Oxidative stress is not required for the induction of apoptosis upon glutamine starvation of Sp2/0-Ag14 hybridoma cells

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Abstract

L-glutamine (Gln) withdrawal rapidly triggers apoptosis in the murine hybridoma cell line Sp2/0-Ag14 (Sp2/0). In this report, we examined the possibility that Gln deprivation of Sp2/0 cells triggers an oxidative stress which would contribute to the activation of apoptotic pathways. Gln withdrawal triggered an oxidative stress in Sp2/0 cells, as indicated by an increased accumulation of reactive oxygen species (ROS) and an increase in the intracellular content in protein carbonyl groups. Gln starvation also caused a decrease in the intracellular levels of glutathione (GSH). However, a decrease in GSH was not sufficient to induce Sp2/0 cell death since reducing GSH levels with DL-buthionine-[S,R]-sulfoximine did not affect cell viability. The antioxidant N-acetyl-L-cysteine (NAC), while effective in inhibiting ROS accumulation and oxidative stress, did not prevent the loss in cell viability or the processing and activation of caspase-3 triggered by Gln starvation. On the other hand, NAC did reduce the formation of apoptotic bodies in dying cells. Altogether these results indicate that in Sp2/0 cells, Gln deprivation leads to the induction of an oxidative stress which, while involved in the formation of apoptotic bodies, is not essential to the activation of the cell death program.
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Introduction

L-glutamine (Gln), the most abundant free amino acid in the blood, is involved in the transport of nitrogen to tissues and organs (Newsholme et al., 2003). It also provides carbon atoms used in metabolism and is a precursor for the synthesis of several biomolecules (Newsholme et al., 2003). Gln has been shown to influence several signal transduction pathways, including the p38 mitogen-activated protein stress kinase pathway (Ko et al., 2001), the cAMP-dependent protein kinase pathway (Xia et al., 2003) and the mammalian target of rapamycin pathway (Xia et al., 2003). Thus, Gln availability can influence several cellular processes, including cell survival (Exner et al., 2002; Fumarola et al., 2001; Paquette et al., 2005; Pithon-Curi et al., 2003). Effectively, Gln withdrawal increases the sensitivity of intestinal epithelial cells to apoptosis, a phenomenon which has been proposed to promote the

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loss of integrity of the intestinal permeability barrier, a contributing factor to sepsis (Evans et al., 2003; Papaconstantinou et al., 1998, 2000). Similarly, Gln starvation induces the spontaneous death of neutrophils (Pithon-Curi et al., 2003), which may explain the increased incidence of infection observed in critical care patients (Labow and Souba, 2000). However, very little is known about the molecular mechanisms by which Gln regulates cell survival.

We have recently shown that the Sp2/0-Ag14 (Sp2/0) mouse hybridoma cell line is acutely sensitive to Gln deprivation (Paquette et al., 2005). Gln starvation rapidly triggers a process of apoptosis in Sp2/0 cells, which are committed to the death program after only 2 h of Gln withdrawal. This effect is specific to Gln, as the removal of any other essential amino acid triggers cell death in a more protracted manner. The rapidity of cell death induction and the specificity of this response make Sp2/0 cells a unique model for the study of the molecular mechanisms responsible for the control of cell survival by Gln.

One possible mechanism whereby Gln could influence Sp2/0 cell viability is through the regulation of the cellular redox status (Matés et al., 2002). Gln is involved in the synthesis of glutathione (GSH; γ-glutamyl-cysteinyl-glycine), the principal intracellular antioxidant (Fig. 1). First, mitochondrial glutaminase converts Gln to L-glutamate (Glu), which is then coupled to L-cysteine (Cys) via the action of the enzyme glutamate cysteine ligase to form L-γ-glutamylcysteine, the first intermediate in GSH synthesis (Amores-Sánchez and Medina, 1999). GSH synthase then completes GSH synthesis by catalyzing the addition of glycine to L-γ-glutamylcysteine. Second, Gln-derived Glu directly participates in the cellular uptake of cystine through the X_c^- antiporter
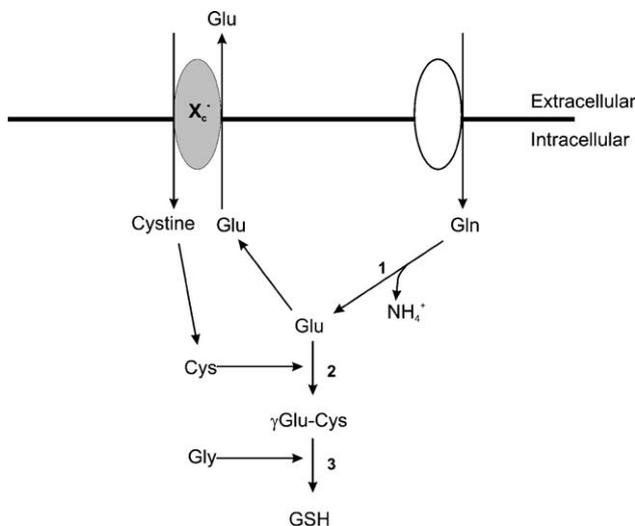
(Bannai and Ishii, 1988; Sato et al., 1999). Cystine is then reduced to Cys in the cytosol. Since Cys is a rate-limiting precursor for GSH synthesis, Gln indirectly contributes to the maintenance of appropriate GSH levels (Sato et al., 1998). Supporting the possibility that Gln controls cell survival through the maintenance of adequate GSH levels, increases in the intracellular concentration of GSH by Gln have been shown to reduce reactive oxygen species (ROS) formation and protect against apoptosis (Chang et al., 2002; Xu et al., 1997). However, whether Gln deprivation induces an oxidative stress which is causal to the onset of apoptosis is unknown.

In this report, we investigated the role which oxidative stress plays in the induction of apoptosis in Gln-starved Sp2/0 cells. We show that Gln starvation triggers an oxidative stress which, while being implicated in the formation of apoptotic bodies, is not essential for the induction of the death program.

Materials and methods

Reagents

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Canada (Oakville, Ont., Canada). DL-buthionine-[S,R]-sulfoximine (BSO), Gln and N-acetyl-L-cysteine (NAC) stock solutions were made fresh in phosphate-buffered saline (PBS; 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) and adjusted to pH 7.4. The pan-caspase inhibitor Z-VAD-fmk (Calbiochem, San-Diego, CA, USA) was dissolved as a 10 mM stock in dimethylsulfoxide (DMSO) and used at a final concentration of 10 μM.

Cell culture

The mouse hybridoma Sp2/0 (CRL 1581) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Iscove's medium supplemented with 5% Fetalclone I (Hyclone, Logan, UT, USA), 4 mM Gln, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture was performed at 37 °C under a humidified atmosphere of 5% CO_2. For the Gln deprivation experiments, exponentially growing Sp2/0 cells were washed twice in warm PBS, resuspended in complete Iscove's medium devoid of Gln, and cultured for the indicated amount of time. For some experiments, NAC was added at the start of the experiment at a final concentration of 15 mM. To determine the viability of the Gln-depleted Sp2/0 cells, an aliquot of the sample was transferred into complete Iscove's medium, the concentration of Gln adjusted to 4 mM and the cells were cultured for 24 h. The number

Fig. 1. Role of Gln in GSH synthesis: (1) glutaminase, (2) glutamate cysteine ligase, (3) GSH synthase. X_c^-: glutamate-cystine antiporter.

of viable and dead cells was then determined using the trypan blue dye exclusion assay.

For the experiments in which BSO was used, Sp2/0 cells were cultured in complete Iscove's medium containing Gln and 50 mM BSO. PBS was used instead of BSO in the control sample. After a 24-h incubation period, a sample was taken for the determination of cell viability using the trypan blue assay. The remainder of the cells was used for the determination of the intracellular GSH levels.

Hoechst assay

Cells (2.5×10^5) were deprived of Gln for 2 h in the presence of NAC or PBS. Gln-supplemented cultures were used as controls. Hoechst 33342 was added to Sp2/0 cells to a final concentration of 2 µg/ml 30 min before the end of the experiment. The cells were then washed twice in PBS, resuspended in PBS and observed under fluorescence microscopy using a Leitz Diaplan microscope (Leica Microsystems, Richmond Hill, Ont., Canada). Cells showing a condensed or fragmented nucleus were counted as apoptotic. At least 200 cells were counted in each experiment.

Acridine orange-ethidium bromide staining

Cells (2.5×10^5) were deprived of Gln for 2 h in the presence of 15 mM NAC or PBS. Gln-supplemented cultures were used as controls. At the end of the incubation period, the cells were put on ice for 10 min and washed once in cold PBS. Cells were then resuspended in PBS containing 6 µg/ml each of acridine orange and ethidium bromide and incubated at room temperature for 1 min before examination by fluorescence microscopy. Photographs were taken using a Zeiss Axiovert 100 fluorescence microscope (Carl Zeiss, Thornwood, NY, USA) with an Optronics 3 CCD cooled color camera and analyzed with Northern Eclipse software (Empix emission, Mississauga, Ont., Canada).

Determination of cellular GSH levels

The cellular levels of GSH in Sp2/0 cells were determined using the ApoGSH GSH detection kit (Biovision Inc., Mountain View, CA, USA), following instructions provided by the manufacturer. This assay is based on the principle that the dye monochlorobimane (3-[chloromethyl]-2,5,6-trimethyl-1H,7H-pyrazolo[1,2-α]pyrazole-1,7-dione – MCB) becomes highly fluorescent upon binding GSH. The concentration of GSH in the samples was determined with a standard curve of reduced GSH. MCB fluorescence (excitation: 380 nm; emission: 460 nm) was measured using a Fluostar microplate spectrofluorometer (BMG-Labtechnologies, Durham, NC, USA). GSH levels were normalized to the protein content of the extracts (determined with the DC-Protein Assay; Bio-Rad Laboratories Ltd., Mississauga, Ont., Canada).

Measurement of oxidative stress

Hydroethidium (HE) assay

The HE experiment was performed according to published procedures (Bindokas et al., 1996). HE was prepared as a 315 µM solution in 50% (v/v) DMSO. Cells (2.5×10^5/ml) were deprived of Gln for 2 h. HE was added to the cells to a final concentration of 5 µM, 15 min before the end of the incubation period. The cells were then incubated on ice for 10 min, washed once and resuspended in PBS. Fluorescence microscopy was performed using a Zeiss Axiovert 100 fluorescence microscope. Photographs were taken as described above.

Protein carbonyl assay

The intracellular content of protein carbonyl residues was determined by the formation of protein hydrazones using 2,4-dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994). Sp2/0 cells (1×10^7) were washed in PBS, resuspended in homogenizing buffer (50 mM phosphate buffer, pH 7.4, 0.1% digitonin, protease inhibitor cocktail [final concentrations: 2 mM aminoethyl-benzene sulfonyl fluoride, 1.6 µM aprotinin, 40 µM leupeptin, 80 µM bestatin, 30 µM pepstatin A, and 30 µM E64], 40 µg/ml phenylmethylsulfonyl fluoride [PMSF] and 1 mM EDTA), incubated for 15 min at room temperature and centrifuged at 6000g for 10 min. Nucleic acids were removed by the addition of streptomycin (1% final concentration), followed by a 15-min incubation at room temperature and centrifugation at 6000g for 10 min. The supernatant was then divided into two aliquots: to one tube, four volumes of a solution of 2.5 M HCl was added, while four volumes of 10 mM DNPH in 2.5 M HCl was added to the other tube. Both samples were incubated for 1 h at room temperature, in the dark, and mixed by vortex every 15 min. After the addition of a 20% trichloroacetic acid (TCA) solution (10% final concentration), the samples were incubated on ice for 10 min and centrifuged (5 min, 6000g, room temperature). The protein pellets were then washed once with 10% TCA and then four times with a 1:1 (v/v) solution of ethanol/ethyl acetate. The pellets were solubilized in 6 M guanidine hydrochloride solution, incubated 10 min at 37 °C with frequent mixing and centrifuged to remove insoluble materials. The spectrum at 355–395 nm of the DNPH-treated sample was obtained (Ultraspec 3000 spectrophotometer, Pharmacia-Biotech, Baie d'Urfé, Que., Canada), using the

sample treated with HCl as blank. The carbonyl content was calculated from the peak absorbance at 355–390 nm, using an absorption coefficient of 22,000/M cm, and normalized according to the protein content. The protein content of the extracts was determined by taking their absorbance at 280 nm, using bovine serum albumin dissolved in 6 M guanidine hydrochloride as standard.

Caspase-3 activity assay

Caspase-3 activity was measured using a colorimetric enzyme assay (Biovision Inc.) following instructions from the manufacturer. Optical density readings were done at 405 nm using a PowerWaveX microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). Caspase activity values were normalized to the protein content of the extract.

Protein extract preparation and Western blot analysis

Protein extracts were prepared in RIPA buffer (1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1 mg/ml PMSF, in PBS) as described previously (Paquette et al., 2005). Protein content was determined using the DC-assay kit (Bio-Rad Laboratories). For poly(ADP-ribose) polymerase (PARP) analysis, soluble protein extracts were prepared in a urea-based lysis buffer (62.5 mM Tris–HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol) as described previously (Shah et al., 1995). The protein extracts were stored at $-80\,^\circ$C until needed. Proteins were fractionated by SDS–PAGE, transferred to a Hybond-P membrane (Amersham Bioscience, Baie-d'Urfé, Que., Canada) and processed for Western blot analysis. Transfer efficiency was determined routinely by staining the membrane with Ponceau S. The following primary antibodies were used: rabbit polyclonal anti-PARP antibody (BioMol, Plymouth Meeting, PA, USA), rabbit anti-ROCK-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-actin antibody. Detection was performed using a goat anti-rabbit IgG secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc.) and the ChemiGlow reagent (Alpha Innotech, San Leandro, CA, USA). Image acquisition was done using a Fluorchem 8000 Imaging System (Alpha Innotech).

DNA fragmentation assay

The analysis of apoptotic DNA laddering was performed as described previously (Charbonneau and Gauthier, 2000). Briefly, 5×10^5 cells were collected, washed once with PBS and lysed with 50 µl of lysis buffer (10 mM Tris–HCl pH 8, 1 mM EDTA pH 8, 0.5% N-lauroyl sarcosine, 0.25 mg/ml proteinase K and 0.02 mg/ml RNase A). After a 5-min incubation at 50 °C, 50 µl of sample buffer (40% sucrose, 0.08% bromophenol blue) was added to the sample, followed by electrophoresis on a 2% agarose gel in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, pH 8). The DNA was then stained for 5 min with ethidium bromide (0.7 µg/ml) and visualized under UV illumination using a Fluorchem 8000 Imaging System (Alpha Innotech).

Transmission electron microscopy (TEM)

TEM analysis was performed at the Electron Microscopy Laboratory, University of New Brunswick (Fredericton, NB, Canada). Sample preparation was done as described previously (Guerin and Gauthier, 2003).

Statistical analysis

Statistical significance was determined using a one-way ANOVA and Scheffe's post hoc test.

Results

Gln deprivation triggers an oxidative stress in Sp2/0 cells

To determine whether oxidative stress plays a role in the induction of apoptosis upon Gln starvation, we analyzed the intracellular accumulation of ROS in Gln-deprived Sp2/0 cells using the HE staining procedure. This assay is based on the intracellular conversion of HE into the fluorescent dye ethidium, a process which is mediated by ROS. While Gln-supplemented cells showed only a weak ethidium signal, Gln deprivation triggered an increase in red fluorescence, suggesting an increase in ROS accumulation (Fig. 2). Importantly, the addition of the antioxidant NAC markedly decreased the ethidium fluorescence in Gln-starved Sp2/0 cells. To determine whether this increase in ROS led to oxidative stress in Sp2/0 cells, the levels of protein carbonyl groups, which increase upon oxidative damage to proteins, were measured. Gln starvation induced a five-fold increase in protein carbonyls ($p < 0.01$), which was prevented in the presence of NAC (Fig. 2G). Therefore, Gln starvation triggers ROS accumulation and oxidative stress in Sp2/0 cells.

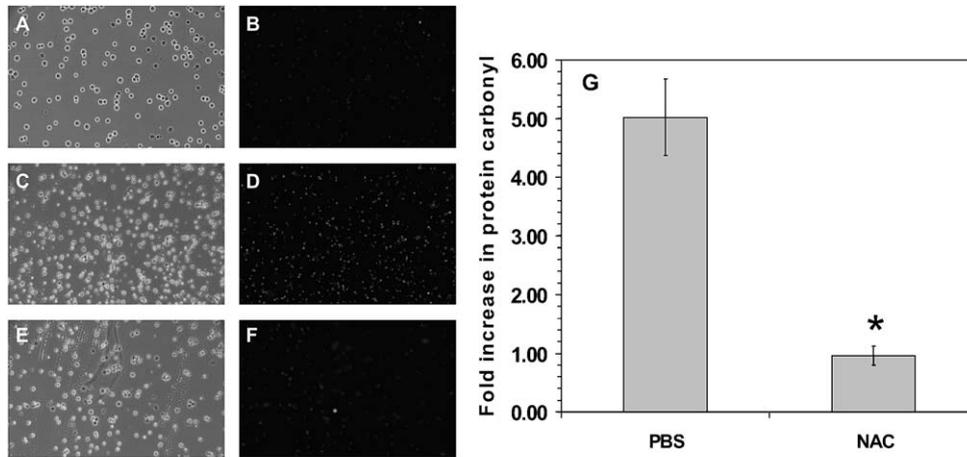The accumulation of ROS and the ensuing oxidative stress in Gln-deprived Sp2/0 cells may be caused by a

Fig. 2. Induction of oxidative stress in Gln-deprived Sp2/0 cells. (A–F) ROS production. Sp2/0 cells were cultured for 2 h in complete Iscove's medium supplemented with Gln (A, B), in medium lacking Gln (C, D) or in medium lacking Gln and supplemented with 15 mM NAC (E, F). Cells were then processed for the detection of ROS formation using the HE staining assay (B, D, F). The corresponding brightfield images are shown (A, C, E). Original magnification × 100. (G) Measurement of protein carbonyls. Sp2/0 cells were transferred to culture medium lacking Gln, supplemented with 15 mM NAC or an equivalent amount of PBS, and incubated for 2 h. Corresponding cultures in which Gln was added (final concentration, 4 mM) were also used as controls. The intracellular content in protein carbonyl groups was then determined as described in Materials and methods. Results are expressed as the fold increase in protein carbonyl in Sp2/0 cells starved of Gln vs. cells incubated in the presence of Gln, and are the average ± standard deviation (SD) of three independent experiments. * $p < 0.01$ vs. PBS control.

diminished ability of the cells to scavenge intracellular oxidants. This possibility was especially attractive, considering the role which Gln plays as a precursor in the synthesis of GSH (Fig. 1). Therefore, we measured the levels of GSH in Gln-deprived and Gln-supplemented Sp2/0 cells. A 50% decrease ($p < 0.05$) in intracellular GSH levels was observed upon Gln withdrawal (Fig. 3A). Importantly, the addition of 15 mM NAC, which was effective in preventing ROS accumulation and protein oxidation in Gln-deprived Sp2/0 cells (Fig. 2), resulted in an increase in the intracellular levels of GSH (Fig. 3B). Since Gln deprivation triggers the rapid activation of caspases in Sp2/0 cells (Paquette et al., 2005), we tested the effect of caspase inhibition on GSH levels. While Z-VAD was effective in protecting Sp2/0 cells against cell death upon Gln starvation (Fig. 3C), the decrease in GSH was still observed (Fig. 3A), indicating that the decline in GSH levels seen in Gln-deprived Sp2/0 cells did not require active caspases.

We next tested whether a decrease in GSH similar to that observed in Gln-deprived Sp2/0 cells was sufficient to trigger cell death. Sp2/0 cells were cultured for 24 h in the presence of the GSH synthesis inhibitor BSO. Determination of the intracellular GSH levels indicated that BSO reduced GSH levels in Sp2/0 cells by 70% (Fig. 4A). However, treatment with BSO did not affect cell viability (Fig. 4B). Together, these results indicate that, while a decrease in GSH is observed in Gln-starved

Sp2/0 cells, lower GSH levels per se are not sufficient to trigger cell death.

Apoptosis induction and caspase activation upon Gln starvation do not require oxidative stress

We next investigated whether the oxidative stress observed upon Gln starvation of Sp2/0 cells was necessary for the induction of apoptosis. In agreement with our previous observations (Paquette et al., 2005), a 2-h Gln deprivation treatment was sufficient to induce nuclear condensation and fragmentation characteristic of apoptosis in over 85% of the cells (Fig. 5). However, the addition of NAC to Gln-deprived cells did not reduce the number of apoptotic cells, even though this treatment significantly decreased oxidative stress as demonstrated by the decrease in ROS production and protein carbonyls (Fig. 2). Confirming these results, NAC was not effective in inhibiting the activation of caspase-3 (Fig. 6A). The cleavage of PARP (Fig. 6C) and the degradation of DNA into oligonucleosome-sized fragments (Fig. 6B), two events catalyzed by active caspase-3, were also observed in NAC-treated, Gln-deprived Sp2/0 cells. Higher concentrations of NAC (up to 60 mM) were ineffective in improving the survival of glutamine-starved Sp2/0 cells (data not shown). Thus, these data demonstrate that the oxidative stress
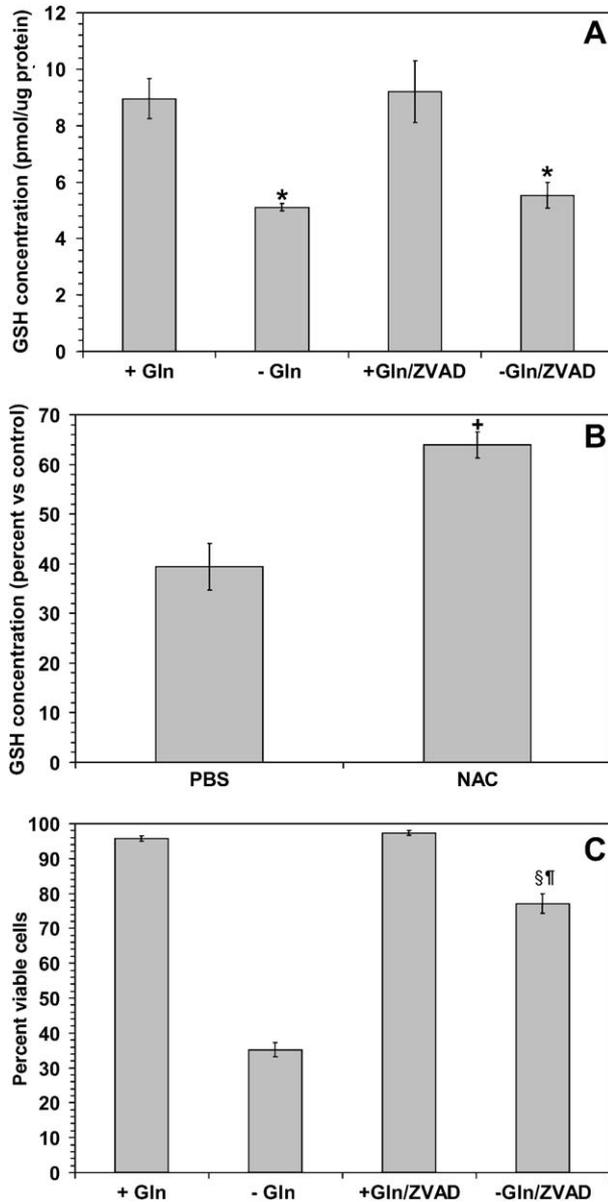
Fig. 3. Gln deprivation triggers a decrease in GSH levels in Sp2/0 cells. Sp2/0 cells were incubated for 3 h in culture medium in the presence or absence of Gln. Where indicated, cultures were also supplemented with 15 mM NAC or 10 μM Z-VAD-fmk. PBS or DMSO was added to the samples incubated in the absence of NAC or Z-VAD, respectively. (A) Effect of Gln starvation and caspase inhibition on GSH levels. Results are expressed as the average $\pm$ SE ($n = 3$). * $p < 0.05$ vs. the respective Gln-containing control. (B) Effect of NAC on GSH levels. Results are expressed as the percent GSH concentration in Gln-deprived cells vs. Gln-containing cultures (average $\pm$ SE of three experiments). $^{+}$ $p < 0.05$ vs. PBS-supplemented control. (C) Cell viability. The percentage of viable cells in the culture was determined using the trypan blue dye exclusion assay. Results are expressed as the average $\pm$ SE ($n = 3$). ¶ $p < 0.01$ vs. samples incubated in the presence of Gln and Z-VAD. § $p < 0.01$ vs. cells deprived of Gln in the absence of Z-VAD.
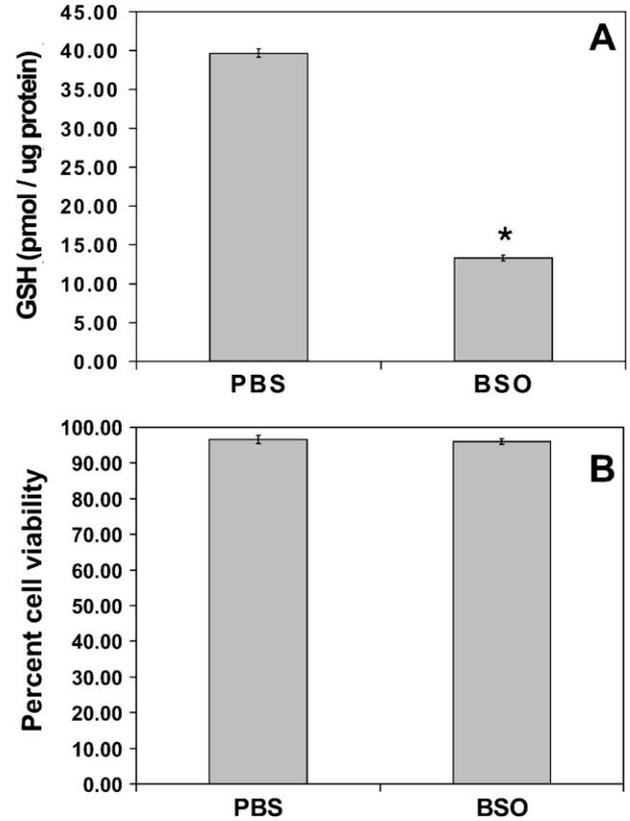

Fig. 4. A decrease in GSH levels is not sufficient to trigger cell death. Sp2/0 cells were cultured for 24 h in the presence of the GSH synthesis inhibitor BSO (final concentration 50 mM). For the control culture, PBS was added in place of BSO. GSH levels and cell viability were then determined as described in Fig. 3. Results are the average $\pm$ SD of triplicate determinations. * $p < 0.01$ vs. PBS-treated control.
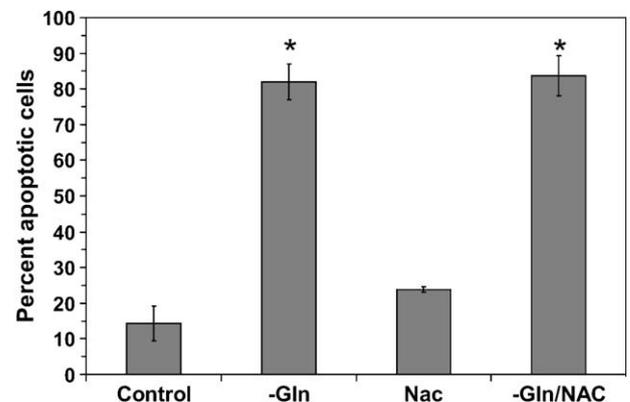

Fig. 5. Effect of NAC on nuclear morphology. Sp2/0 cells were incubated for 2 h in medium in the presence or absence of Gln and supplemented with either PBS or NAC (15 mM). The cells were then processed for a Hoechst assay as described in Materials and methods. Cells exhibiting a condensed or fragmented nucleus were counted as apoptotic. Results are the average $\pm$ SD of three independent determinations. * $p < 0.01$ vs. Gln-supplemented control.
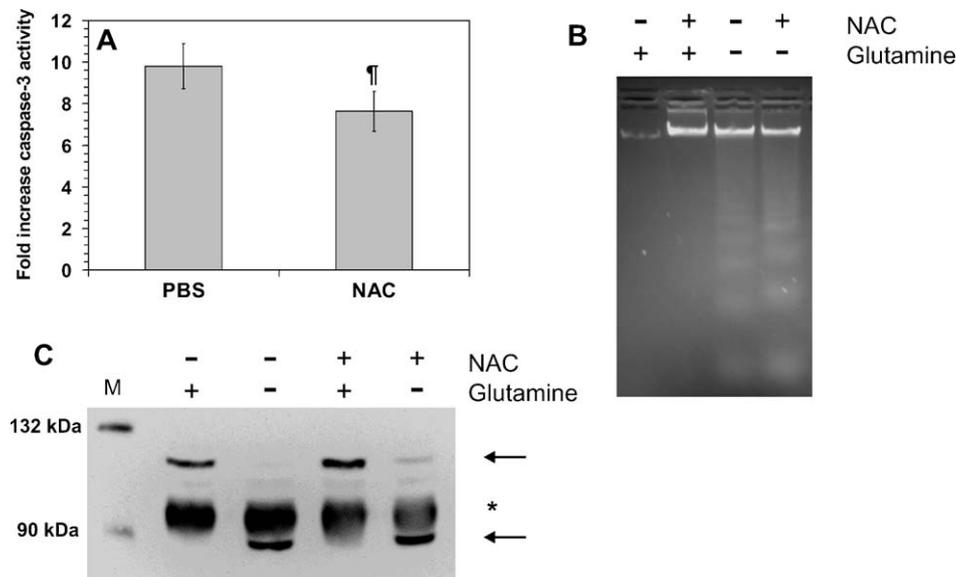
Fig. 6. NAC does not prevent caspase-3 activation in Gln-starved Sp2/0 cells. Cells were incubated in the presence of PBS or NAC (15 mM) and in the presence or absence of Gln for 2 h and processed for (A) the measurement of caspase-3 activity, (B) DNA fragmentation analysis and (C) the determination of PARP cleavage. (A) Activation of caspase-3. Results are expressed as the fold increase in caspase-3 activity in cells deprived of Gln vs. cells incubated in the presence of Gln. Results are the average ± SD of three independent experiments. ¶ $p < 0.05$ vs. PBS control. (B) DNA fragmentation analysis. (C) PARP cleavage. The arrows indicate the 116- and 85-kDa bands corresponding to the intact and cleaved PARP protein, respectively. The asterisk indicates an unknown protein cross-reacting with the primary antibody. M: Cruz marker.

triggered upon Gln starvation of Sp2/0 cells was not required for the activation of the cell death machinery.

Requirement of oxidative stress for the formation of apoptotic bodies

We wished to determine whether the oxidative stress triggered in Gln-starved Sp2/0 cells could be involved in the formation of apoptotic bodies, another morphological feature characteristic of apoptotic cells. In contrast to the lack of effect on apoptotic nuclear morphology, NAC treatment of Gln-deprived Sp2/0 cells significantly reduced the number of cells with apoptotic bodies (Fig. 7A–C). While over 35% of the cells showed the formation of apoptotic bodies after a 2-h Gln deprivation period, this number dropped to 22% in the presence of NAC (Fig. 7D). Examination of ultrastructure of the cells using TEM revealed that, with the exception of a reduction in the number of cells with apoptotic bodies, the addition of NAC to Gln-starved cells did not result in other gross morphological alterations in the dying cells (Fig. 7E–G). In fact, apoptotic chromatin condensation was clearly visible in the NAC-treated cells, in agreement with the Hoechst data (Fig. 5). Finally, we examined whether NAC interfered with the processing of ROCK-1, a 160-kDa Rho GTPase effector protein known to be a key player in the formation of apoptotic membrane blebbing (Leverrier and Ridley, 2001; Sebbagh et al., 2001). Only the 160-kDa form of

ROCK-1 was observed in the Gln-supplemented cells, while Gln deprivation led to ROCK-1 cleavage. Interestingly, NAC treatment did not interfere with ROCK-1 processing in Gln-starved Sp2/0 cells (Fig. 8). Altogether these data show that Gln starvation triggers an oxidative stress in Sp2/0 cells which, while not required for the induction of the death machinery, plays a role in the formation of apoptotic bodies.

Discussion

A number of studies have reported that the antioxidant properties of Gln underlie its pro-survival function. Gln deprivation triggers a decrease in intracellular GSH and promotes oxidative stress in Jurkat T cells (Chang et al., 2002) and HuH-7 hepatoma cells (Xu et al., 1997), with an associated increase in sensitivity to death triggers. Similarly, the oxidative stress initiated by Gln starvation rendered rat bone marrow cells more sensitive to the toxic effect of the chemotherapeutic drugs doxorubicin (Tavares et al., 1998) and cisplatin (Mora Lde et al., 2002). In some cell types (RIE-1, rat neutrophils, human CEM and HL-60), Gln deprivation alone is sufficient to trigger apoptosis (Fumarola et al., 2001; Papaconstantinou et al., 1998; Pithon-Curi et al., 2003). Furthermore, interfering with Gln metabolism by reducing glutaminase activity sensitized Erlich ascites tumor cells to the induction of apoptosis by methotrexate and hydrogen peroxide (Lora et al., 2004). However,
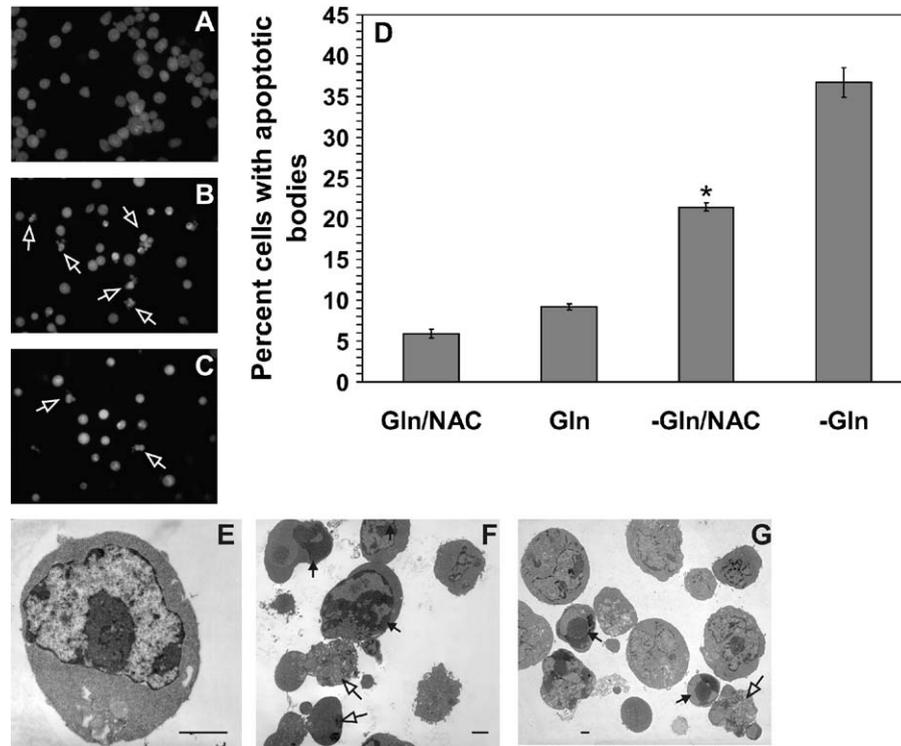
Fig. 7. NAC interferes with apoptotic body formation in Gln-deprived cells. Cells were incubated for 2 h in the presence of PBS or NAC (15 mM) and in the presence or absence of Gln and processed for fluorescence (A–C) or electron (E–G) microscopy. (A–C) Photomicrographs of acridine orange/ethidium bromide-stained Sp2/0 cells. (A) Gln-containing control, (B) Gln-deprived cells and (C) Gln-deprived, NAC-supplemented cells. Open arrows show cells with apoptotic bodies. Original magnification × 100. (D) Quantification of the data shown in (A–C). Data are the average ± SD of five independent experiments. At least 500 cells were enumerated for each experiment. * $p < 0.01$ vs. Gln-deprived cells. (E) Cells cultured in the presence of Gln. (F) Gln-deprived cells. (G) Gln-deprived, NAC-supplemented cells. Open arrows show cells with apoptotic bodies. Solid arrows show cells with condensed or fragmented nucleus. Bars = 1 μm.
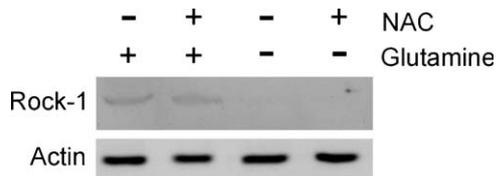

Fig. 8. ROCK-1 cleavage in NAC-treated, Gln-deprived cells. Cells were incubated for 2 h in the presence of PBS or NAC (15 mM) and in the presence or absence of Gln and processed for Western blot analysis with the indicated antibodies. The anti-ROCK-1 antibody is specific for the C-terminal portion of the protein and reacts with the full-length, 160-kDa ROCK-1 protein. An anti-actin antibody was used to demonstrate that equal amounts of proteins were loaded on the gel.

the role of oxidative stress induced by Gln starvation in triggering spontaneous apoptosis has not been demonstrated.

Because Gln deprivation results in the rapid and specific induction of apoptosis, the Sp2/0 cell line constitutes a unique model in order to define the molecular and cellular processes associated with Gln-mediated cell survival

(Paquette et al., 2005). We have demonstrated that Gln starvation results in a decreased level of GSH, an increase in ROS, and a higher level of protein carbonyl groups, indicating that Gln withdrawal triggers an oxidative stress in Sp2/0 cells. Exposure to BSO did not reduce Sp2/0 cell viability, indicating that lower cellular GSH levels are not sufficient to trigger apoptosis in this cell line. Similar observations were recently reported with HT-29 human intestinal cells, where Gln supplementation increased resistance to TRAIL-induced cell death even when GSH synthesis was blocked by BSO (Evans et al., 2003). More importantly, we showed that while the antioxidant NAC was effective in reducing oxidative stress in Gln-starved Sp2/0 cells, it did not inhibit the induction of several apoptosis hallmarks, such as nuclear condensation and fragmentation, caspase activation, and PARP cleavage. Furthermore, TEM data clearly showed that NAC treatment did not lead to alternative death pathways (e.g. necrosis) in Gln-deprived Sp2/0 cells. Therefore, we conclude that oxidative stress is not essential to the induction and execution of cell death in Gln-starved Sp2/0 cells.

In some cell models, ROS has been shown to be necessary for the translocation of the pro-apoptotic protein Bax to the mitochondria, the release of cytochrome *c*, the dissipation of the mitochondrial inner transmembrane potential ($\Delta\psi$m) and caspase activation (Sidoti-de Fraisse et al., 1998). We have recently shown that in Sp2/0 cells, the mitochondrial proteins cytochrome *c* and SMAC/DIABLO are released into the cytosol as early as 15–30 min following Gln withdrawal, preceding caspase-3 activation and the commitment to the death program (Paquette et al., 2005). Therefore, Gln starvation triggers an early disruption of mitochondrial function in Sp2/0 cells. However, the data reported here clearly indicate that signaling events other than oxidative stress must be triggered following Gln starvation to initiate apoptosis in Sp2/0 cells. Other mechanisms leading to alterations in mitochondrial integrity and the release of apoptogenic proteins include members of the Bcl-2 family of apoptosis-modulating proteins and the opening of the permeability transition pore (mPTP) (Green and Kroemer, 2004). The pro-apoptotic Bcl-2 family member Bax has been shown to translocate from the cytosol to the mitochondria upon apoptosis induction. Mitochondrion-associated Bax participates in the formation of channels which allow the release of the mitochondrial proteins involved in caspase activation (Dejean et al., 2005). We have previously shown that Bax is recruited to the mitochondria in Gln-deprived Sp2/0 cells, although only after cytochrome *c* and SMAC/DIABLO release and caspase-3 activation, making it an unlikely candidate as the death-inducing factor in this model (Paquette et al., 2005). We are currently investigating other mechanisms of mitochondrial outer membrane permeabilization, such as the involvement of the so-called BH3-only proteins of the Bcl-2 family, which can also trigger the cytosolic release of mitochondrial apoptogenic proteins (Green and Kroemer, 2004).

In some situations, the accumulation of ROS occurs only following caspase-mediated disruption of mitochondrial integrity (Ricci et al., 2003). Our results revealed that oxidative stress plays a part in the demise of Gln-starved Sp2/0 cells by participating in the formation of membrane blebs and apoptotic bodies, a late event in the apoptotic program. The observation that caspase-3 activation was not prevented in Gln-deprived Sp2/0 cells incubated with NAC, while apoptotic body formation was reduced suggests that ROS contribute to apoptotic membrane blebbing in a pathway located either downstream or in parallel to caspase-3 activation. Several caspase-3-independent pathways that participate in apoptotic body formation have been identified. A kinase dead mutant of the short form of caspase-2 (caspase-2S) is able to block the maturation of apoptotic bodies without interfering with caspase-3 activity or DNA and PARP cleavage,

suggesting that caspase-2 may play a critical role in the generation of apoptotic bodies (Droin et al., 2001). Interestingly, caspase-2 has also been shown to be activated in cells undergoing oxidative stress (Annunziato et al., 2003; Takahashi et al., 2004). Mono-ADP ribosylation of actin is also an important event in the genesis of apoptotic bodies, and the ADP-ribosylation inhibitor meta-iodobenzylguanidine can block apoptotic body formation without perturbing caspase-3 activation (Lodhi et al., 2001). A signaling pathway leading to apoptotic membrane blebbing and involving oxidative stress-mediated GSH oxidation, mobilization of intracellular calcium and p38 kinase activation has been described (van Gorp et al., 2002). Finally, both the structure and spatial organization of actin can be modulated by oxidative stress, suggesting that increased ROS levels could directly act on the cytoskeleton to trigger membrane blebbing (Dalle-Donne et al., 2001). More investigations are therefore required in order to define the pathways modulated by ROS and which contribute to apoptotic body formation in Gln-starved Sp2/0 cells.

In conclusion, the data presented in this paper show that, while oxidative stress is involved in the formation of apoptotic bodies, it does not play a major role in triggering apoptosis in Gln-deprived Sp2/0 cells. Further, studies are warranted in order to define the signaling pathways contributing to the induction of cell death in Gln-starved Sp2/0 cells, and to determine whether these observations can be extended to other cell models.

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