The *pag* gene product, a physiological inhibitor of c-*abl* tyrosine kinase, is overexpressed in cells entering S phase and by contact with agents inducing oxidative stress

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Abstract The human *pag* gene product is an inhibitor of the c-*abl* tyrosine kinase and belongs to a new family of proteins. We show here that higher levels of *pag* gene expression are observed following induction of proliferation and contact with compounds inducing oxidative stress such as diethyl maleate and sodium arsenate. A weaker overexpression is seen in a macrophage cell line using hydrogen peroxide or menadione as inducers. *Pag* gene expression increases in synchronized cells entering the S phase. This raises the possibility that elevated levels of *pag* counteract the cytostatic activity of *abl*. Treatment of growth arrested cells with diethyl maleate and sodium arsenate induces *pag* gene overexpression, independently of cell proliferation. Thus, enhanced *pag* gene expression occurs in two cellular events: proliferation and response to oxidative stress.

Key words: *Pag* gene expression; Oxidative stress; S phase

1. Introduction

The cDNA of the *pag* gene has been initially identified by differential cloning between untransformed and *ras*-transformed human cells. This gene is constitutively expressed in most human tissues, but its expression is higher in tissues having a higher level of proliferation. In cell culture, higher levels of expression are achieved when cells are induced to proliferate. Its expression decreases when cells are induced to differentiate and cease proliferation. Therefore the level of *pag* gene expression is tightly associated with cell proliferation [1]. It codes for a single cytoplasmic protein of 199 amino acids (Ferbus and Goubin, unpublished) sharing similarity with highly conserved proteins found in pro- and eukaryote. These proteins were identified as gene products associated with cell proliferation or overexpressed in response to oxidative stress. The first group of proteins includes, besides the PAG protein, the murine MER5 protein that is preferentially expressed in erythroleukemia cells during the early period of cell differentiation [2] and the batrachian ABP25 protein which is expressed during the early stages of embryogenesis [3]. The second group includes MSP23 which was identified as a stress-induced mouse peritoneal macrophage protein [4], the yeast and human thioredoxin-dependent peroxide reductase [5,6] and the C22 component of alkyl hydroperoxide reductase of *Salmonella typhimurium* [7]. Related proteins were found in *Entamoeba histolytica* [8] and *Helicobacter pylori* [9]. Although their function is unknown, their association with the plasmic membrane suggests that they are conferring some protection against oxidative stress generated by macrophages during infection by these pathogens. In addition, a closely related plant protein (bas1) displays higher levels of expression in highly proliferating tissues and in some tissues following oxidative stress [10]. Recently, the PAG protein was shown to bind to the *Abelson* (*abl*) SH3-binding domain and to inhibit the *abl* tyrosine kinase activity [11]. *Abl* is a key regulator of cell cycle and *abl* kinase activity is activated by DNA damage suggesting a role of *abl* in the stress response to genotoxic insults [12].

In this paper we show that higher levels of *pag* gene expression are achieved using compounds inducing oxidative stress. The levels of induction are compared between a macrophage and an epithelial cell line. To determine whether *pag* gene overexpression can be observed independently of cell proliferation, we have generated stress in absence of cell proliferation. Finally, we compare the levels of expression following contact with stress inducing agents and induction of proliferation.

2. Material and methods

2.1. Cell culture conditions

Cells were propagated in Dulbecco's modified minimum essential medium (DMEM), supplemented by 10% newborn calf serum for the human mammary cell line HBL100 cells and 10% fetal calf serum for the murine macrophage RAW cell line. Serum starved cells were obtained by seeding 3×10^6 cells for at least 60 h, in 100-mm Petri dishes containing 10 ml DMEM with 0.2% newborn calf serum for HBL100 cells and 0.5% for RAW cell. Nb2 rat lymphoma cell line was propagated in RPMI supplemented with 5% fetal calf serum. For cell cycle experiments, 10^6/ml Nb2 cells were growth arrested by seeding them in 60-mm Petri dishes containing 5 ml RPMI with 3% horse serum for 24 h (stationary medium). Stimulation of proliferation was achieved by adding 5 ng/ml prolactin (Sigma).

2.2. Northern blot analysis

Cytoplasmic RNA isolated from cell cultures [1] was denatured by formaldehyde, size separated in 1% agarose gel and transferred onto an Amersham Hybond N^+ nylon membrane (France). Hybridization was performed using nick-translated probes as recommended by the supplier. A phosphorimager (Molecular Dynamics) was used for quantitative evaluation of hybridization signals.

2.3. Purification of pag antibody

Hyperimmune serum obtained from female New Zealand rabbits was passed over an immunoabsorbant column consisting of MBP-PAG coupled to Sepharose 4B (Pharmacia). Anti-MBP-PAG antibodies were eluted with 0.1 M glycine-HCl buffer pH 2.6 and neutralized to pH 7.0 with 2 M Tris. To remove the anti-MBP antibodies, the eluted antibodies were then passed over a second immunoabsorbant column of MBP-OZF [13]. The purified anti-PAG antibodies were finally concentrated by Centricon concentrators (Amicon).

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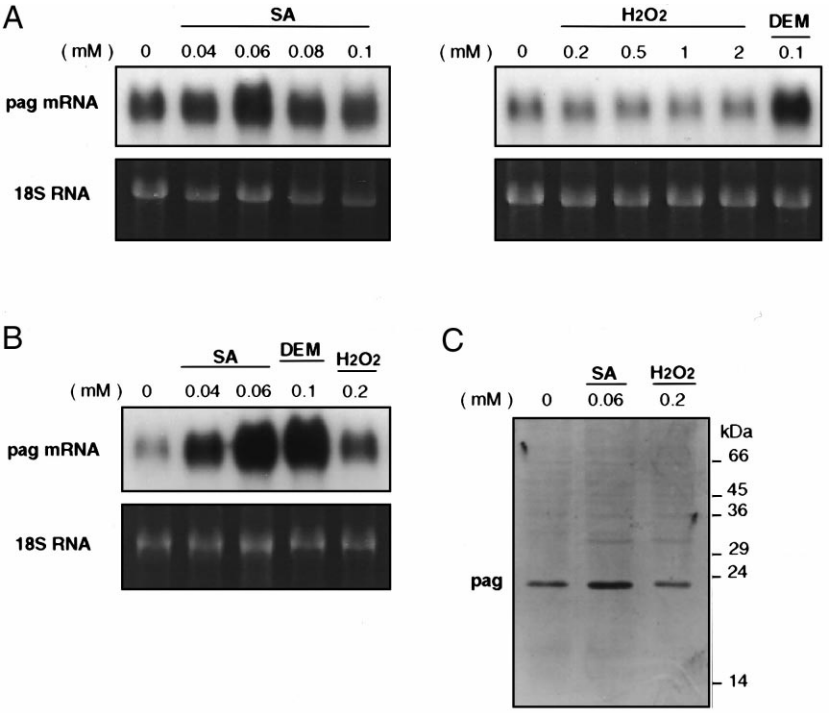


Fig. 1. Effect of stress agents on the induction of the *pag* gene expression. Cytoplasmic RNA (5 μg) was prepared from HBL100 cells (A) or RAW cells (B) after incubation for 16 h with each agent and analyzed by Northern blot. Proteins extracts were prepared from RAW cells and the expression of PAG protein analyzed by Western blot using a rabbit polyclonal antibody directed against the *E. coli* produced protein (C). The molarity and the name of the stress agent are indicated on the top of each figure: SA, sodium arsenate; H_2O_2, hydrogen peroxide; DEM, diethyl maleate. The integrity and the amount of RNA loaded onto the gel were checked by ethidium bromide staining of 18S ribosomal RNA.

2.4. Western blot analysis

Cell extracts were solubilized in boiling SDS-containing buffer in the presence of reducing agent β-mercaptoethanol. Equal amount of proteins (10 μg) was loaded into each lane. Proteins were separated by PAGE and transferred to nitro-cellulose membrane. Western blot analysis was carried out as described previously [1]. Blots were probed with the anti-PAG protein antibodies and then with alkaline phosphatase-conjugated anti-rabbit antibodies (Sigma), diluted 400- and 6000-fold, respectively.

2.5. Cell cycle analysis

The distribution of Nb2 rat cells in the different phases of the cell cycle was established by pulse-labelling cells with 5-bromodeoxyuridine (BrdU) 30 μM for 15 min. The cells were consequently harvested using trypsin/EDTA and fixed overnight (or longer) in 70% ethanol. Fixed cell suspensions were treated with pepsin, denaturated in 2 M HCl and incubated successively with α-BrdU (Seralab) diluted 1/20 and FITC-labelled goat anti-rat IgG (Southern Biotech) at 20 μg/ml. The nuclear suspensions were counterstained with propidium iodide at 25 μg/ml and analyzed on a Facscan (Beckton-Dickinson) flow cytometer. Cells were considered in S phase if they had incorporated the DNA precursor BrdU. G1 and G2/M cells (BrdU negative) were discriminated on the basis of DNA content with doublet discrimination. Bivariate analysis (BrdU/DNA) and histogram representations of the distribution in the cell cycle phases were performed with the PROCYT software (INSERM, Grenoble).

3. Results

3.1. Oxidative stress induces pag gene overexpression

The expression of the *pag* gene following oxidative stress was first studied at mRNA and protein levels in HBL100 cells. We chose this cell line because most of the work concerning cell proliferation was done using this human mammary cell line [1]. Since the PAG protein has similarity with proteins involved in the response to oxidative stress, we examined whether higher levels of expression can be observed following oxidative stress. To reduce proliferation and consequently the level of *pag* expression, HBL100 cells were propagated in a low serum medium then treated with compounds known to induce oxidative stress: sodium arsenate, hydrogen peroxide and diethyl maleate. Preliminary experiments were performed to determine for each compound the appropriate dose and time of contact (data not shown). Maximum levels of expression were observed 12–16 h following contact with the drugs. Fig. 1A shows that *pag* gene expression increased after treatment with sodium arsenate to reach a maximum with 0.06 mM. Overexpression was also observed with 0.1 mM diethyl maleate. In contrast hydrogen peroxide did not induce significant overexpression of the *pag* gene in the range of 0.2 to 2 mM. Higher concentrations were cytotoxic.

MSP23 which is closely related to PAG (96% identity) is significantly overexpressed in mouse peritoneal macrophages following exposure to hydrogen peroxide [4]. Since the response to oxidative stress can be dependent of the cell type, we studied *pag* gene expression in the mouse macrophage cell line, RAW [14]. In contrast to HBL100 cells, mRNA overexpression was observed following hydrogen peroxide treatment. However, it was much lower than with sodium arsenate and diethyl maleate (Fig. 1B). mRNA overexpression induced accumulation of PAG protein. An example is given of a Western blot analysis showing PAG protein accumulation following contact with sodium arsenate (Fig. 1C). No accumulation of PAG protein was seen in RAW after treatment with hydrogen peroxide. This discrepancy between mRNA and protein accumulation is likely to be due to a higher sensitivity of

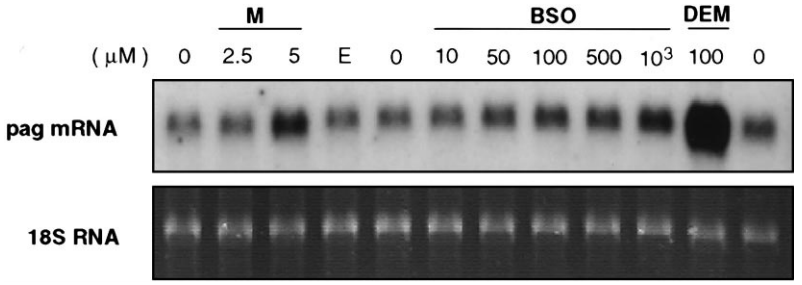


Fig. 2. Effect of menadione and buthionine sulfoximine on the induction of the *pag* gene expression. Cytoplasmic RNA was prepared from RAW cells after incubation with menadione and buthionine sulfoximine for 16 and 24 h respectively then analyzed by Northern blot analysis. The molarity and the name of the stress agent are indicated on the top of each figure: M, menadione in ethanol solution; E, 0.05% ethanol; BSO, buthionine sulfoximine; DEM, diethyl maleate. The integrity and the amount of RNA loaded onto the gel were checked by ethidium bromide staining of 18S ribosomal RNA.

Northern blot analysis as compared to Western blot analysis to detect slight variations of expression. We conclude from these experiments that oxidative stress as well as proliferation induces *pag* gene overexpression. Levels of expression are related to the cell type since higher levels of expression were achieved in RAW cells as compared to HBL100 cells.

Stress induction by diethyl maleate and sodium arsenate was reported to occur by decreasing the intracellular levels of glutathione [15,16]. To determine whether a decreased concentration of glutathione is responsible for *pag* gene overexpression, RAW cells were treated with increasing concentrations of buthionine sulfoximine, a compound which blocks glutathione biosynthesis [17]. A weak accumulation was observed when cells were treated with 1 mM buthionine sulfoximine as compared to cells treated with diethyl maleate (Fig. 2). Thus, a decrease of intracellular concentration of glutathione is not responsible for the strong induction observed following treatment with diethyl maleate and sodium arsenate.

Since the weak response observed following treatment with hydrogen peroxide could result from the experimental conditions, we examined the activity of menadione, a compound producing reactive oxygen species. Treatment of RAW cells with 5 μM menadione induced a moderate overexpression of the *pag* gene (Fig. 2) similar to that observed with hydrogen peroxide. Higher concentrations killed the cells. Radiations, like hydrogen peroxide and menadione, are known to generate free radicals. That accounts for the deleterious effects observed after irradiation. No overexpression was observed when RAW cells were irradiated with 0.1–10 Grays of γ rays and 0.2 MJ/m^2 of UVA radiations (data not shown). This contrasts with the strong overexpression observed after treatment with diethyl maleate (Fig. 2). Hydrogen peroxide appeared to be a weak inducer of *pag* gene overexpression as compared to sodium arsenate and diethyl maleate. Thus, levels of expression depend on the stress inducer and the cell type.

3.2. *Pag gene mRNA accumulates during the S phase*

Most of the studies concerning the *pag* gene expression were done using unsynchronized cells. We took advantage of the prolactin-dependent Nb2 rat lymphoma cell line to study *pag* gene expression during the cell cycle [18]. These cells, cultured in the stationary medium for 24 h, became synchronized with approximately 90–95% cells in the G0/G1 phase of the cell cycle as determined by flow cytometry. Addition into the medium of 5 ng/ml prolactin induced cell division with a doubling time of 25–30 h. Thus, this cell line was particularly appropriate: (1) to study the pattern of *pag* gene expression during progression from G0/G1 to the S phase, (2) to uncouple oxidative stress from proliferation.

Pag gene expression was analyzed by Northern and Western blot at various times after addition of prolactin. Fig. 3A shows that *pag* mRNA was found to accumulate after 7 h, to reach a maximum after 19–21 h, and this in three independent experiments. Accumulation of the protein was also observed from 15 to 24 h (Fig. 3B). Flow cytometry cell cycle studies indicate that Nb2 cells arrested in G0/G1, enter S phase 7 h after prolactin stimulation. A maximum of cells in S phase is observed 19 h post stimulation, then decreases (Fig. 3C–D). Thus, *pag* gene expression is linked to the cell cycle: it is induced in cells entering the S phase of the cell cycle and reaches a maximum when most of the cells has entered the S phase (Fig. 3D).

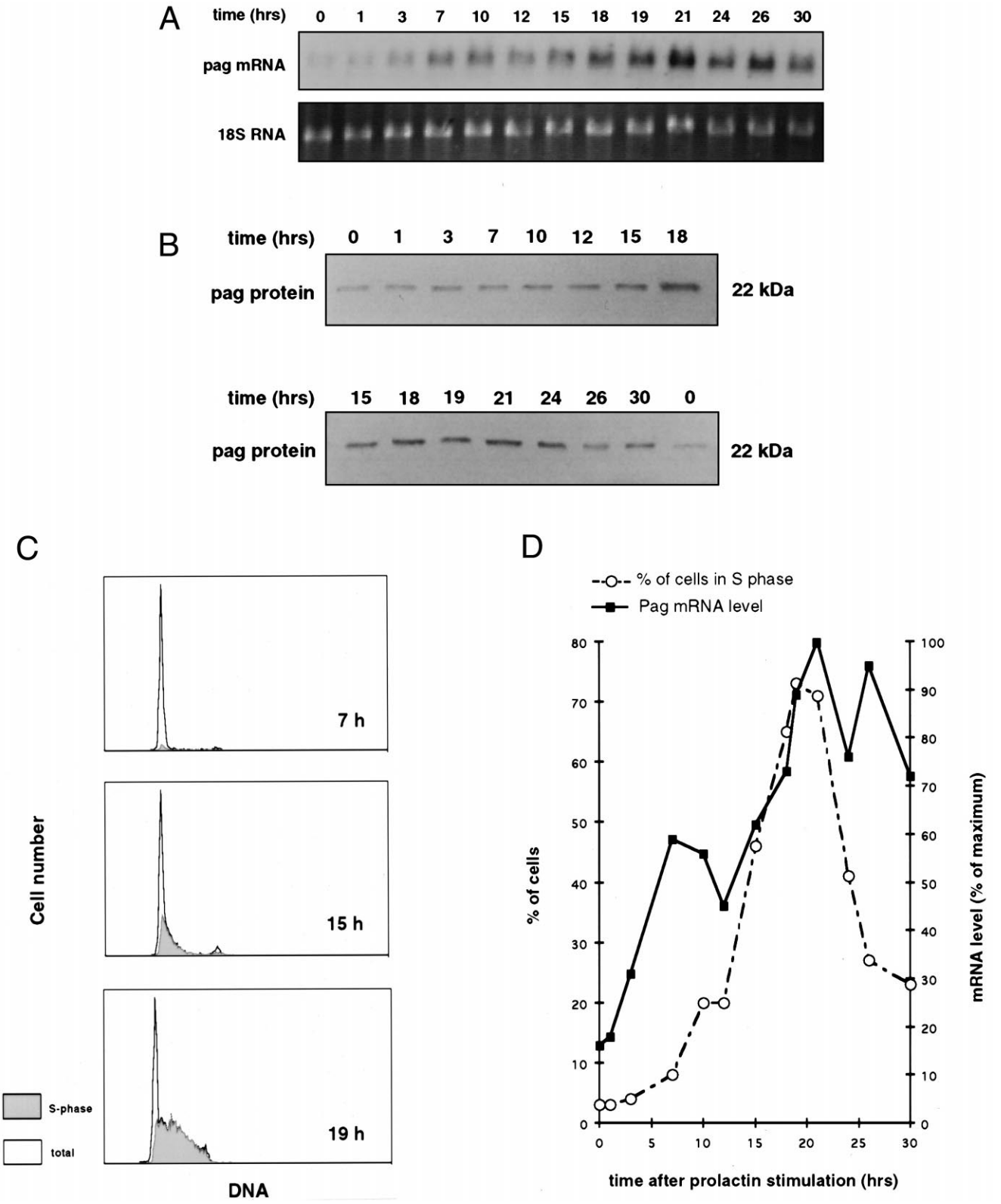
3.3. *Comparison of pag gene expression in Nb2 cells following stimulation of proliferation and oxidative stress*

In an attempt to study separately *pag* gene expression following oxidative stress and induction of proliferation, we examined the expression of *pag* mRNA in growth arrested Nb2 cells. Stationary Nb2 cells were treated separately with prolactin, sodium arsenate, diethyl maleate for 16 h, and *pag* gene expression was analyzed by Northern blotting. Prolactin stimulation induced proliferation, and 16 h post stimulation 65% cells were in the S phase (Fig. 4A). *Pag* overexpression was observed with 0.06 mM sodium arsenate or 0.5 mM diethyl maleate. However, the abundance of the *pag* transcript is approximately 5-fold higher in cells treated with prolactin as compared to cells treated with sodium arsenate or diethyl maleate (Fig. 4B).

4. Discussion

The *pag* gene was initially characterized because of its close association with cell proliferation. The sequence similarity of the PAG protein with a family of stress-induced proteins prompted us to examine its expression following contact with stress inducing agents. In HBL100 epithelial cells, treatment with compounds such as diethyl maleate or sodium arsenate strongly enhanced the level of *pag* mRNA. Treatment with hydrogen peroxide has no detectable effect on the accumulation of *pag* mRNA in HBL100 cells.

Ishii et al. [4] have isolated and characterized a 23-kDa



stress-induced protein (MSP23) from peritoneal macrophages. Its sequence similarity (96%) with the PAG protein indicates that it is the murine counterpart of PAG. Overexpression of MSP23 protein was observed following treatment by diethyl maleate or sodium arsenite but also with hydrogen peroxide. Since the response of the *pag* gene to oxidative stress may be dependent of the cell type, the similar experiments were repeated in RAW cells, a mouse macrophage cell line [14]. In contrast to HBL100 cells, *pag* gene overexpression was observed with hydrogen peroxide albeit at lower levels as com-

Fig. 3. Time course of *pag* mRNA accumulation during the cell cycle. Resting Nb2 cells were induced to proliferate upon prolactin addition (5 ng/ml). Triplicate plates were analyzed by Northern and Western blotting and cytometric flow at various times following induction of proliferation. A: Cytoplasmic RNA (5 μg) was analyzed by Northern blotting. The integrity and the amount of RNA loaded onto the gel were checked by ethidium bromide staining of 18S ribosomal RNA. B: Protein extracts (10 μg) of Nb2 cells were separated through SDS/13.5% polyacrylamide gel and transferred to nitrocellulose membrane using standard immunoblotting techniques. Immunoblot was treated with purified anti-PAG antibodies. C: DNA histograms redrawn according to the data of BrdU/DNA bivariate cytograms, representing the amount of cells in S phase and the amount of cells in G0/G1 and G2/M (total). D: Comparison of *pag* mRNA accumulation with the percentage of Nb2 cells in the S phase of the cell cycle. A phosphorimager was used for quantitative evaluation of hybridization signals presented in A. These results were used to plot the curve in arbitrary units according to the time after stimulation of proliferation.
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pared to that observed following contact with diethyl maleate or sodium arsenate. Activated macrophages are known to produce reactive oxygen species. Since these cells resist those reactive oxygen radicals and subsequent reactive by-products, they must have potent defense systems to minimize the deleterious effects of those insulting agents. The generation of radicals is much lower in epithelial cells than in macrophages. They are essentially metabolic by-products and are not specifically produced to kill other cells. The higher levels of *pag* gene expression found in macrophages, as compared to HBL100 epithelial cells, is consistent with a protective action of the PAG protein against oxidative damage.

It was also observed that the level of PAG accumulation depends on the inducing agent. In RAW cells, diethyl maleate and sodium arsenate appeared to be better inducers of *pag* gene expression than hydrogen peroxide and menadione. Hydrogen peroxide is an agent which is an oxidant itself and menadione is able to generate reactive oxygen intermediates [15–19] whereas diethyl maleate and sodium arsenate are involved in several pathways including the decrease of intracellular glutathione concentration [15,16]. To examine whether the glutathione pathway is indeed involved in *pag* gene expression, we used buthionine sulfoximine which blocks an essential step of the glutathione synthesis and is therefore more specific of the glutathione pathway than other compounds [17]. Since buthionine sulfoximine is a weaker inducer of *pag* gene expression than diethyl maleate and sodium arsenate, the contribution of the glutathione pathway appears to be a minor in this process. It is likely that *pag* gene overexpression is observed in both HBL100 and RAW cells, fol-

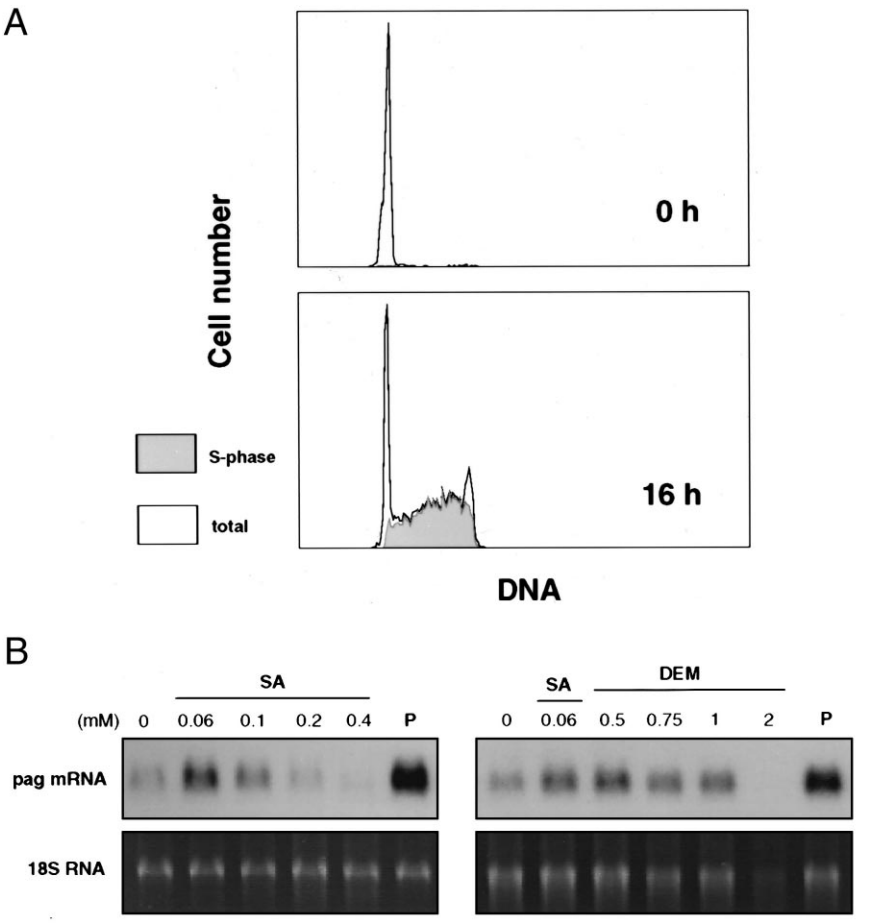

Fig. 4. Comparison between the effect of stress agents and proliferation on the induction of the *pag* gene expression. A: DNA histograms redrawn according to the data of BrdU/DNA bivariate cytograms, representing the cycle distribution before and 16 h post prolactin stimulation. B: Cytoplasmic RNA (5 μg) was prepared from Nb2 cells treated with sodium arsenate (SA), diethyl maleate (DEM) and prolactin (P) and analyzed by Northern blotting. SA, DEM and prolactin (5 ng/ml) were added for 16 h and RNA extracted. The integrity and the amount of RNA loaded onto the gel were checked by ethidium bromide staining of 18S ribosomal RNA.

lowing treatment with diethyl maleate and sodium arsenate results, besides to the activation of the oxidative stress pathway, from the activation of other pathways involved in the response to toxic stress.

Pag gene overexpression is observed in two cellular contexts: proliferation and oxidative stress. To compare the response of the *pag* gene following induction of cell proliferation versus induction of stress, we examined its expression in the murine Nb2 cell line which can be efficiently synchronized. We found that expression of the *pag* gene increased 7 h following induction of proliferation, thus corresponding to entry into the S phase and reached a maximum after 19–21 h when most of the cells had entered the S phase. Therefore, in the absence of oxidative stress, the level of *pag* gene expression varies according to the position of the cells in the cell cycle and is higher during the S phase. Recently, Wen and Van Etten [11] showed that the *pag* gene product is a physiological inhibitor of c-*abl*, a nonreceptor tyrosine kinase, interacting with its SH3 domain. *Abl* proteins with deletion or point mutation in the SH3 domain induce transformation and increase phosphotyrosine levels in a variety of cell types [20,21], suggesting that the SH3 domain suppresses the intrinsic transforming ability of *abl*. These observations lead to the hypothesis that in c-*abl* the tyrosine kinase activity is reversibly inhibited by proteins interacting with the SH3 domain. C-*abl* tyrosine kinase activity was shown to induce a cytostatic effect by an unknown mechanism [22]. Since *pag* expression increases when cells enter the S phase, this raises the possibility that *pag* overexpression counteracts the physiological role of *abl*. Elevated levels of *pag* would inhibit the *abl* kinase activity and abrogate the G1/S cell cycle block.

Finally, we show that *pag* gene expression in growth-arrested Nb2 cells is induced following contact with diethyl maleate and sodium arsenate, thus in absence of cell proliferation. These data show that in addition to proliferation enhanced *pag* gene expression is achieved with compounds inducing oxidative stress and possibly toxic stress.

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