Biochemical and Biophysical Research Communications 378 (2009) 145–148

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ALG-2 knockdown in HeLa cells results in G2/M cell cycle phase accumulation and cell death

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ARTICLE INFO

Article history:
Received 3 November 2008
Available online 14 November 2008

Keywords:
Calcium-binding proteins
Apoptosis-linked gene-2 (ALG-2)
siRNA
Apoptosis
Cell cycle
Caspase

ABSTRACT

ALG-2 (apoptosis-linked gene-2 encoded protein) has been shown to be upregulated in a variety of human tumors questioning its previously assumed pro-apoptotic function. The aim of the present study was to obtain insights into the role of ALG-2 in human cancer cells. We show that ALG-2 downregulation induces accumulation of HeLa cells in the G2/M cell cycle phase and increases the amount of early apoptotic and dead cells. Caspase inhibition by the pan-caspase inhibitor zVAD-fmk attenuated the increase in the amount of dead cells following ALG-2 downregulation. Thus, our results indicate that ALG-2 has an anti-apoptotic function in HeLa cells by facilitating the passage through checkpoints in the G2/M cell cycle phase.

The calcium-binding protein ALG-2 was initially identified as a factor needed for TCR mediated apoptosis in 3DO mouse cells [1]. Since ALG-2 binds calcium at the micromolar (μM) level [2], it has been proposed that ALG-2 could be involved in calcium mediated signaling processes regulating cell death (reviewed in [3,4]). Induction of cell death by various means was partially impaired in the ALG-2 depleted 3DO clones [1,4] and it was proposed that ALG-2 has a pro-apoptotic function in the murine Apaf$^{-/-}$ cell line [5]. In addition, it was shown that the death-inducing effect of Alix (apoptosis-linked gene-2 (ALG-2)-interacting protein X) on chicken neuroepithelial cells is dependent on its ability to bind ALG-2 [6]. However, the pro-apoptotic function of ALG-2 has not been confirmed in human cells. Recent findings that ALG-2 is overexpressed in a variety of human tumors [7–10] and that ALG-2 may contribute to cancer cell viability [9] challenge the concept that ALG-2 is a general pro-apoptotic factor. The present study provides evidence that ALG-2 depleted HeLa cells accumulate in the G2/M phase of the cell cycle and that depletion of ALG-2 increases the fraction of early apoptotic and dead cells.

Materials and methods

Cell lines and siRNA transfection. The human cervical carcinoma cell line, HeLa, was grown as earlier described [11]. A day prior to siRNA transfection the cells (10,400 cells/cm^2) were seeded in medium containing 10% FBS. Cells were transiently transfected with 50 nM siRNA using Oligofectamine reagent (Invitrogen) according to the manufacturer's transfection protocol. For investigations of cell viability/apoptosis the cells were reseeded the following day for a second round of siRNA transfection. The previously described ALG-2 siRNA (siALG-2) duplexes targeting position 112-130 and 197-215 of the ALG-2 transcript (GenBank Accession No. NM_013232) [9] were used to achieve ALG-2 downregulation and the siRNA against the firefly luciferase gene (siGL2) was included as a non-targeting control [12]. All siRNA duplex oligonucleotides were obtained from Dharmacon (Lafayette, CO, USA).

Western blot analysis. Following trypsinization cells were harvested with spent medium to include nonadherent cells. Cells were washed with PBS and extracts were prepared in lysis buffer and further processed for Western blotting as described [9]. The polyclonal anti-ALG-2 antibodies were purified in our laboratory [8] and monoclonal mouse anti-cyclin B1 and polyclonal goat anti-Hsc70 antibodies were from Santa Cruz Biotechnology (Santa Cruz). Secondary HRP conjugated antibodies (Goat anti-rabbit, goat anti-mouse and rabbit anti-goat) were obtained from Dako. As loading controls immunoblotting using Hsc70 antibodies were used.

Cell cycle profile. Adherent and nonadherent cells were collected and a subset of the cells were isolated for Western blot analysis of ALG-2, cyclin B1, and Hsc70 content. The remaining cells were washed once and resuspended in cold PBS containing 1% FBS (PBS/FBS). The cells were fixed and permeabilized by adding ice-cold ethanol to a final concentration of 84% followed by incubation at 4 °C for at least 30 min. DNA staining was done by resuspending approximately 1×10^6 cells/ml in PBS/FBS containing 50 μg/ml

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doi:10.1016/j.bbrc.2008.11.021

propidium iodide (PI; Sigma–Aldrich), 10 μg/ml RNase-A (Sigma–Aldrich) and incubated for 30 min at 37 °C. Samples were kept in dark on ice until analysis by flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ, USA). The distribution of cells in the cell cycle was analysed using the ModFit LT software (BD Biosciences).

Annexin V assay. Detached cells were collected and attached cells were briefly trypsinized. The cells were pooled, washed once in PBS and resuspended to approximately 1×10^6 cells/ml in a buffer containing 0.01 M HEPES–NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$. The cells were stained by incubation for 15 min with 2% v/v fluorescein isothiocyanate (FITC)-conjugated Annexin V (ANNEX-INV01, Invitrogen) and 8.0 μg/ml 7-amino-actinomycin (7-AAD; Sigma–Aldrich) at room temperature. Samples were kept in the dark on ice until analysed by flow cytometry on a BD FACScan. The populations of viable (Annexin V-FITC negative; 7-AAD negative), early apoptotic (Annexin V-FITC positive; 7-AAD negative) and dead (7-AAD positive) cells were determined using the Summit software (Dako, Glostrup, Denmark) [13].

Caspase inhibition. Twenty-four hours following siRNA transfection 25 or 50 μM of the pan-caspase inhibitor z-VAD-fmk (Bachem) was added and the cells were incubated for 48 h in the presence of inhibitor. The fractions of early apoptotic and dead cells were determined using the Annexin V assay.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test. A *p* value less than 0.05 was considered significant.

Results

ALG-2 deficiency leads to G2/M cell cycle phase accumulation

Previous investigations showed that knockdown of ALG-2 in HeLa and U2OS cells resulted in a decreased proliferation rate of cells [9]. This could indicate that they were arrested in a specific cell cycle phase or progressed more slowly through the entire cell cycle. To address this question two different siRNAs against the ALG-2 transcript were used (siALG-2 112 and siALG-2 197) to downregulate ALG-2 expression [9]. Cells, either non-transfected or transfected with 50 nM siGL2, siALG-2 112 or siALG-2 197, were analysed by FACS three days following siRNA transfection. A decrease in the 2N and an increase in the 4N DNA content was observed (Fig. 1A) following partial downregulation of ALG-2 (Fig. 1B). Compared to control siGL2 transfected cells a significant increase in the G2/M (4N) population of 51% was observed following partial ALG-2 knockdown using siALG-2 112 and 197, respectively (Fig. 1C). To further examine whether partial knockdown of ALG-2 resulted in G2/M arrest, the protein level of a known G2/M cell cycle phase marker, cyclin B1 [14], was examined by Western blot analysis. Immunoblotting of lysates from cells harvested from the same cultures as used for FACS analysis showed elevated levels of cyclin B1 (Fig. 1B) supporting our FACS analysis studies, which point to a G2/M phase arrest as a result of partial ALG-2 downregulation. Cyclin B1 levels were 5- and 3.5-fold higher in the siALG-2 112 and 197 transfected cells, respectively as compared to the cyclin B1 levels in siGL2 transfected cells (Fig. 1D).

ALG-2 downregulation leads to induction of caspase dependent cell death pathways

In order to evaluate the effect of ALG-2 downregulation on cell viability, the exposure of phosphatidylserine on the outer cell membrane (Annexin V staining), and cell permeability (7-AAD staining) were examined by flow cytometry. Partial depletion of ALG-2 led to a marked increase in the number of early apoptotic and dead cells as compared to non-transfected or control (siGL2) transfected cells (Fig. 2). Comparing single and double siRNA transfected cells it was clear that more early apoptotic cells were pres-

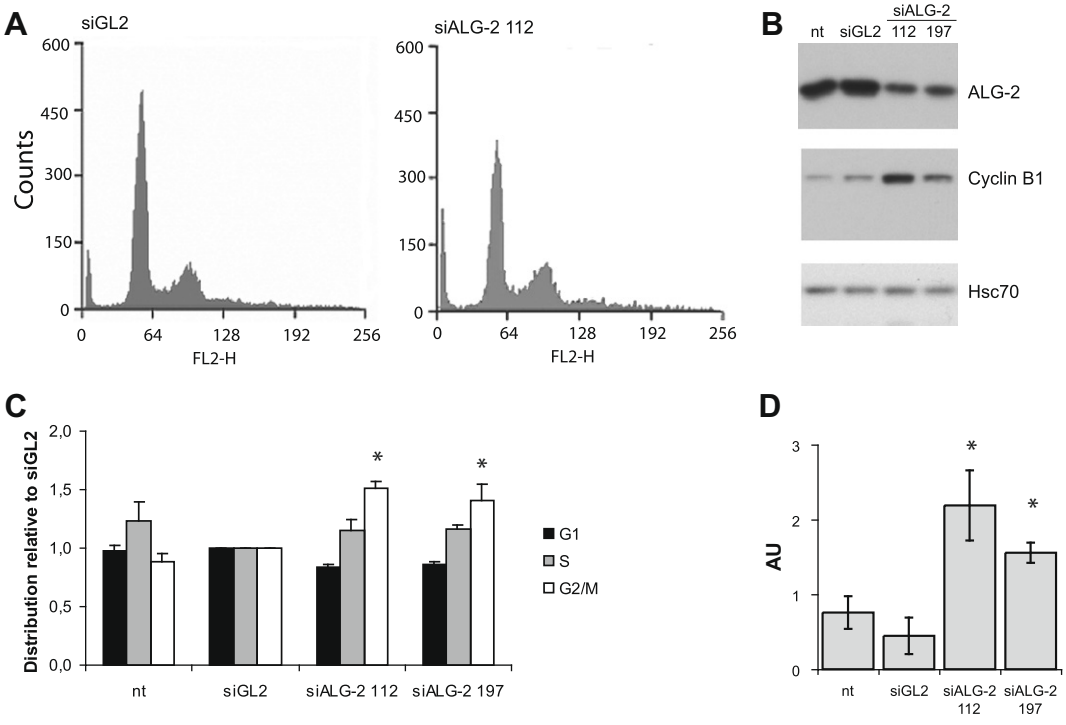

Fig. 1. Downregulation of ALG-2 increases the population of cells in the G2/M phase of the cell cycle. (A) Representative histograms showing the cell cycle profile of siGL2 and siALG-2 112 transfected cells. (B) Western blot analysis of ALG-2, cyclin B1, and Hsc70 levels in siRNA transfected cells. (C) Cell cycle phase distribution of cells relative to siGL2 transfected cells. Data represent the mean of three independent experiments and bars indicate standard error of the mean. (D) Quantification in arbitrary units (AU) of cyclin B1 levels relative to the loading control, Hsc70. Measurements were done on three independent experiments. Error bars indicate standard error of the mean. Asterisks indicate *t*-test *p*-values less than 0.05 when the results obtained with the ALG-2 siRNAs were compared with those obtained with the siGL2 control RNA. Increase in the G2/M population, $p = 0.003$ and 0.04; increase in cyclin B1 level, $p = 0.03$ and 0.016 for siALG-2 112 and 197, respectively.

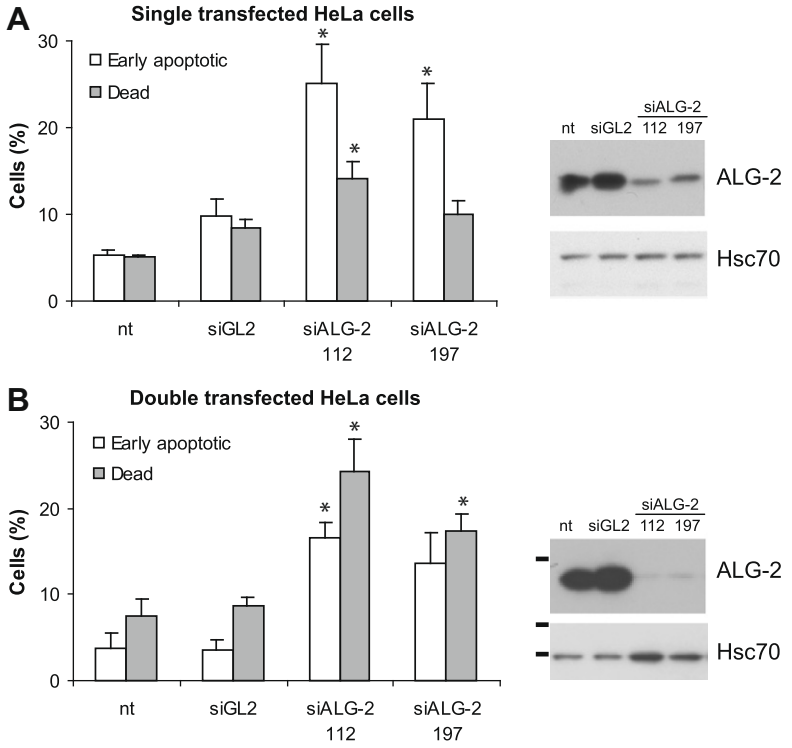

Fig. 2. Downregulation of ALG-2 increases the number of early apoptotic and dead cells. (A) Single siRNA transfected cells (Annexin V assay and Western blot). (B) Double siRNA transfected cells (Annexin V assay and Western blot). The population of early apoptotic (Annexin V positive) and dead (7-AAD positive) cells increased when ALG-2 was downregulated. Data represent the mean of three independent experiments and bars represent the standard error of the mean. Asterisks indicate *t*-test *p*-values less than 0.05 when the results obtained with the ALG-2 siRNAs were compared with those obtained with the siGL2 control RNA in double transfected cells (in parenthesis values obtained with single transfected cells). Increase in early apoptotic cells, $p = 0.011$ and 0.079 (0.023 and 0.033); increase in dead cells, $p = 0.014$ and 0.011 (0.031 and 0.158) for siALG-2 112 and siALG-2 197, respectively.

ent in the cultures of single transfected cells whereas more dead cells were seen in the double transfected cells. This demonstrates that HeLa cell survival is dependent on ALG-2 concentration and indicates that the cellular demise is triggered through the apoptotic pathway. The pan-caspase inhibitor z-VAD-fmk was used to investigate the caspase dependency on the observed elevated levels of early apoptotic and dead cells following ALG-2 knockdown. The viability of the cells was examined using the Annexin V assay three days after transfection of the cells and after incubation for 48 h with two different concentrations of z-VAD-fmk. Transfection of cells with siALG-2 duplexes led to an increase in the populations of early apoptotic cells and dead cells (Fig. 2). Addition of z-VAD-fmk reduced the percentages of early apoptotic and dead cells in all situations in a concentration-dependent manner with the largest effect observed in siALG-2 transfected cells (Fig. 3).

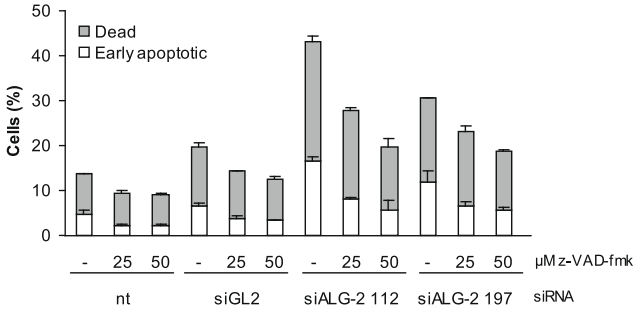

Fig. 3. Caspase-dependent cell death is induced following ALG-2 downregulation. Cells were transfected with either siGL2, siALG-2 112 or siALG-2 197. As an additional control non-transfected (nt) cells are shown. The day after siRNA transfection the medium was changed to control medium (untreated) or medium containing 25 or 50 μM z-VAD-fmk. Early apoptotic and dead cells were measured by the Annexin V assay and assessed by flow cytometry three days after siRNA transfection. Empty bars represent early apoptotic (Annexin V positive) cells and gray bars represent dead (7-AAD positive) cells. Data are the means of three measurements and the bars represent standard error of the mean.

Discussion

This study demonstrates that the decrease in proliferation rate previously observed in HeLa cells transiently transfected with ALG-2 siRNA constructs [9] is at least partly an effect of accumulation of cells in the G2/M cell cycle phase and an increase in the amount of apoptotic and dead cells. Activation of the Cyclin B1/Cdk1 complex is necessary for the progression of cells from G2 to M phase, but it must be inactivated for mitosis to proceed beyond anaphase [15]. The Cyclin B1 level which oscillates during the cell cycle, partly regulates the activity of Cyclin B1/Cdk1 (reviewed in [16]), and cells arrested in mitosis have previously been demonstrated to maintain high levels of Cyclin B1 [14]. Thus, the elevated levels of Cyclin B1 observed as a consequence of ALG-2 downregulation substantiate our observation that ALG-2 depleted cells accumulate in the G2/M cell cycle phase (Fig. 1). This finding together with the observed increased number of early apoptotic and dead cells in ALG-2 depleted cells could be explained by the fact that cells are arrested at checkpoints, and hereby delay the progression through the cell cycle.

We have observed that the presence of the caspase inhibitor z-VAD-fmk reduces the amount of early apoptotic and dead cells following ALG-2 downregulation, indicating that the increase in the population of early apoptotic and dead cells following reduction in the ALG-2 level is dependent on caspase activity. This anti-apoptotic function of ALG-2 in human cancer cells is conflicting with previously published results demonstrating pro-apoptotic functions of ALG-2 [1,5,6,17]. Though ALG-2 was discovered by screening of transiently transfected cells [1], the further examination of ALG-2 was made in stable murine 3DO clones expressing antisense ALG-2

RNA. This examination revealed that cell death induced by various means was impaired in ALG-2 depleted clones [1,4]. A study performed by Wong et al. in fibroblast hamster CHO cells also involved stable clones that expressed ALG-2 targeting siRNA and showed that at high cell densities ALG-2 deficient cells had a higher viability manifested by a delay in the onset of apoptosis [17]. The experimental setup for our work involved transient transfection of human cancer cells with siRNA, thus avoiding selection of cells being able to overcome the ALG-2 downregulation. Accumulation of compensatory effects in stably siRNA expressing clones towards both the intended and the unintended targets of siRNA has been reported previously [18]. In addition, Shibata et al. recently described inconsistency of Sec31A distribution in ALG-2 deficient cells [19], when comparing results obtained with transient siRNA transfected [20] and stably ALG-2 depleted HeLa cells [19]. The observed absence of an apoptotic phenotype in a ALG-2 knockout mouse [21] could as well be explained by functional redundancy.

Another prospect of the opposing results regarding ALG-2 function could be that ALG-2 function is species or cell-type dependent. It is therefore important in further research to address the question of whether downregulation of ALG-2 will impair the viability of other cancer cells. Work by our group showing that ALG-2 is upregulated in certain cancer types [8,9] has recently been substantiated and extended by genomic work on pulmonary adenocarcinomas [10] showing that the ALG-2 gene is amplified and upregulated in the latter cancer type. In addition, the ALG-2 gene was identified as a prognostic marker for early stage adenocarcinoma with poor prognosis. As our present study indicates a functional significance of ALG-2 for cell survival in the HeLa carcinoma cell model it is tempting to speculate that downregulation of ALG-2 could be of therapeutic relevance.

Acknowledgments

We acknowledge Casper Hølmkjær and Ulla Mortensen for technical assistance. This work was supported by funding from the Danish Cancer Society, the Danish Research Council and the Lundbeck Foundation to M. W. Berchtold, and by a NovoNordisk scholarship to Berit R. Høj.

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