gln deprivation triggers the spontaneous apoptosis of several cell types, including neutrophils (Pithon-Curi et al., 2003), intestinal epithelial cells (Papaconstantinou et al., 1998), leukemia/lymphoma

cell lines (Petronini et al., 1996; Fumarola et al., 2001), and murine hybridomas and myelomas (Mercille and Massie, 1994; Gauthier et al., 1996; Charbonneau and Gauthier, 2000).

In spite of the demonstrated importance of Gln in promoting cell survival, very little is known about the underlying intracellular signaling pathways. In this report, we show that the viability of the murine hybridoma cell line Sp2/0-Ag14 (Sp2/0) is strictly dependent on the presence of Gln. We demonstrate that Gln deprivation triggers intracellular events, which rapidly target the mitochondria, activating an intrinsic pathway of apoptosis leading to a commitment to the death program less than 2 h after Gln withdrawal. This work introduces the Sp2/0 hybridoma as a unique model for the study of the pro-survival function of Gln.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Oakville, Ont., Canada).

Cell culture

The murine hybridoma cell line Sp2/0 (ATCC CRL 1581) was purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in Iscove's-modified Dulbecco's media (IMDM) supplemented with 5% Fetalclone I (Hyclone, Logan UT), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 4 mM Gln. Cell culture was performed at 37°C under an atmosphere of 5% CO₂. For glutamine deprivation studies,

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Sp2/0 cells were washed twice in warm phosphate-buffered saline (PBS: 9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) and cultured in complete IMDM lacking Gln.

MTT assay

The MTT assay was done according to published procedures (Hansen et al., 1989). Sp2/0 cells were washed twice in warm PBS and transferred in amino acid-free minimal essential media (MEM) containing 5% Fetalclone, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cultures were then supplemented with an amino acid mixture in which one of the 20 amino acids had been omitted. A control sample was included where cells were cultured in the presence of all 20 amino acids. The final amino acid concentrations were as reported for complete MEM media. Cells (1×10^5) were placed in the wells of a 96-well plate and incubated at 37°C for 4 or 24 h. Twenty-five microliters of a MTT solution (5 mg/ml in PBS) was then added and the plate was incubated for 2 h at 37°C. Subsequently, 100 µl of lysis buffer (20% SDS in 50% *N,N*-dimethylformamide, pH 4.7) was added to each well, and the incubation was resumed for 18 h at 37°C. Optical density readings were then taken at 570 nm using a PowerWave-X microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT).

Hoechst assay

Sp2/0 cells (4×10^5 cells/ml) were cultured in IMDM lacking Gln for the indicated time intervals. Hoechst 33342 (2 µg/ml final concentration) was added 30 min prior to the end of the incubation period. The cells were then incubated on ice for 15 min, washed twice in cold PBS and visualized using a Leitz Diaplan fluorescence microscope (Leica Microsystems, Richmond Hill, Ont., Canada). Cells showing a pycnotic or fragmented nucleus were scored as apoptotic.

Rescue assay

Cells (2×10^5 /ml) were cultured in a 24-well plate in complete IMDM media deprived of Gln. At the indicated time intervals, Gln was added to a final concentration of 4 mM and cell culture was resumed for 24 h. The number of viable cells was then determined using the trypan blue dye exclusion assay. When caspase inhibitors were used, Z-VAD-fmk, Z-DEVD-fmk, or Z-LEHD-fmk (Calbiochem, 10 mM stocks in DMSO) were added to the cells to a final concentration of 10 µM at the start of the experiment. Cells receiving an equivalent amount of DMSO were used as controls.

Caspase activity assay

Caspase activity was determined using colorimetric (caspase-3) or fluorometric (caspase-8 and -9) assay kits from Biovision, Inc. (Mountain View, CA) following the manufacturer's instructions. Sp2/0 cells (1×10^7) were cultured in complete IMDM media lacking Gln for the indicated amount of time. Cells were then placed on ice for 15 min, washed twice with cold PBS, and the cell pellets were processed for the determination of caspase activity. The protein levels of each cell extract were determined using the DC-Protein Assay kit from Bio-Rad (Mississauga, Ont., Canada), and the same amount of extract was used for the enzymatic assays. Absorbance readings were obtained using a PowerWave X microplate spectrophotometer. Fluorescence readings were collected using a Fluostar Optima microplate spectrofluorometer (BMG Labtechnologies, Inc., Durham, NC).

Subcellular fractionation

Cell fractionation into cytosolic and mitochondria-enriched fractions was based on a previously described procedure (Gottlieb and Granville, 2002). Cells (5×10^7) were incubated under Gln deprivation conditions for the indicated amount of time, placed on ice for 15 min and washed twice with cold PBS. Cell pellets were then resuspended in 250 µl of buffer A (75 mM KCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, 230 µg/ml digitonin) and incubated on ice for 10 min. To monitor the extent of cell permeabilization, an aliquot was taken and the number of permeabilized cells was determined using the trypan blue dye exclusion assay. The cells were then

centrifuged (15,000g, 4°C) for 5 min. Both the pellet and supernatant were kept to obtain the mitochondrial and cytosolic fractions, respectively. For the cytosolic fraction, the supernatant obtained after the centrifugation of the permeabilized cells was collected and centrifuged again (15,000g, 4°C, 5 min). The supernatant of this last centrifugation constituted the cytosolic fraction, which was mixed with an equal volume of 2× RIPA buffer (1× RIPA is 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1 mg/ml phenyl methyl sulfonyl fluoride [PMSF], in PBS) and stored at -80°C. For the isolation of the mitochondrial (heavy membrane) fraction, the cell pellet obtained after cell permeabilization was resuspended in 500 µl of buffer B (75 mM KCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose), passed 30 times through a 26 G½ needle, centrifuged twice at 2,500g (4°C, 5 min) to remove intact cells and nuclei and centrifuged again (15,000g, 4°C, 30 min). The pellet, which contains the mitochondrial heavy membrane fraction, was washed once in buffer B, resuspended in 40 µl RIPA buffer and placed on ice for 60 min. The mitochondrial extract was finally centrifuged twice (15,000g, 4°C, 20 min) to remove insoluble debris, and stored at -80°C.

Western blot analysis

Whole cell extracts were prepared by lysis in RIPA buffer, as described previously (Charbonneau et al., 2003). Protein content of the total, cytosolic and mitochondrial extracts was determined using the DC-Protein Assay kit (Bio-Rad). Proteins were fractionated on a PAGE-SDS gel in a Tris-glycine buffer (Ausubel et al., 1995). For cytochrome c (Cyt c) analysis, proteins (25 µg) were separated on a 16% PAGE-SDS gel in a Tris-Tricine buffer system (Schagger and von Jagow, 1987). For poly(ADP ribose) polymerase (PARP) analysis, cells (5×10^5) were washed twice in cold PBS and resuspended in urea sample buffer (62.5 mM Tris/HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol) (Shah et al., 1995). Samples were then sonicated for 15 sec and heated at 65°C for 15 min before being fractionated on a 10% PAGE-SDS gel. Proteins were transferred onto a PVDF membrane (Amersham Bioscience, Baie-d'Urfé, Que., Canada) using a TransBlot SD Semi-Dry Transfer Cell (Bio-Rad). Transfer efficiency was routinely monitored by staining the membrane with Ponceau S. Primary polyclonal antibodies raised against the following proteins were used: PARP (Bio-Mol, Plymouth Meeting, PA), caspases-3, -6, -7, -9, -10, -12 (New-England Biolabs, Pickering, Ont., Canada), caspases-2 and -8, X chromosome-linked inhibitor of apoptosis (XIAP) (Stressgen Bioreagents, Victoria, BC), Bax, Cyt c, glucose-related protein 75 (Grp75), HSP60 (Santa Cruz Biotechnologies, Santa Cruz, CA), SMAC/DIABLO (Calbiochem/EMD Biosciences, La Jolla, CA), and lactate dehydrogenase (LDH, Fitzgerald Industries International, Inc., Concord, MA). Detection was carried out using HRP-coupled secondary antibodies (Santa Cruz Biotechnologies) and the Chemiglow Western Blot Substrate kit (Alpha Innotech, San Leandro, CA). Image capture and analysis were performed using a Fluorchem 8000 Imaging System (Alpha Innotech) and the AlphaEase software (Alpha Innotech).

DNA fragmentation analysis

DNA fragmentation analysis was performed as described (Charbonneau et al., 2003). Briefly, 5×10^5 cells were washed once in PBS and resuspended in 50 µl of lysis buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8.0, 0.5% *N*-lauroyl sarcosine, 0.02 mg/ml RNase A, and 0.25 mg/ml proteinase K). The mixture was incubated for 5 min at 50°C, 50 µl of sample buffer (40% sucrose, 0.08% bromophenol blue) was then added and 30 µl of the sample was loaded into the wells of a 2% agarose gel. Electrophoresis was then performed in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0), the gel was stained with ethidium bromide and visualization was done under UV transillumination.

Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) and Scheffe's post-hoc test.

RESULTS

Gln deprivation rapidly triggers apoptosis in Sp2/0 cells

Our previous studies indicated that Sp2/0 hybridoma cells are sensitive to Gln deprivation (Charbonneau and Gauthier, 2000; Charbonneau et al., 2003). To determine whether this is a general phenomenon, Sp2/0 cells were deprived of any one of the 20 amino acids and cell viability was measured after 4 and 24 h. While the viability of Sp2/0 cells was significantly decreased following the 24-hr deprivation of any of the essential amino acids, only the removal of Gln could lead to a reduction in cell viability after a 4 h withdrawal period (Fig. 1). With the notable exception of cysteine and tyrosine, the removal of non-essential amino acids did not affect cell viability. Gln-starved Sp2/0 hybridomas exhibited a morphology characteristic of apoptotic cells (Fig. 2a) and over 85% of the cells showed pycnotic or fragmented nuclei after only 2 h of Gln deprivation (Fig. 2b). Most of the increase in the number of apoptotic cells occurred between the first and second hour of Gln deprivation (Fig. 2b), indicating that Sp2/0 cells underwent cell death in a synchronous manner. To estimate the time of commitment to cell death, cultures starved of

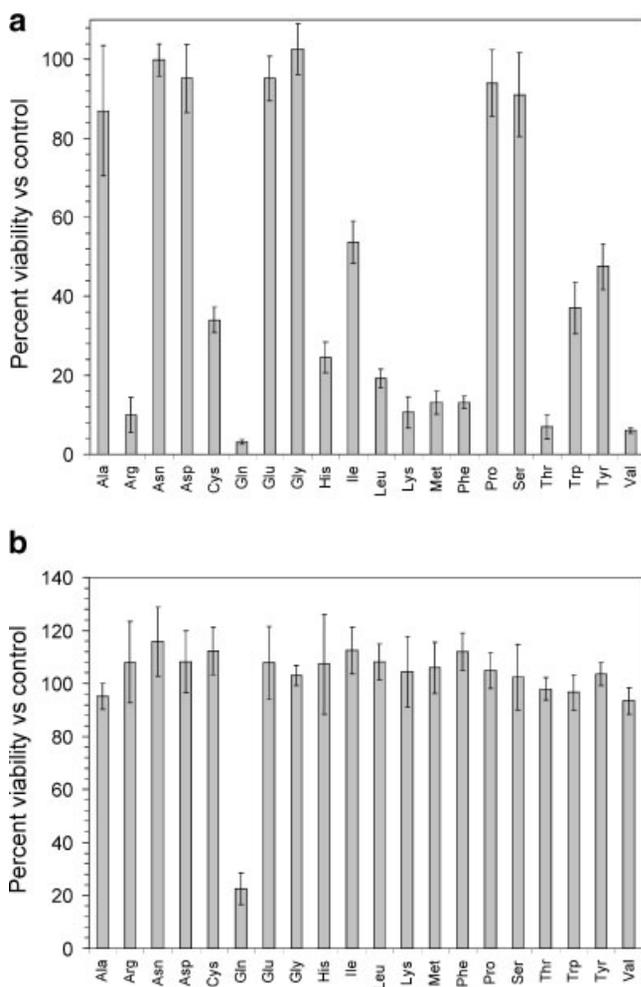


Fig. 1. Viability of Sp2/0 cells upon amino acid deprivation. Sp2/0 cells were cultured for 24 h (a) or 4 h (b) in the absence of the indicated amino acids. Cell viability was then determined using the MTT assay. The results are expressed as the percent viability versus a control sample cultured in the presence of all amino acids. Data are the average \pm standard deviation (SD) of three independent experiments.

Gln for different time periods were rescued by the addition of Gln. Addition of Gln to cells starved for 1 h or less successfully aborted the death program and most of the cells remained viable (Fig. 2c). However, cultures deprived of Gln for longer time periods could not be rescued as efficiently and a 2-hr glutamine deprivation period resulted in 60% of the cells being committed to die. Therefore, Gln deprivation rapidly triggers apoptosis in Sp2/0 cells, which are committed to die less than 2 h after Gln withdrawal.

Effector caspase processing and activation triggered by Gln starvation

Effector caspases are responsible in large part for the protein processing events which lead to the morphological changes characteristic of apoptotic cells (Salvesen, 2002). Executioner caspases are initially synthesized as a zymogen precursor, which is activated upon processing into a large (20 kDa) and small (10 kDa) subunit (Salvesen, 2002). To determine whether executioner caspases were activated in Gln-starved Sp2/0 cells, total cell extracts were processed for Western blot analysis and probed with anti-caspase antibodies. A decrease in the levels of the proenzyme form of the executioner caspases-3 and -6 was observed in cells deprived of Gln for 2 h (Fig. 3). An increase in the level of the processed, 17 kDa large subunit of caspase-3 was also observed as early as 1 h after Gln withdrawal (Fig. 3). We also observed a decrease in the level of caspase-7 β , an enzymatically inactive isoform of caspase-7 (Fernandes-Alnemri et al., 1995). Caspase-3 cleavage occurred concomitantly with an increase in caspase-3-like enzymatic activity (Fig. 4c). DNA fragmentation and PARP cleavage, two events associated with the activation of caspase-3, were detected after 60 min of Gln starvation (Fig. 4a,b) and coincided with the commitment to the death program. Cells incubated in the presence of Gln did not show caspase activation or PARP processing (Figs. 3 and 4). Therefore, effector caspase activation is an early event of apoptosis triggered by Gln starvation, occurring concurrently with the time of commitment to cell death.

Initiator caspase processing and activation triggered by Gln deprivation

Effector caspases are activated by upstream initiator caspases, which are generally triggered by specific death cues (Salvesen, 2002). For instance, receptor-mediated cell death leads to caspase-8 and -10 activation (Muzio et al., 1996; Wang et al., 2001; Chen and Wang, 2002; Micheau and Tschopp, 2003), caspases-2 and -9 are triggered by intracellular stresses targeting the mitochondria (Li et al., 1997; Chen and Wang, 2002; Guo et al., 2002; Lassus et al., 2002) and caspase-12 is involved in apoptosis prompted by endoplasmic reticulum stress (Nakagawa et al., 2000; Yoneda et al., 2001; Chen and Wang, 2002). Here, we determined that Gln starvation leads to the processing of caspase-9, with a decrease of the 49 kDa precursor and a concomitant accumulation of the 37 and 39 kDa cleavage products being detectable as early as 60 min after Gln deprivation (Fig. 5). Processing of caspase-9 was not seen when Gln was present in the culture (Fig. 5). We also investigated the possibility that other initiator caspases could be processed. Only the precursor form of caspases-2 (both long [L] and short [S] forms), -10 and -12 was observed in Gln starved cells. However, proteolytic fragments characteristic of processed caspase-8 were detected throughout the incubation period (Fig. 5). These

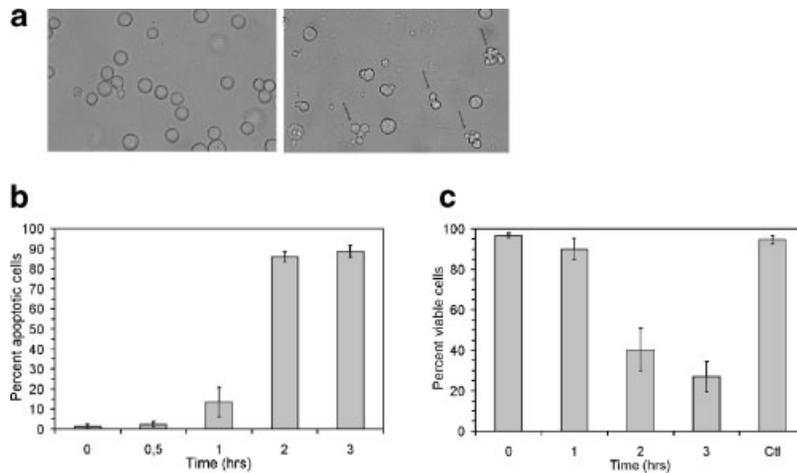


Fig. 2. Gln deprivation induces rapid apoptotic cell death in Sp2/0 cells. **a:** Sp2/0 cells were cultured for 2 h in the absence of Gln and examined under brightfield microscopy. Arrows show cells with morphological characteristics of apoptosis. **b:** Sp2/0 cells were cultured in the absence of Gln for the indicated time periods, stained with Hoechst 33342, and examined under fluorescence microscopy. Cells exhibiting pycnotic or fragmented nuclei were classified as apoptotic. At least 200 cells were enumerated for each time point. **c:** Sp2/0 cells

were cultured in Gln-deprived media. At the indicated time periods, Gln was added at a concentration of 4 mM, and cell culture was resumed for 24 h. The number of dead and viable cells was then assessed by the trypan blue dye exclusion assay. Ctl: control Sp2/0 cells cultured in the presence of Gln for the entire duration of the experiment. The data in (b) and (c) are expressed as the average \pm SD of at least three independent experiments.

processed fragments were observed whether the cells were cultured in the presence of Gln or not, indicating that caspase-8 processing is not affected by Gln deprivation.

To compare the activation status of both caspases-8 and -9 in Gln-deprived Sp2/0 cells, we performed *in vitro* enzymatic assays. A 2.5-fold increase in caspase-9 activity was detected as early as 60 min after Gln deprivation (Fig. 6a). By 2 h of Gln starvation, caspase-9 activity had increased more than sevenfold compared to

the Gln-fed control (Fig. 6a). By contrast, caspase-8 activity was increased by only 1.5-fold after 2 h of Gln withdrawal (Fig. 6a). To further confirm the importance of caspases in the death program triggered by Gln starvation, Sp2/0 cells were deprived of Gln for 3 h in the presence of different synthetic, peptide-based caspase inhibitors. Gln was then added and cell culture was resumed for 24 h, at which time the number of viable cells was assessed. The presence of the pan-caspase inhibitor Z-VAD-fmk and the caspase-3 inhibitor DEVD-fmk resulted in a significant reduction in the number of cells committed to the death program (Fig. 6b). However, the caspase-9 inhibitor LEHD-fmk provided only a limited protection against cell death. The addition of an equivalent amount of DMSO had no effect on the reduction of cell viability upon Gln starvation (Fig. 6b). All together, these data indicate that caspases play an important role in the cell death process induced by Gln starvation. Our results also demonstrate that caspase-9 is the primary initiator caspase which is processed and activated in Gln-deprived Sp2/0 cells. However, caspase-9 inhibition is clearly not sufficient, on its own, to block the progression to cell death, an observation which is in line with other studies (Oppenheim et al., 2001; Yaginuma et al., 2001).

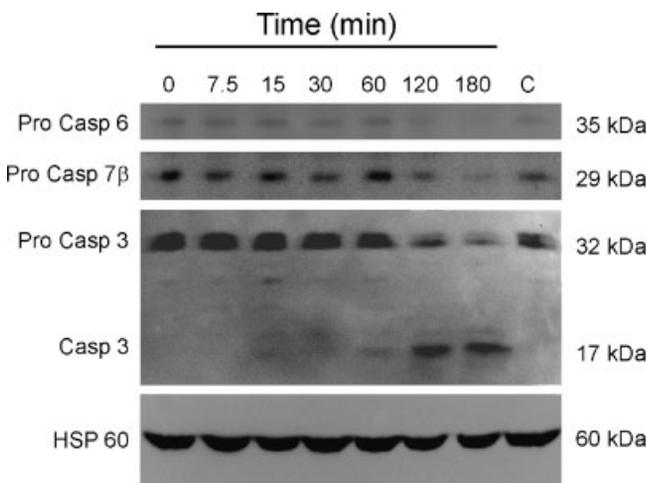


Fig. 3. Processing of executioner caspases in Gln-starved Sp2/0 cells. Sp2/0 cells were cultured in the absence of Gln for the indicated time periods. Chaps cell extracts were then prepared and processed for PAGE-SDS/Western blot analysis using polyclonal antibodies against caspase-3, -6, and -7. An anti-HSP-60 antibody was also used to confirm that equal amounts of proteins were loaded in each lane. Lane C: Extracts from control Sp2/0 cells cultured in the presence of Gln for the entire duration of the experiment. The proforms of caspases-6 (35 kDa), -7 β (29 kDa), and -3 (32 kDa), as well as the processed, 17 kDa fragment of caspase-3 are shown. The precursor and processed forms of the functional caspase-7 α enzyme, as well as the processed fragments of caspases-6 and -7 β could not be detected despite of the use of several polyclonal antibodies. Data are representative of duplicate time course experiments.

Rapid cytoplasmic release of Cyt c and SMAC/DIABLO upon Gln withdrawal

The activation of caspase-9 suggested that Gln deprivation triggered a mitochondria-dependent, intrinsic apoptotic pathway. One of the hallmark features of this pathway is the cytoplasmic release of mitochondrial proteins such as Cyt c and SMAC/DIABLO (van Gurp et al., 2003). We, therefore, performed cellular fractionation experiments in order to examine the release of mitochondrial proteins upon Gln deprivation. A time course experiment indicated that Gln deprivation caused the rapid release of both Cyt c and SMAC/DIABLO into the cytoplasm (Fig. 7a). Both proteins were released in two waves: a modest, early leakage 15–30 min after Gln deprivation followed by a more significant release at 60 min. In keeping with the known

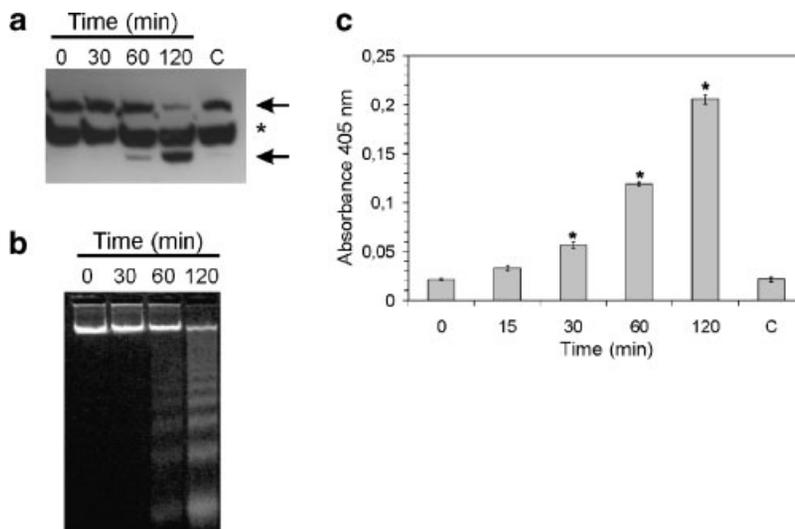
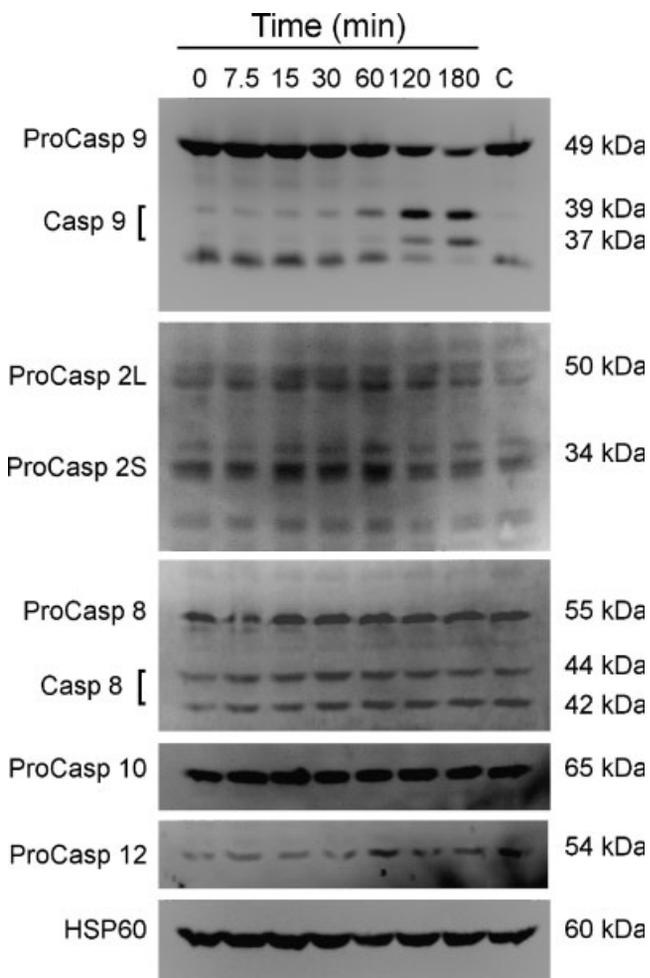


Fig. 4. Activation of caspase-3-like enzymes in Gln-starved Sp2/0 cells. Sp2/0 cells were incubated for the indicated time periods in the absence of Gln. **a:** Poly (ADP ribose) polymerase (PARP) cleavage analysis. Arrows indicate the 116 and 85 kDa fragments characteristic of the native and processed forms of PARP, respectively. The star shows a protein, which cross-reacted with the PARP antibody. **b:** Electrophoretic analysis of DNA fragmentation. **c:** Determination of

caspase-3-like enzymatic activity. Stars show statistical significance ($P < 0.01$) versus samples incubated in the presence of Gln. Lane C: control Sp2/0 cells cultured in the presence of Gln for the entire duration of the experiment. Data in parts a and b are representative of three independent experiments. Data in part c is expressed as the average \pm SD of triplicate determinations and is representative of three independent experiments.

role of Cyt c as a cofactor for the apoptosome (a large protein complex responsible for caspase-9 activation (Li et al., 1997; Srinivasula et al., 1998)), the timing of the larger Cyt c release in the cytoplasm coincided with

caspase-9 processing (Fig. 5) and activation (Fig. 6a). Interestingly, while low levels of processed caspase-3 could be seen as early as 30 min after Gln deprivation, the full activation of caspase-3 followed the second wave of SMAC/DIABLO and Cyt c release into the cytoplasm (Fig. 7a).



Downregulation of XIAP upon Gln starvation

To prevent the disastrous effect the spontaneous activation of caspases would have on the cell, proteins of the inhibitor of apoptosis (IAP) family act as endogenous caspase inhibitors (Salvesen and Duckett, 2002). SMAC/DIABLO has been shown to target and inhibit IAPs, resulting in full caspase activation and progression through the death pathway (Du et al., 2000). As expected, the intracellular level of the IAP family member XIAP was decreased after 2 h of Gln withdrawal (Fig. 7b), following the second wave of SMAC/DIABLO release into the cytoplasm. Notably, the decrease in XIAP levels coincided with the increase in caspase-3 processing (Fig. 7a) and commitment to cell death (Fig. 2). All together, our results show that in Sp2/0 cells, Gln deprivation rapidly triggers intracellular events leading to the release of mitochondrial apoptogenic molecules such as Cyt c and SMAC/DIABLO, the decrease in XIAP levels, the activation of caspases-3 and -9, and cell death.

Cleavage and mitochondrial translocation of Bax

The release of Cyt c and SMAC/DIABLO can be triggered by the recruitment at the mitochondria of the

Fig. 5. Processing of initiator caspases in Gln-starved Sp2/0 cells. Sp2/0 cells were incubated in the absence of Gln for the indicated time periods. Chaps protein extracts were then prepared and processed for PAGE-SDS/Western blot analysis using polyclonal antibodies against caspases-2, -8, -9, -10, and -12. The precursor and processed forms of the caspases are indicated, along with their molecular masses. An anti-HSP-60 antibody was used to confirm that similar amounts of proteins were loaded in each lane. Lane C: Extracts from control Sp2/0 cells cultured in the presence of Gln for the entire duration of the experiment.

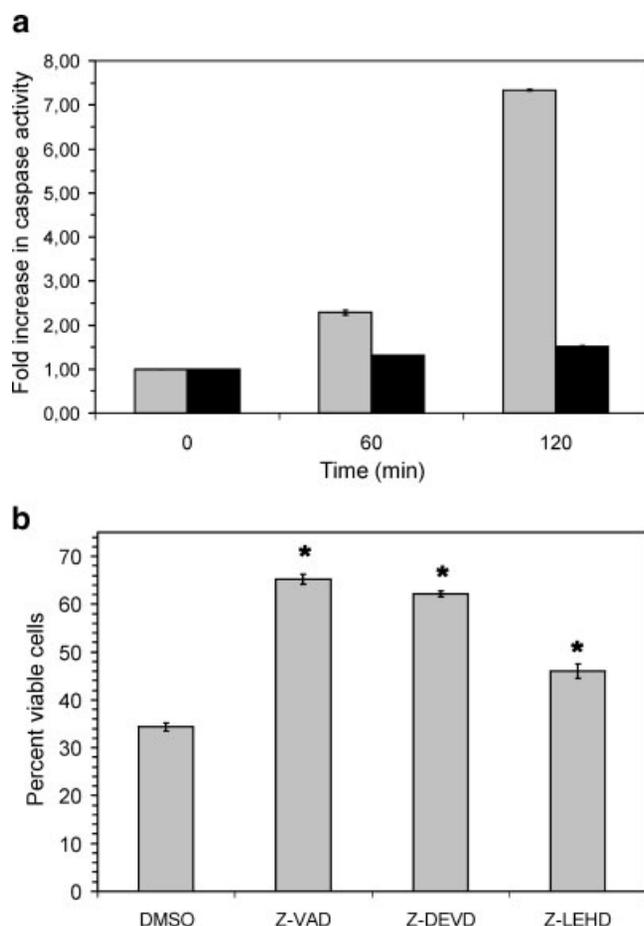


Fig. 6. Activation of caspases-8 and -9 in Gln-deprived Sp2/0 cells. **a:** Activation of caspases-8 and -9 in Gln-starved SP2/0 cells. SP2/0 cells were cultured in the absence of Gln for the indicated time periods and processed for the determination of caspase-9 (gray bars) and caspase-8 (black bars) activity. Data are the average \pm SD of triplicate determinations and are representative of two independent experiments. **b:** Effect of caspase inhibitors. Sp2/0 cells were transferred in culture media deprived of Gln and supplemented with 10 μ M of the indicated synthetic caspase inhibitors or with DMSO. After 3 h, Gln was added to a final concentration of 4 mM, and the incubation was resumed for 24 h. The number of dead and viable cells was then assessed by the trypan blue dye exclusion assay. Data are expressed as the average \pm standard error (SE) of five independent experiments. Stars indicate statistical significance ($P < 0.01$) versus the DMSO-supplemented control.

pro-apoptotic Bcl-2-related protein Bax (Wei et al., 2001). In viable cells, Bax exists as a 21 kDa cytosolic protein which migrates to the mitochondria upon apoptosis induction, where it undergoes oligomerization and contributes to the formation of channels through which mitochondrial factors can leak into the cytosol (Esposti and Dive, 2003). Bax can also be cleaved at its amino terminus to generate an 18 kDa protein, which exhibits a greater affinity for the mitochondria and an increased apoptogenic potency (Gao and Dou, 2001; Cao et al., 2003). Here, we show that the larger release of Cyt c and SMAC/DIABLO observed after 60 min of Gln starvation coincided with the generation of the 18 kDa Bax cleavage product and the translocation of both the p18 and p21 forms of Bax to the mitochondria (Fig. 8a). This was accompanied by a decrease in the level of p21 Bax in the cytoplasm (Fig. 8b). The quality of the mitochondrial extracts was monitored by the absence of

LDH, a cytosolic enzyme, and the presence of Grp75, a mitochondrial matrix protein (Fig. 8a). These data suggest that Gln deprivation causes the cleavage of Bax into its 18 kDa form, which migrates to the mitochondria. There, Bax would contribute to the release of SMAC/DIABLO and Cyt c to the cytoplasm, leading to the activation of caspases.

DISCUSSION

The induction of apoptosis following Gln deprivation has been described for several cell lines, including intestinal (RIE-1) (Papaconstantinou et al., 1998), hepatoma (HuH-1) (Xu et al., 1997), leukemia (HL-60, CEM) (Petronini et al., 1996; Fumarola et al., 2001), and lymphoma (U937, Daudi) (Petronini et al., 1996). In all these cell lines, morphological and biochemical indicators of apoptosis are detectable after 12 to 48 h of Gln withdrawal. We report here that Gln starvation induces an apoptotic program of cell death in the murine hybridoma Sp2/0. What distinguishes the Sp2/0 cell line from other cell models is the remarkable rapidity with which Gln deprivation triggers apoptosis. Sp2/0 cells were irreversibly committed to die after only 2 h of Gln starvation, at which time caspase-3 activation was clearly detectable (Figs. 3 and 4). This constitutes, to our knowledge, the most rapid instance of induction of cell death upon amino acid starvation to be reported for a mammalian cell line.

The acute sensitivity of Sp2/0 cells to the effects of Gln deprivation may be explained by a number of factors. Firstly, like several murine B cell hybridomas and myelomas, Sp2/0 cells express low levels of glutamine synthetase (Bebbington et al., 1992), making glutamine an essential amino acid for these cells. Thus, the removal of external sources of Gln would lead to a rapid decrease in the intracellular concentration of the amino acid, accelerating the onset of signaling pathways triggered by Gln starvation. In fact, the growth of hybridomas expressing higher levels of glutamine synthetase, either because of gene amplification or transfection, shows increased Gln-independence (Bebbington et al., 1992; Bell et al., 1995). Secondly, Sp2/0 cells express low endogenous levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL, resulting in sensitization to several cytotoxic agents (Gauthier et al., 1996). Accordingly, overexpression of Bcl-xL significantly increases the viability of Gln-deprived Sp2/0 cells (Charbonneau and Gauthier, 2000; Charbonneau et al., 2003). Because Bcl-xL and Bcl-2 promote cell survival by maintaining the integrity of the mitochondria (Cory and Adams, 2002), the observation that Bcl-xL increased the survival of Sp2/0 cells following Gln starvation also validates our findings of a central role for this organelle in the induction of Sp2/0 cell death. Finally, Sp2/0 cells constitutively express *c-myc*, a feature inherited from the plasmacytoma tumors from which mouse myelomas and hybridomas were originally derived (Shen-Ong et al., 1982). The inappropriate regulation of the expression of *c-myc* sensitizes cells to apoptosis (Evan et al., 1992), in a process, which can be inhibited by Bcl-2 (Bissonnette et al., 1992; Fanidi et al., 1992). In that regard, it is interesting to note that Gln has been shown to inhibit apoptosis induced by *c-myc* overexpression (Xu et al., 1997). We postulate that the combination of these three factors contributes to sensitizing Sp2/0 cells towards Gln deprivation-induced cell death.

We demonstrate here that in Sp2/0 cells, an immediate outcome of Gln starvation is the targeting of the mitochondria. The limited release of apoptogenic factors

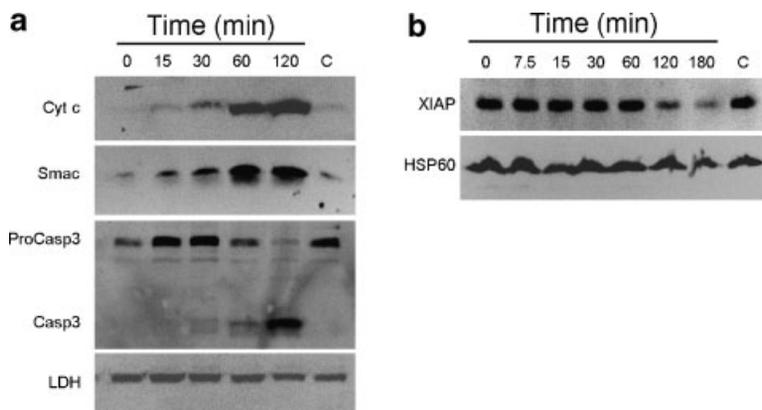


Fig. 7. Cytosolic release of mitochondrial proteins upon Gln deprivation. **a:** Cytochrome c (Cyt c) and SMAC/DIABLO release. Sp2/0 cells were cultured in the absence of Gln for the indicated time periods, and cytosolic protein extracts were prepared. Samples were then processed for SDS-PAGE/Western blot analysis with the following polyclonal antibodies: anti-Cyt c, anti-SMAC/DIABLO, and anti-caspase-3. An anti-lactate dehydrogenase (LDH) antibody was used to show that similar amounts of proteins were present in each lane. The anti-caspase-3 antibody detected the 32 kDa proform and

the processed 17 kDa form of the protease. **b:** Downregulation of X chromosome-linked inhibitor of apoptosis (XIAP). Sp2/0 cells were cultured in the absence of Gln for the indicated time periods, and total cell extracts were prepared. Samples were then processed for SDS-PAGE/Western blot analysis with an anti-XIAP antibody. An anti-HSP 60 antibody was used to show that similar amounts of proteins were present in each lane. Lane C: Extracts from control Sp2/0 cells cultured in the presence of Gln for the entire duration of the experiment. Data is representative of two time course experiments.

into the cytoplasm was the earliest event detected in Sp2/0 cells upon Gln deprivation, with SMAC/DIABLO and Cyt c being detected in the cytosol only 15–30 min after Gln withdrawal (Fig. 7a). This initial release of Cyt c and SMAC/DIABLO occurred before Bax cleavage and its translocation to the mitochondria, preceded caspase-9 and -3 activation and did not commit the cells to the death program. Since our data clearly shows that the

induction of cell death in Sp2/0 cells occurs in a synchronous fashion (Fig. 2), the early presence of SMAC/DIABLO and Cyt c in the cytoplasm may indicate a gradual or stepwise release of mitochondrial proteins, with a critical cytosolic concentration being required to commit the cell to die. Moreover, the first wave of Cyt c and SMAC/DIABLO release may play an active role in the cell death process, as described recently for Cyt c (Boehning et al., 2003).

The second wave of cytosolic release of apoptogenic proteins in Gln-starved Sp2/0 cells could be caused, at least in part, by the pro-apoptotic protein Bax, the cleavage and translocation of which being early events observed in Gln-starved Sp2/0 cells. Interestingly, Bax cleavage into its 18 kDa form has been shown to be catalyzed by calpains, a family of calcium-activated proteases (Wood et al., 1998; Wood and Newcomb, 2000; Gao and Dou, 2001). Whether calcium release and calpain activation are involved in triggering Sp2/0 cell death and whether this process requires the prior release of mitochondrial factors are important issues that are still unresolved. In that regard, preliminary work performed in our laboratory suggests that calpain inhibition significantly reduces the extent of Sp2/0 cell death upon Gln deprivation (Paquette et al., unpublished observations), suggesting that these proteases play an important role in this process.

One of the outcomes of the cytosolic release of Cyt c and SMAC/DIABLO in Gln-starved Sp2/0 cells is the formation of the apoptosome and the inactivation of XIAP, leading to caspase-9 and -3 activation and the commitment to the death program. While our results show that caspase-9 is undergoing processing and activation upon Gln starvation, confirming the activation of an intrinsic apoptotic pathway, we also noted a lack of effect of caspase-9 inhibition on cell survival. This situation is reminiscent of studies on the developmental death of mouse spinal cord neurons, in which the deletion of caspase-9 changed the mode of cell death without affecting the number of dead cells (Oppenheim et al., 2001; Yaginuma et al., 2001). Our observation that the release of both Cyt c and SMAC/DIABLO was

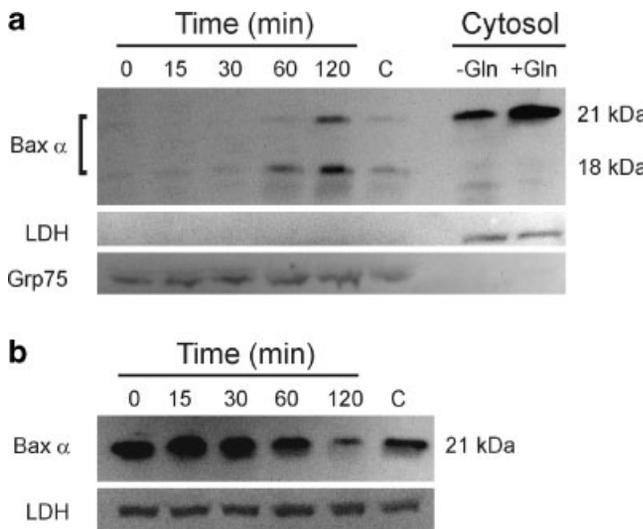


Fig. 8. Cleavage and translocation of Bax to the mitochondria. Sp2/0 cells were cultured in the absence of Gln for the indicated time periods, and cytosolic and mitochondrial protein extracts were prepared. Samples were then processed for SDS-PAGE/Western blot analysis with the following polyclonal antibodies: anti-Bax α , anti-LDH, and anti-glucose-related protein 75 (GRP75). The anti-LDH and anti-GRP75 antibodies were used to show that similar amounts of proteins were present in each lane and to confirm the quality of the extracts. Lane C: Extracts from control Sp2/0 cells cultured in the presence of Gln for the entire duration of the experiment. **a:** Mitochondrial extracts. As a control, cytosolic extracts of SP2/0 cells cultured for 2 h in the presence or absence of Gln were included. **b:** Cytosolic extracts prepared from the same cultures as those shown in (a).

maximal 1 h after Gln withdrawal (Fig. 7a) indicates that mitochondrial integrity was already compromised at the time of caspase-9 activation. Under such conditions, the inhibition of caspase-9 is expected not to affect the progression to cell death but rather to lead to the activation of alternative, caspase-independent death mechanisms (Lockshin and Zakeri, 2002).

Our data also revealed an increase in caspase-8 activity in Sp2/0 cells upon Gln deprivation. While this increase in caspase-8 activity was limited, the actual contribution of this caspase to the death program triggered in Gln-starved Sp2/0 cells remains to be determined. Also of particular interest is our observation of the constitutive processing of caspase-8 in Sp2/0 cells. The existence of a processed initiator caspase in otherwise healthy cells may seem surprising. However, it should be kept in mind that, while processing is sufficient to activate executioner caspases, initiator caspase activation requires dimerization of the enzyme (Boatright et al., 2003). It is, therefore, conceivable that processed caspase-8 is present in an inactivated form in viable Sp2/0 cells as long as the enzyme exists as a monomer.

While Sp2/0 cells underwent cell death upon the removal of any one of several amino acids, the rate of apoptosis induction was considerably slower than following Gln starvation (Fig. 1). This indicates that specific cellular mechanisms are responsible for sensing the intracellular Gln levels in Sp2/0 cells and for triggering the death program. Such a sensing device would allow a rapid response to Gln deprivation and at least in the case of Sp2/0 cells, would target the mitochondria and participate in inducing the cytosolic release of apoptogenic proteins. In that regard, three possibilities can be proposed based on the current understanding of the role of Gln in cell survival. Firstly, Gln has been shown to directly modulate the activity of JNK, a mitogen activated protein kinase (MAPK) involved in stress response and known to participate in the induction of cell death following exposure to a variety of cellular insults. Hence, a recent study by Ko et al. (2001) showed that association of Gln with glutamyl-tRNA synthetase (QRS) resulted in the QRS-mediated inhibition of the apoptosis signal-regulating kinase (ASK-1), a JNK activator (Ko et al., 2001). However, Gln withdrawal relieved the QRS-mediated ASK-1 inhibition, leading to an increase in ASK-1 and JNK enzymatic activity and an increased sensitivity towards FAS-induced apoptosis. As JNK signals cell death by targeting the mitochondria (Tournier et al., 2000), the possible involvement of this stress kinase pathway in the induction of apoptosis in Gln-deprived Sp2/0 cells clearly needs to be investigated.

Secondly, because it participates in the synthesis of GSH, a major cellular antioxidant, Gln deprivation can indirectly trigger cell death signals via the induction of an oxidative stress. Effectively, Gln supplementation has been shown to promote cell survival by reducing the level of oxidative stress (Xu et al., 1997). In addition, a recent study has revealed that Gln supplementation led to an increase in cellular GSH levels, a reduction in oxidative stress, elevated levels of the pro-survival protein Bcl-2, and a decreased sensitivity to apoptotic triggers (Chang et al., 2002). Finally, the increased accumulation of reactive oxygen species observed upon Gln starvation has been shown to sensitize cells to death triggers (Goossens et al., 1996). However, whether Gln withdrawal can be sufficient, on its own, to cause an oxidative stress that would trigger

pathways leading to spontaneous apoptosis remains to be demonstrated.

Lastly, Gln has been recently shown to signal cell survival via its effect on the regulation of cellular volume. Effectively, because its intracellular transport frequently involves sodium-dependent mechanisms and its metabolism results in cytosolic accumulation of non-permeant catabolic products, Gln is involved in cell volume homeostasis. Therefore, signaling pathways responsive to changes in cell volume could be triggered upon Gln deprivation, resulting in the activation of a death program. Indeed, Gln starvation of CEM and HL-60 cultures was shown to lead to cell death in a mechanism, which was dependent on a reduction in cell volume (Fumarola et al., 2001). The Gln-deprivation-induced death of CEM and HL-60 cells could be prevented in the presence of D-Gln and other compatible osmolytes, confirming that Gln starvation triggered apoptosis in these cells via the induction of an osmotic stress (Fumarola et al., 2001). Of major interest is the finding by Fumarola et al. (2001) that the initiation of apoptosis by Gln deprivation in both CEM and HL-60 cells required the activation of caspase-8, and that cell death could be blocked by preventing the recruitment of caspase-8 to the Fas receptor (Fumarola et al., 2001). Whether the activation of caspase-9 in Gln-starved Sp2/0 cells is the result of an osmotic stress should be examined, as well as the possible involvement of the intrinsic pathway in the death of Gln-deprived CEM and HL-60 cells. Most importantly, however, what the Fumarola study and our current findings clearly demonstrate is that at least two distinct apoptotic pathways can potentially be activated upon Gln starvation: a death receptor-dependent pathway, observed in HL-60 and CEM cells, in which the activation of caspase-8 triggers cell death, and an intrinsic pathway, observed in Sp2/0 cells, in which the release of mitochondrial proteins leads to caspase-9 activation. A pressing issue, therefore, will be to understand how the cellular context can influence the type of death pathway triggered upon Gln starvation.

Several diseases, including inflammatory bowel disease as well as sepsis, have been associated with reduced physiological levels of Gln (Ziegler et al., 2003). Accordingly, Gln administration has been shown to have marked beneficial effects in several models of critical illness, and the use of Gln as a drug or nutraceutical is actively being investigated (Kelly and Wischmeyer, 2003). A better understanding of the molecular mechanisms responsible for the cellular effects of Gln is essential in order to understand the role which this amino acid (or the lack thereof) plays in pathogenesis, as well as to evaluate its potential usefulness in therapy. In that regard, our findings on the regulation of apoptosis in Sp2/0 hybridoma clearly demonstrate that this cell line constitutes a unique model for the study of the signaling pathways responsible for the pro-survival properties of Gln.

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