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Cell Growth-Dependent Subcellular Localization of p8

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Abstract p8 is a stress-induced protein, biochemically related to the architectural factor HMG-I/Y, overexpressed in many cancers and required for tumor expansion. The molecular mechanisms by which p8 may exert its effect in aspects of growth is unknown. Using immunocytochemistry, we found that p8 presents nuclear localization in sub-confluent cells, but it localizes throughout the whole cell in high density grown cells. Cells arrested in Go/G1, either by serum deprivation or by hydroxyurea treatment, show a nucleo-cytoplasmic localization of p8, whether in the rest of the cell cycle stages of actively dividing cells the localization is nuclear. A comparison of p8 sequences from human to fly predicts a conserved bipartite nuclear localization sequence (NLS). The putative NLS has been demonstrated to be functional, since nuclear import is energy dependent (inhibited by sodium azide plus 2-deoxyglucose), and fusion proteins GFP–p8 and GFP–NLSp8 localize to the nucleus, whereas GFP–p8NLSmut in which with Lys 65, 69, 76, and 77 mutated to Ala localized to the whole cell. p8 localization does not involve the CRM1 transporter, since it is insensitive to leptomycin B. Inhibitors of MAPK pathways did not affect p8 subcellular localization. The inhibition of deacetylation with Trichostatin A promotes cytoplasmic accumulation of p8. The results suggest that p8 growth stage-dependent localization is regulated by acetylation, that p8 is not free within the cell but forming part of a complex and that it may exert a role in both subcellular localizations. J. Cell. Biochem. 97: 1066–1079, 2006.

Key words: p8; NLS; cell density; acetylation

The p8 gene, coding for an 82 aminoacid protein, was first identified in rat, while studying the molecular response of the injured pancreas and found to be overexpressed in acinar cells, during the acute phase of pancreatitis [Mallo et al., 1997]; later on, it was characterized in human, mouse, and frog [Vasseur et al., 1999a,b; Igarashi et al., 2001]. It was also isolated as an overexpressed gene in rats, in endothelin-induced mesangial cell hypertrophy, as well as in diabetic kidney [Goruppi et al., 2002]. Further experiments have shown that p8 mRNA is activated in numerous cell types in response to several stresses [Jiang et al., 1999], including a minimal stress such as routine change of the culture medium in the absence of any added substance [García-Montero et al., 2001], indicating that p8 is an ubiquitous protein induced by cellular stress.

Several functions, sometimes difficult to reconcile, have been attributed to p8 such as growth promotion following forced-overexpression in pancreatic-derived and HeLa cells [Mallo et al., 1997; Vasseur et al., 1999a] or growth inhibition in MEF (murine embryonic fibroblasts) and breast cancer-derived cells [Bratland et al., 2000; Vasseur et al., 2002b]. These functions seem to involve regulation of p27 at its cellular level [Vasseur et al., 2002b]. In addition, p8 contributes to adriamycin-induced apoptosis

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[Vasseur et al., 2002b] but protects liver against LPS- (lipopolysaccharide) and CCl_4-induced injuries [Vasseur et al., 2003; Taieb et al., 2005]. A particularly attractive role in tumor progression has recently been proposed for p8 [Vasseur et al., 2002a]. Fibroblasts obtained from p8-expressing or p8-knock-out animals were transformed with a retroviral vector expressing both the ras^{VAL12} mutated protein and the E1A adenoviral oncogene. In soft-agar assays, transformed p8-expressing cells formed colonies at high frequency, as expected, but transformed p8-deficient fibroblasts were unable to form colonies. Similarly, transformed p8-expressing cells produced tumors in all athymic nude mice when injected subcutaneously or intraperitoneally, whereas transformed p8-deficient fibroblasts did not.

Homology searching in databases did not reveal significant similarity of p8 with other proteins of known function. However, p8 shares some biochemical properties with High Mobility Group proteins (HMG) [Bustin and Reeves, 1996], particularly with the HMG-I/Y family. The overall identity of human p8 with human HMG-I/Y is only about 35%, but the molecular mass, isoelectric point, hydrophilicity plot, resistance to denaturation after heating at 100°C and charge separation are very similar [Encinar et al., 2001]. The p8 protein seems to bind DNA weakly, as shown by electrophoretic mobility shift assay, without preference for DNA sequences. Finally, human p8 has also been shown to be a substrate of protein kinase A in vitro and phosphorylated p8 has a higher content of secondary structure and binding to DNA is highly increased [Encinar et al., 2001]. An architectural role in transcription has been suggested for this protein, in analogy with the HMG-I/Y proteins, and recent work seems to confirm this hypothesis [Hoffmeister et al., 2002].

After the initial discovery of p8 by the Iovanna group, other laboratories have independently identified p8, as a gene related to tumor progression. Studies by Ree et al. [1999] revealed that expression of the Com1 (candidate of metastasis) protein, which is identical to human p8, mediates the growth of human breast cancer cells after metastatic establishment in a secondary organ, indicating that activated expression of Com1/p8 in metastatic cells is required for tumor progression. Mohammad et al. [2004] found p8 highly expressed in pituitary tumors and conducted experiments that indicated a linkage of p8 with pituitary tumorigenesis.

The distribution pattern of a protein within the cell can provide important clues as to its function. Contradictory results are obtained regarding p8 subcellular localization. Preliminary results using COS-7 cells, transiently transfected with p8 cDNA have shown a nuclear localization [Vasseur et al., 1999a]. However, in human thyroid cancer cells a predominant nuclear or cytoplasmic localization of p8 was dependent on the stage of the disease [Ito et al., 2003]. Very recently Päth et al. [2004] showed that in human cultured pancreatic islets p8 was mainly cytoplasmic, while in INS-1 β cells it was mostly nuclear.

We were therefore interested in studying in more detail and systematically the subcellular localization of this 9 kDa protein, which is small enough to passively diffuse through nuclear pores. Using cultured cells, we found that p8 was nuclear when cells were growing at low density and was distributed in nucleus and cytoplasm in high density cultures. It has an active transport to the nucleus mediated by a NLS (nuclear localization signal, NLS), conserved among its paralogs. This NLS is both necessary and sufficient for p8 nuclear localization. Nuclear localization of p8 can be regulated directly or indirectly by acetylation.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

HeLa, HEK 293T, and human pancreatic cancer Panc-1 and MiaPaCa cell lines were routinely cultivated at 37°C in 5% CO_2 95% air atmosphere in Dulbecco's modified Eagle's medium (Invitrogen, Argentina) containing 10% (v/v) fetal bovine serum (Invitrogen), 1% non-essential amino acids, 4 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Expression Vector Constructions and Transfection Assays

Proliferating HeLa cells, grown at 60% confluence in 10 cm dishes, were transfected with a total of 8 μg pcDNA3/hp8 [Vasseur et al., 1999a], using the FuGENE 6 reagent following the manufacturer's instructions (Roche Diagnostics, Meylan, France). To select for stable transfectants, the transgenic cells were cultivated for over three weeks in media containing G418 (600 μg/ml) (Sigma Chemical Co., St Louis, MO)

starting 48 h after transfection. Surviving colonies were expanded in standard culture medium supplemented with G418 (400 μg/ml).

Transient transfections were performed in cells growing in 0.8 cm^2 chambers (see below), using Escort™ transfection reagent (Sigma Chemical Co.), and 100–500 ng plasmid DNA/well.

pGFP–p8 was generated by in frame ligation of p8 to the 3′-end of the sequence encoding green fluorescent protein (GFP) in pEGFP–C2 (Clontech, Palo Alto, CA). The p8 containing sequence in pcDNA3/hp8 plasmid, was amplified by PCR with the following pair of primers: forward 5′-GGGCTCGAGGATGGCCACCTTC-CCACCAGCA-3′ and reverse 5′-GGGGGA-TCCTCAGCGCCGTGCCCCTCGCTT-3′, flanked by *Xho*I-*Hind*III restriction sites.

pGFP–p8NLS was generated by in frame ligation of the putative p8 NLS sequence: ATCTCGAGTAGGAAACTGGTGACCAAGCT-GCAGAATTCAGAGAGGAAGAAGCGAGATCC (from aa 64–78) to the 3′-end of the sequence encoding GFP in pEGFP–C2. The p8 NLS was inserted in the *XhoI-BamHI* restriction sites.

pGFP–p8NLS mut was generated by in frame ligation of a mutated form of p8 in which lysines 65, 69, 76, and 77 were changed for alanines using Stratagene Quick Change Site directed mutagenesis kit. The p8 NLSmut was inserted in the *XhoI-BamHI* restriction sites to the 3′-end of the sequence encoding GFP in pEGFP–C2.

The Smurf1 NES mutant fused to GFP (GFP-NES(606–614)), used as a positive control for leptomycin B nuclear retention effect, was kindly provided by Dr. Takeshi Imamura (The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan).

Immunofluorescence

Cells were seeded onto tissue culture chamber slides with a surface area of 0.8 cm^2 (Lab-Tek II, 8 chamber slides, Nunc) at low (30,000 cells per well) or high (300,000 cells per well) cell density as indicated, and allowed to grow for 24 h before manipulation (transfection, fixation, or drug treatment). For immunofluorescence, cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Following two 5 min PBS rinses, cells were permeabilised with 0.1% Triton X-100 in PBS for 10 min, washed with PBS, and incubated for 30 min with 3% bovine serum albumin (BSA), 10% fetal calf serum, 0.2% Triton X-100 in PBS. Cells were then rinsed with PBS and incubated with primary antibody b (raised in rabbit against recombinant human p8) diluted 1:200 when used to detect endogenous p8, and 1:600 when used to detect overexpressed recombinant p8 in antibody buffer (3% BSA, 0.2% Triton X-100 in PBS) for 2 h at room temperature. Controls were performed using preimmune serum instead of primary antibody b. Cells were rinsed twice with PBS and once with wash solution (50 mM Tris-HCl pH 7.2, 100 mM NaCl, 0.2% Tween, 0.2% Triton X-100) prior to incubation for 1 h with the secondary antibody (goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Santa Cruz Biotech., Santa Cruz, CA)), diluted 1:100 in the antibody buffer at room temperature in the dark. Anti SF2/ASF antibody, used in Figure 7 as a control, was a generous gift of Dr. A. Kornblihtt, University of Buenos Aires. The addition of 0.2% Triton X-100 throughout the whole immunocytochemistry procedure turned to be necessary in order to avoid the artifact of nuclear exclusion when cells are at superconfluence. After rinsing with PBS and washing solution, nuclei were co-stained with Hoechst 33258 solution (3 μg/ml in PBS) or propidium iodide (50 μg/ml), and finally cells were mounted with Mowiol 4–88 antifading agent (Calbiochem, San Diego, CA). Slides were visualized using an Olympus Fluoview FV 300 scanning laser biological inverted microscope IX70 and images acquired with a Zeiss AxioCam digital camera, operated by the AxioVision 3.1 software. Confocal microscopy was performed with an Olympus FV300 microscope, and images acquired with Fluowiew 3.3.

Flow Cytometry Data Acquisition and Analysis

Cells were trypsinised and pelleted, washed once with PBS, resuspended in cold 1% formaldehyde in PBS, and left at 4°C for 1 h. The cells were pelleted and washed with PBS, fixed with 70% ethanol, centrifuged, and resuspended in PBS containing RNAse (4 U/ml) and propidium iodide (50 μg/ml). Reagents were obtained as a kit from Coultronics France (Margency, France). After vortexing, samples were allowed to equilibrate at room temperature in the dark for at least 1 h before analysis. Data were acquired on a BD FACSCalibur System using BD CELLQuest software.

Drug Treatment

For cell synchronization sub-confluent cells growing in multiwell chambers (Lab-Tek) were serum starved for 24 h; the cells in one well were fixed, while the remaining wells were replenished with serum and cells were fixed every 4 h. Alternatively, cells were arrested at G1/S stage through treatment with 1 mM hydroxyurea (Sigma Chemical Co.) for 12 h.

For energy charge depletion studies, sub-confluent HeLa and 3T3 cells, transfected transiently with GFP, GFP–p8, and GFP–NLSp8, or HeLa–p8 cells, were treated with 10 mM sodium azide and 6 mM 2-deoxyglucose in Hanks glucose free medium supplemented with 0.1% FBS during 4 h. A control experiment was performed without the addition of sodium azide or 2-deoxyglucose.

For nuclear export studies, high density growing cells or sub-confluent cells plated at high densities, were incubated with D-MEM 10% SFB medium containing 20 ng/ml of Leptomycin B (Sigma Chemical Co.) 8–15 h prior to fixation.

The following stress-inducing drugs were added to HeLa–p8 cells growing in Lab-Teks at low or high cell density and incubated for the indicated times before fixation: 100 nM dexamethasone (Sigma Chemical Co.) for 20 h, 0.1 μM staurosporine (Sigma Chemical Co.) for 20 h, 100 ng/ml TNFα (Boehringer Mannheim, Germany) for 1 h, 120 mM NaCl for 20 h, 0.8 μM doxorubicin (Sigma Chemical Co.) for 20 h, and 100 μM H_2O_2 for 1 h.

Drugs affecting MAP kinase pathways were added at the indicated concentrations under the following conditions. Sub-confluent HeLa–p8 cells were plated at high densities with D-MEM 10% SFB medium containing the indicated drug and were incubated for 8–15 h prior to fixation. Superconfluent cells were plated at low densities with D-MEM 10% SFB medium containing the drug and were incubated for 8 h prior to fixation. The drugs used were 10 μM U0126 (Calbiochem; MEK1/2 inhibitor), 10 μM SP600125 (Calbiochem; JNK inhibitor), and 10 μM SB503280 (Calbiochem; p38 inhibitor).

The influence of the protein acetylation state of the cell was assayed by addition of 2 μM Trichostatin A (TSA, Calbiochem) to low density growing HeLa–p8 cells, and further incubation during 4 h previous to fixation and immunofluorescence. As a control, the same assay was performed on low density growing HeLa cells which, after fixation, were stained using a monoclonal anti SF2/ASF antibody.

RESULTS

Distribution of p8 in the Nucleus and Cytoplasm of Low- and High-Density Cells

As a first step toward determining the distribution pattern for p8, HeLa–p8 cells expressing p8 stably under CMV (cytomegalovirus) promoter and cells from the human pancreatic cancer lines Panc-1 and MiaPaCa, were stained with an antibody raised in rabbit against recombinant human p8, using indirect immunofluorescence microscopy. Figure 1 shows that the staining was specific and that p8 localization was dependent on cell density. At low cell density (sub confluent) both endogenous and overexpressed p8 was concentrated in the nucleus. By contrast, in Hela–p8, Panc-1, and MiaPaCa cells grown to high cell density (confluent), p8 was more evenly distributed in nucleus and cytoplasm. The same results were observed for the distribution of endogenous p8 in human HEK 293T cells, although with fainter images, since the level of p8 expression is much lower (data not shown).

Subcellular Distribution of p8 and Cell Cycle

As a first approach to analyze whether the localization of p8 was related to cell cycle, we closely inspected p8 immunofluorescence on around 500 HeLa–p8 cells grown at low density, with a distribution, according to FACS analysis (not shown) of 43.8% G1, 19.8% S, 36.3% G2/M. p8 location was always nuclear indicating that, in active cycling cells, the distribution did not change during the cell cycle.

A second approach was to synchronize HeLa–p8 cells, growing asynchronically at low density, by serum starvation (0.1% FBS) for 24 h, or by 12 h treatment with 1 mM hydroxyurea, which enriched the cell culture population in cells arrested at G1/Go (70% G1, 15% S, 15% G2/M). The results of the immunocytochemistry shown in Figure 2, indicate very clearly that after either serum starvation or hydroxyurea treatment, p8 was more evenly distributed between cytoplasm and nucleus. Once cells arrested by serum starvation were relieved from the arrest by serum re-addition, the usual nuclear localization observed in cycling cells began to be

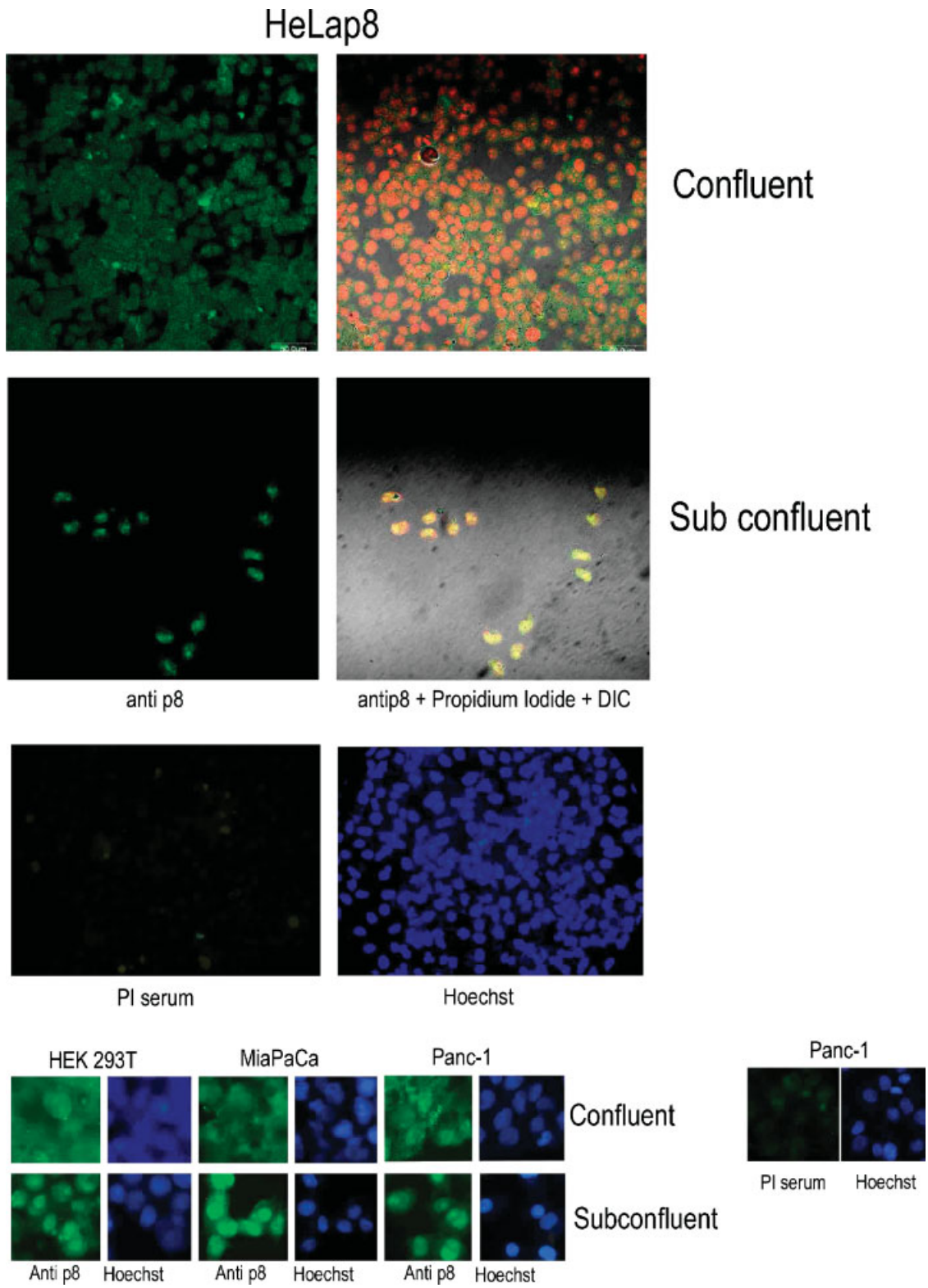

Fig. 1. p8 localization is influenced by cell density. HeLa–p8, Panc-1, and MiaPaCa cells were grown to either low (sub confluent) or high density (confluent), as indicated. p8 was visualized by immunofluorescence microscopy using a polyclonal antibody anti-human p8. HeLap8 cells, with nuclei stained with propidium iodide, were visualized through fluorescent confocal microscopy and DIC (differential interference contrast). The rest of the cells were visualized using regular fluorescence microscopy, with nuclei stained with Hoechst 33248. Representative results are shown. Controls in HeLap8 and Panc-1 cells were performed using preimmune serum (PI) instead of anti-human p8 antibody.

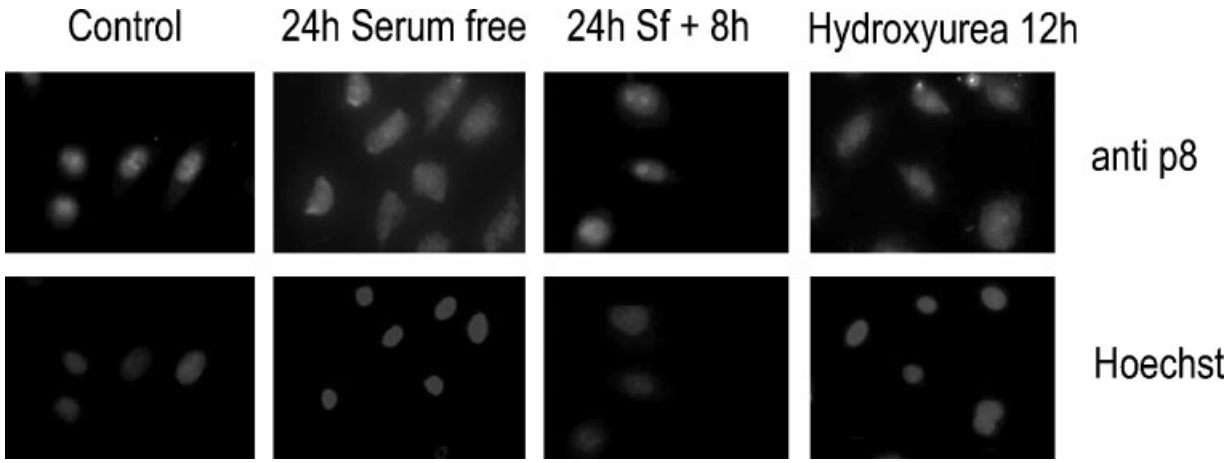

Fig. 2. Subcellular localization of p8 in populations enriched in different stages of the cell cycle. Sub-confluent HeLa–p8 cells were arrested by serum starvation during 24 h; complete medium was added to allow cell cycle progression and cultures were fixed. Sub-confluent HeLa–p8 cells were arrested at G1/S by treatment with 1 mM hydroxyurea during 12 h. p8 localization was analyzed by immunocytochemistry and nuclei were visualized by Hoechst 33248 staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

evident by 4 h (not shown) and was complete by 8 h (Fig. 2).

The results obtained by hydroxyurea treatment and serum deprivation, together with those observed with cells grown to high density, suggest that the cytoplasmic appearance of p8 correlates with an arrested Go state of the cells, while cells in active cell division localize p8 almost exclusively to the nucleus.

Nuclear Localization Signal in p8

Nuclear translocation occurs through the nuclear pore complex (NPC) [Macara, 2001]. Small proteins (<45 kDa) can diffuse into and out of the nucleus freely, whereas nuclear translocation of larger proteins usually requires the presence of a NLS. Most transport events through the NPC are mediated by soluble receptors that specifically recognize their cargoes and facilitate the passage of receptor–substrate complexes. Despite the impressive number of receptor–cargo interactions that have been studied, the prediction of nuclear localization and nuclear export signals in candidate proteins remains extremely difficult. The best studied examples are the lysine-rich "classical" NLS, originally defined in the SV40 T antigen, recognized by the importin α/β dimer and the leucine-rich nuclear export sequence (NES), originally identified in HIV Rev and Protein kinase A inhibitor (PKI), recognized by Crm1. There are two classical NLSs: a monopartite NLS composed of a single cluster of basic aminoacids, and a bipartite NLS composed of two basic aminoacids, a spacer region of 10–12 aminoacids, and a basic cluster in which three of five aminoacids are basic.

Even though p8, due to its size, apparently would not need a NLS to enter the nucleus, human p8 sequence was predicted to have a potential NLS by analysis through the PSORTII program at http://psort.nibb.ac.jp. Figure 3 shows the alignment of p8 sequences from human to fly. The similarity between human, rat, and mouse p8 is higher than 70%; while there is an overall similarity of around 40% with the other species. However it is evident that partial strings of sequences are highly conserved among the seven species, begining from aminoacid 25 of human p8 and that even the p8 orthologs from mosquito and fly do not have the aminoterminal sequences at all. Among those conserved sequences a classical bipartite NLS, of the type $B_2x_{10}(B_3x_2)$ (where B stands for basic residue K or R, and x for any residue) is strictly conserved in human, rat, mouse, and even *Xenopus*, and more loosely in fish, mosquito, and fly. A potential NES, rich in hydrophobic aminoacids, particularly leucine in mammals, is also conserved.

Two p300 consensus acetylation motifs (one lysine with a charged residue at +4 or at −3; [Thompson et al., 2001]) are also conserved, with one of those motifs embedded in the putative NLS.

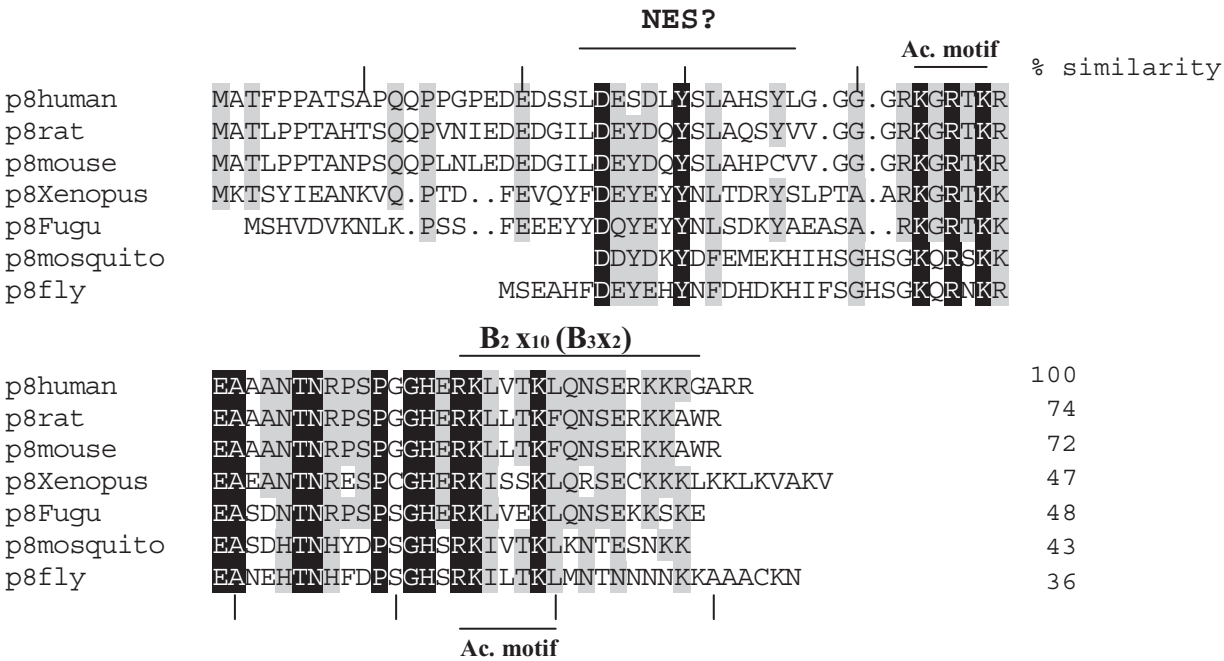

Fig. 3. Homology in p8 sequences from higher eukaryotes. Human p8 was BLAST tested against the full genome of different species using the National Center of Biotechnology Information (NCBI) with all the protein and genome available databases. Similar sequences to hp8 found in 100% of the potential orthologs are highlighted in black and 70% in gray. p8 human: *Homo sapiens* NP_036517; p8 rat: *Rattus norvegicus* NP_446063; p8 mouse: *Mus musculus* NP_062712; p8 frog: *Xenopus laevis* BAB33387; p8 Fugu: *Fugu rubripes* JGI_27767; p8 mosquito: *Anopheles gambiae* XP_315506; p8 fly: *Drosophila melanogaster* NP_609539. The boxes above the alignment show the bipartite NLS sequence ($B_2x_{10}(B_3x_2)$), a potential NES sequence rich in hydrophobic aminoacids, and two acetylations motifs. % similarity is indicated in the right margin. The ticks above and below the sequence, every 10 aminoacids, are included to facilitate the identification of the mutated aminoacids in the GFP–p8NLSmut (see Fig. 5).

Nuclear Import and Export of p8

The import process can be dissected into two distinct steps: first, docking of the nuclear protein destined for import to the NPC periphery; and second, active translocation of the nuclear protein through a gated channel residing in the central pore of the NPC. The first step does not require nucleotide hydrolysis and is temperature independent, whereas the second step does require nucleotide hydrolysis and is attenuated upon lowering the temperature. Nuclear transport of NLS-containing proteins is dependent on energy charge in vivo [Richardson et al., 1988]. In order to ascertain whether p8 import to the nucleus is an active process, the energy charge of the HeLa–p8 cells was decreased by treatment with 10 mM sodium azide (inhibitor of mitochondrial electron transport) plus 6 mM 2-deoxyglucose (inhibitor of glycolysis) during 4 h (Fig. 4); these compounds decrease the energy state of the cell. Figure 4 shows that after 4 h, p8 although still within the nucleus, could also be observed in the cytoplasm, suggesting an active nuclear import process in which the newly synthesized p8, and/or p8 that has been shuttling from the nucleus to the cytoplasm is retained in the cytoplasm due to the impairment of the active process.

As a second approach we used a popular method used nowadays for the identification of a functional NLS, consisting in following the localization of a recombinant fusion protein in which the protein of interest is fused to the GFP [Damelin et al., 2002]. Figure 5 shows the direct visualization of GFP in living HeLa and 3T3 cells transfected with pGFPp8 as compared to those transfected with pGFP. It is evident that while GFP was localized throughout the whole cell (although with a certain higher nuclear localization), the GFP–p8 fusion localized almost exclusively in the nucleus, suggesting that p8 contains a strong NLS within its sequence, that is able to shift the nuclear/cytoplasmic equilibrium toward a predominant nuclear localization. In order to map the NLS sequence more accurately, we made a GFP–p8NLS

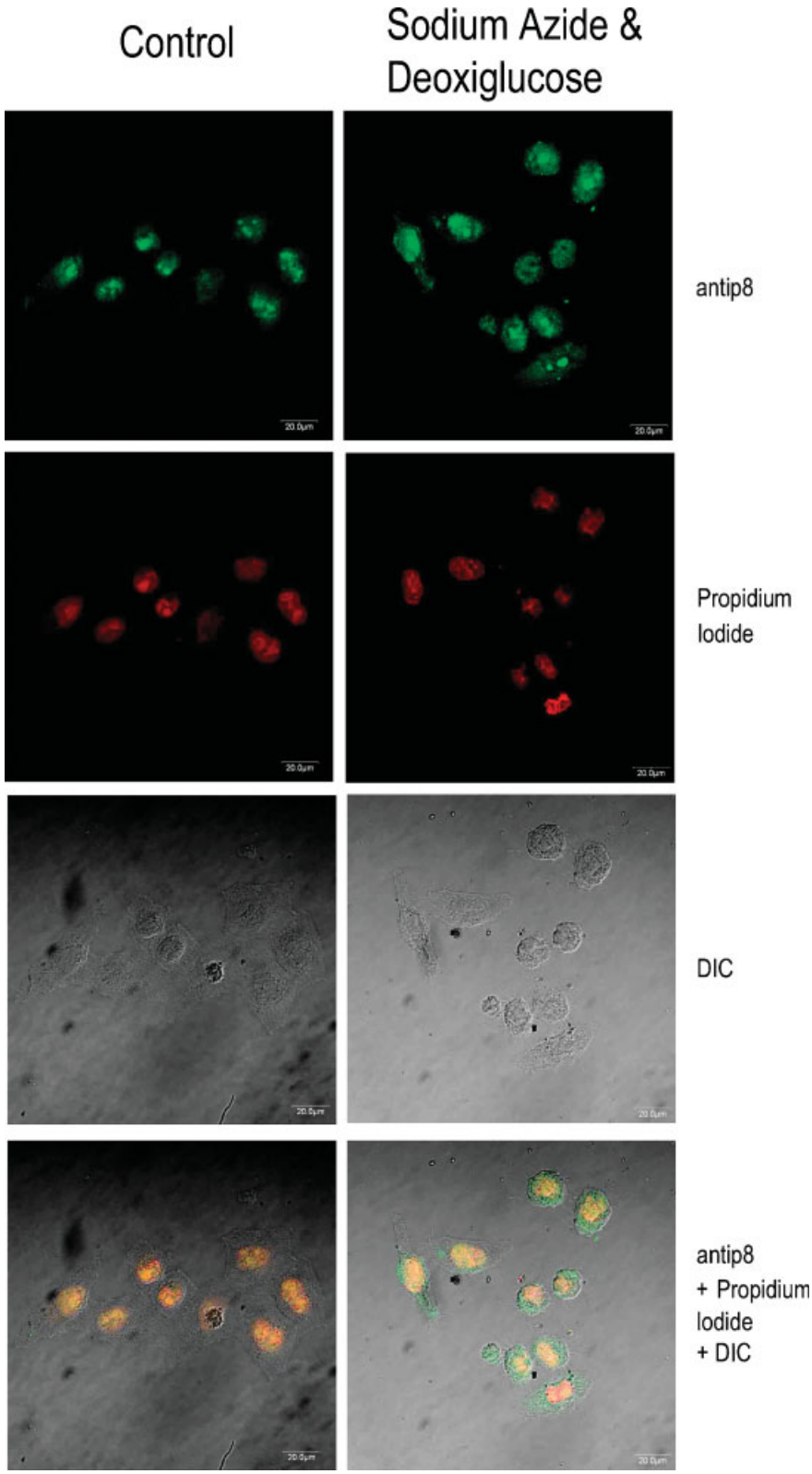

Fig. 4. Effect of energy depletion on nuclear localization of p8. Sub-confluent HeLa–p8 cells were treated with 10 mM sodium azide plus 6 mM 2-deoxyglucose during 4 h. Cells were visualized by confocal microscopy after fixation and immunostaining for p8 and nuclear staining with propidium iodide.

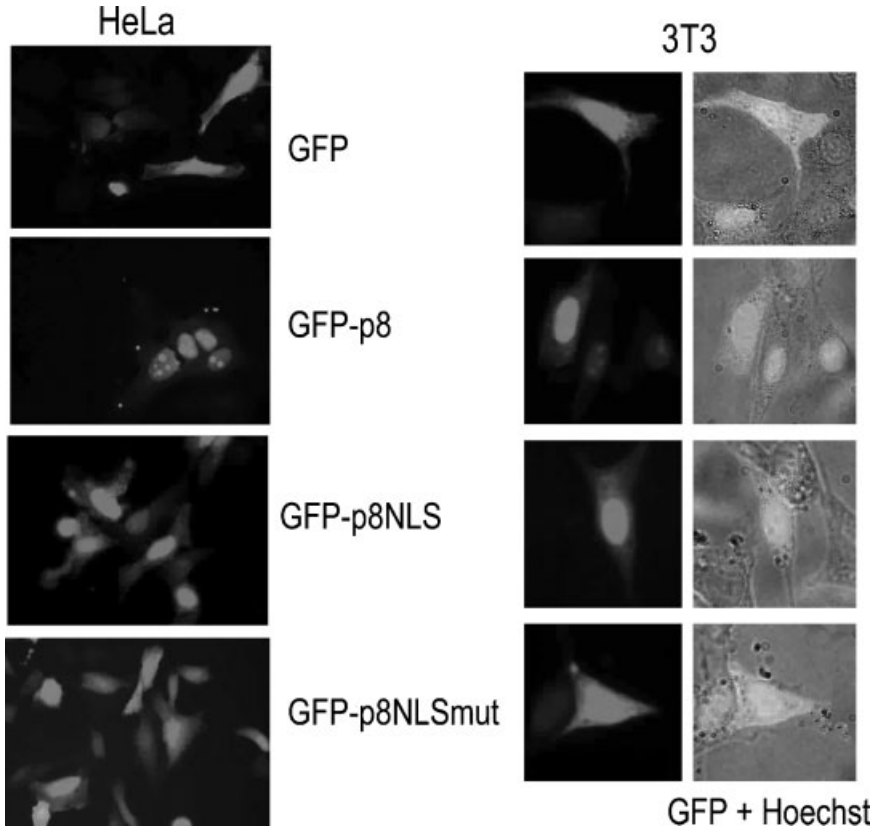

Fig. 5. p8 NLS is necessary and sufficient for retention of a heterologous protein. HeLa cells, cultured at low density and expressing either GFP, GFP–p8, GFP–p8NLS, or GFP–p8NLSmut (**left panels**) were visualized for GFP fluorescence. The localization of these GFP fusion proteins was also analyzed in 3T3 cells (**right panels**). These cells were visualized for GFP fluorescence, and also through phase contrast microscopy (**right panel**). Nuclei were visualized using Hoechst 33248 staining. The relative nuclear/cytoplasmic intensity was scored in 300 cells for each condition for each of two separate transfection experiments. In all the experiments the time of exposure for the fluoresecence acquired for the pictures was the same. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

construction in which we fused the putative p8–NLS sequence comprising aminoacids 64–78 to the carboxy end of GFP. Figure 5 shows that this construction also localized to the nucleus, indicating that this NLS sequence is sufficient for driving GFP to the nucleus. Decreasing the energetic charge of the cell by treatment with sodium azide and 2-deoxyglucose caused both the GFP–p8 and the GFP–p8NLS fusion proteins to re-equilibrate across the nuclear envelope; on the contrary, energy depletion had no effect on GFP localization (data not shown). In order to evaluate whether the basic residues in the 64–78 p8 sequence are necessary as determinants to make this sequence an NLS, we mutated the lysines 65, 69, 76, and 77 to alanine to generate a GFP–p8NLSmut construction. The results, shown in Figure 5 show, that both in HeLa and 3T3 cells, the GFP distribution is again more uniform throughout the whole cell, although, as with GFP alone, with still a slightly higher nuclear localization. The combined results obtained with GFP–p8, GFP–p8NLS, and GFP–p8NLSmut indicate that p8NLS is both necessary and sufficient to drive the localization of a fusion protein to the nucleus.

Since p8 is a protein with nuclear–cytoplasmic localization, it is possible that the cell density-related accumulation of cytoplasmic p8 depends on sustained nuclear export. To determine whether p8 is actively exported from the nucleus, the distribution of p8 in HeLa cells, at high cell density, was examined after 8 h treatment with 20 ng/ml Leptomycin B (LMB), a specific inhibitor of Crm1-dependent nuclear export (Fig. 6) [Fornerod et al., 1997]. If p8 shuttles between nucleus and cytoplasm, and its nuclear export is dependent on Crm1, then LMB, added to high density cells, should induce accumulation of p8 in the nucleus. A positive control for LMB effect in HeLa cells was included (shown in Fig. 6), in which cells were

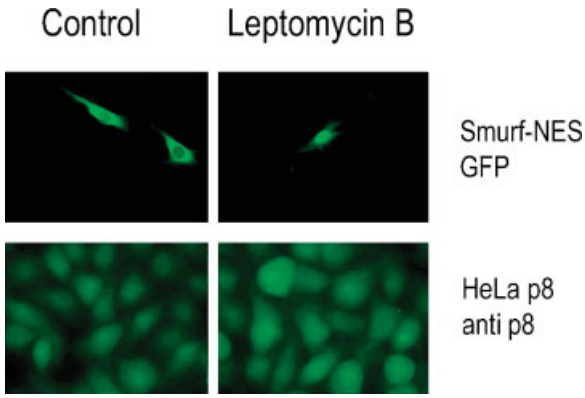

Fig. 6. Leptomycin B has no effect on p8 localization. **Upper panel**: HeLa cells transiently transfected with pSmurf–NES–GFP; **lower panel**: HeLa–p8 cells at high density. Both cell cultures were incubated with 20 ng/ml Leptomycin B for 8 h prior to fixation. The Smurf–NES–GFP fusion was visualized by GFP fluorescence, and p8 by immunofluorescence.

transfected with Smurf1 NES–GFP plasmid. The NES sequence of Smurf1 has been demonstrated to be an export sequence clearly dependent on Crm1 for active export, and therefore sensitive to inhibition by LMB [Tajima et al., 2003]. Figure 6 shows that p8 localization did not change after the 8 h treatment. This result has two possible interpretations: either p8 does not shuttle continuously between cytoplasm and nucleus when the cells are at high density or the export of p8 to the cytoplasm is independent of Crm1. In addition, LMB did not prevent the translocation of p8 from the nucleus to the cytoplasm when low-density cells were replated at high density (data not shown). This result again has two interpretations: either p8 exits the nucleus by a mechanism independent of Crm1, and therefore LMB insensitive, or at high cell density the cytoplasmic localization results from a block to nuclear import and not from an active export process.

Signal Transduction Pathways and Subcellular Localization of p8

Previous experiments have shown that p8 mRNA is activated in almost all cells in response to several stresses [Jiang et al., 1999], including minimal stresses such as routine change of the culture medium in the absence of any added substance [García-Montero et al., 2001], indicating that p8 is an ubiquitous protein induced by cellular stress. We chose to test whether some stress promoting agents had some influence on p8 subcellular localization when added to low or high density growing cells. Results (not shown) indicate that the following stress-inducing treatments had no effect when applied either to human 293T cells nor to HeLa–p8 cells: 20 h in the presence of 100 nM dexamethasone, 0.8 μM doxorubicin, 0.1 μM staurosporine or 120 mM NaCl, and 1 h in the presence of 100 μM H_2O_2 or 100 ng/ml TNF-α. Under all the above conditions p8 localization did not change during the time of the experiment.

It has been demonstrated recently that two pathways that stimulate growth, such as the Ras → Raf → MEK → ERK and JNK pathways, down-regulate p8 expression in pancreatic cancer cells, while the p38 pathway, which inhibits proliferation in pancreatic cells, up-regulates p8 expression [Malicet et al., 2003]. Typical inhibitors of Erk1/2 (U0126), of JNK (SP 600125), and of p38 (SB 203580) were assayed on HeLa p8 cells under two conditions: addition of the drugs for 8–15 h when plating high density cells at low density, and inversely, when plating low density growing cells at high density. Even though we are visualizing mainly p8 expressed from the CMV promoter we expected to evaluate whether a postranslational modification triggered by one of those pathways, and inhibited by the selective drugs, could have an effect on p8 subcellular localization, either nuclear or cytoplasmic. No difference with control cells could be observed upon immunofluorescence visualization of p8 (data not shown).

Finally, since the conserved domains of p8 (see Fig. 3) include two acetylation motifs and it has been demonstrated that p8 can be acetylated in vitro by p300 [Hoffmeister et al., 2002], we assayed the effect of adding 2 μM trichostatin A (TSA), a known inhibitor of deacetylases [Yoshida et al., 1990], to HeLa-p8 cells growing at low or high density. A short time (4 h) incubation with TSA was chosen for this experiment, since during this time and up to 12 h incubation, TSA is known to exert no effect on cell growth on HeLa cells [Hoshikawa et al., 1994; Wu et al., 2005]. Figure 7 shows that in the presence of TSA, the almost exclusively nuclear localization observed in control low density cells was lost, and p8 was evenly distributed throughout the whole cell. p8 localization in high density cells did not change upon incubation with TSA for even up to 8 h (not shown). To ensure that TSA effect on p8 localization was

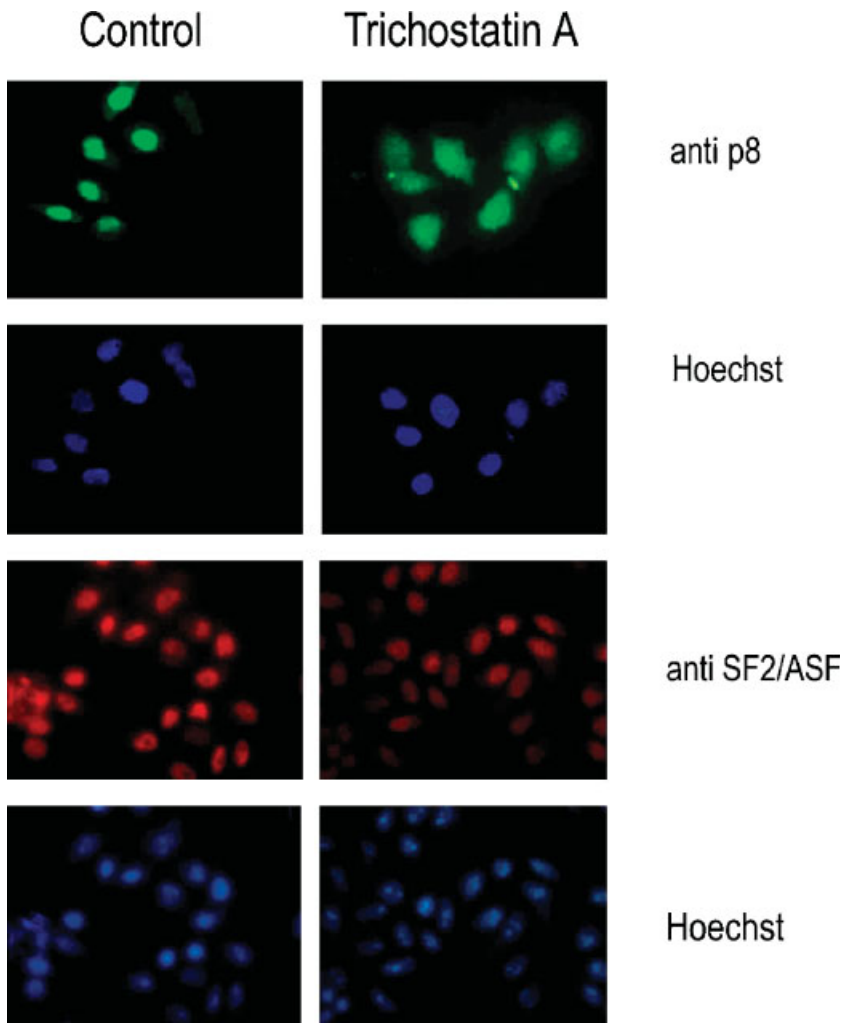

Fig. 7. p8 changes subcellular localization following deacetylation inhibition. HeLa–p8 cells, growing at low density, were treated with 2 μM TSA during 4 h. Cells were fixed and immunostained for p8 (**upper panels**) and for SF2/ASF (**lower panels**); nuclei were stained with Hoechst 33248.

not due to a general effect on nuclear proteins, HeLa–p8 cells treated with TSA for 4 h were immunostained against SF2/ASF (splicing regulator, belonging to the SR family), a constitutively nuclear protein. As Figure 7 (bottom) shows, TSA had no effect on the localization of this protein. These results suggest that an increase in the acetylation state of proteins in the cell, either directly or indirectly affects the nuclear/cytoplasmic equilibrium for p8.

DISCUSSION

This study shows that endogenous p8, as well as p8 stably expressed from a heterologous promoter accumulates in the nucleus of human cells (HeLa, HEK 293T, Panc-1, MiaPaCa) when cultured at low density. However when cells became confluent, were starved for serum or were treated with an inhibitor of DNA synthesis, all conditions that presumably arrest the cells in G_0, p8 was found to localize both to nuclei and cytoplasm. The literature shows that a similar change in subcellular distribution in response to cell density has been described for a variety of proteins with different sizes and function [Lee et al., 1996; Sierra-Honigmann et al., 1996; Petridou et al., 2000; Sussman et al., 2001; Zhang et al., 2001; Bui et al., 2002; Dietrich et al., 2002; Fagman et al., 2003].

A close inspection of p8 homology from fly to human indicated that a NLS corresponding to the classical bipartite type could be predicted (Fig. 3). In fact, the import of p8 has been shown

to be an energy-dependent process, and the predicted NLS turned to be a bonafide NLS, necessary and sufficient to drive and heterologous protein to the nucleus. Even though a potential NES, rich in leucine, could also be predicted from sequence homology, the export of p8 does not seem to depend, at least, on the Crm1 export pathway, since its localization was unmodified when treated with leptomycin B (Fig. 6). Active NLS sequences have already been described for other small sized proteins, such as histones [Baake et al., 2001] and High Mobility Group 1 proteins (HMGB1) [Bonaldi et al., 2003].

A protein may contain a NLS but fail to enter the nucleus at a significant rate because of a high affinity interaction with a cytosolic anchor protein that does not enter the nucleus and may even occlude the NLS of its binding partner. Similarly, a protein may reside stably in the nucleus even though it contains a NES if it interacts tightly with a nuclear protein. In either case, the intracellular distribution of such a protein can be regulated by signal-mediated changes in its affinity for or the availability of its anchor(s) [Cyert, 2001]. The fact that both p8 expressed from its endogenous gene and from a recombinant construction under the control of a viral promoter display the same distribution, suggests that the regulation of its localization is not very much influenced by the level or timing of its expression, but that most probably is the result of a posttranslational effect exerted directly or indirectly over the protein. The participation of several transduction pathways in the localization of p8 has been assayed. None of the following seem to be involved: stress induced by several agents, ERK1/2, JNK, nor p38 mediated pathways. However, the impairment of protein deacetylation by incubation with TSA, shifts the exclusively nuclear localization of p8 in low cell density cells to the cytoplasmic side (Fig. 7).

The results obtained in this paper lead us to a working model in which we propose that at low cell density, p8 is synthesized and transported actively to the nucleus either alone or forming part of a complex (see below), where it is retained. The import/export equilibrium under these conditions is completely shifted toward a nuclear accumulation. We can not decide whether the export of p8 is passive or active, depending on a particular sequence, and on an export machinery different from Crm1. The difference in localization observed in confluent cells, as well as low density cells treated with TSA, could be attributed to an increase in export, a decrease in import or a combination of both processes with a balance toward the cytoplasmic side. A tempting speculation is that under certain physiological conditions the acetylation state of p8 is modified, having as a consequence a decrease on the positive charge of the bipartite NLS, making it inactive, and inhibiting therefore the active import process of p8. Support for this speculation comes on one side from the existence of consensus acetylation motifs in the conserved region of p8 sequence, embedded in part in the NLS (Fig. 3), and on the other side from the demonstration that p8 interacts in vivo and in vitro with p300, a transcriptional coactivator with histone acetylase activity, and is also acetylated in vitro by p300 [Hoffmeister et al., 2002]. A mechanism such as the one proposed has been demonstrated for the High Mobility Group 1 protein (HMGB1) [Bonaldi et al., 2003], to which we have demonstrated that p8 resembles both chemically and structurally [Encinar et al., 2001].

The fact that p8 has a clear nuclear localization when cells are in active growth, suggests that the protein is not free within the nucleus, but complexed to other protein(s) or nucleic acids. Otherwise it would be possible to find p8 more evenly distributed, since a passive nuclear/cytoplasmic transport for free monomeric p8 could not be avoided. Nuclear pores allow passive diffusion of proteins with a mass up to around 40–50 kDa, which raises the question of why would p8, a 9 kDa protein, contain a functional NLS? One possible explanation is that p8 exists in the cell as part of a protein complex and that the NLS of p8 is responsible for mediating the nuclear translocation of the entire complex. Certain intracellular proteins are only active when built into larger protein complexes that coordinate multiple biochemical activities. The interactions between proteins in these complexes are frequently regulated in response to environmental stimuli or to changes in cell state. This might be the case for p8, which may be active in association to other protein partners directing their subcellular localization. In support of this proposal is the fact that purified recombinant p8 is unstructured in solution, and acquires some degree of secondary structure

upon phosphorylation and/or DNA binding [Encinar et al., 2001]. The interaction of p8 with its putative partners is another possible target of direct or indirect regulation by acetylation, with an impact on its subcellular localization.

Recent studies have established that protein function is often regulated at the level of localization during signaling. There are cases where proteins are retained in the nucleus to ensure their inactivity prior to signaling and are released from the nucleus to allow their activation in the cytosol or at the surface of intracellular membranes, or inversely are sequestered and inactive in the cytoplasm, and active within the nucleus. However, it is also possible that proteins may have a particular function in both subcellular compartments. This might be the case for p8 where localization seems to be an important clue to its physiological or pathological role. An architectural role in transcription, already proposed for p8 [Hoffmeister et al., 2002], is compatible with a nuclear localization. It has recently been shown that p8 is detected in the cytoplasm of cultured human pancreatic islets, poorly or non proliferating, whereas an actively dividing INS-1 β-cell line, shows translocalization of p8 into the nucleus [Päth et al., 2004]. A hint to the role in cytoplasm comes from a recent study on the immunohistochemical localization of p8 in thyroid neoplasms [Ito et al., 2003], in which it is suggested that a cytoplasmic localization might reflect disease progression of papillary carcinomas.

Cell growth related control of p8 localization opens new avenues of study into the role of p8, particularly in its participation in tumorigenesis; further investigations on the molecular mechanism underlying the nuclear-cytoplasmic localization of p8 are of fundamental importance.

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REFERENCES

Baake M, Bauerle M, Doenecke D, Albig W. 2001. Core histones and linker histones are imported into the nucleus by different pathways. Eur J Cell Biol 80:669–677.

Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME. 2003. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it toward secretion. EMBO J 22:5551–5560.

Bratland A, Risberg K, Maelandsmo GM, Gutzkow KB, Olsen OE, Moghaddam A, Wang MY, Hansen CM, Blomhoff HK, Berg JP, Fodstad O, Ree AH. 2000. Expression of a novel factor, com1, is regulated by 1,25-dihydroxyvitamin D3 in breast cancer cells. Cancer Res 60:5578–5583.

Bui M, Myers JE, Whittaker GR. 2002. Nucleo-cytoplasmic localization of influenza virus nucleoprotein depends on cell density and phosphorylation. Virus Res 84:37–44.

Bustin M, Reeves R. 1996. High-mobility-group chromosomal proteins: Architectural components that facilitate chromatin function. Prog Nucleic Acids Res Mol Biol 54: 35–100.

Cyert MS. 2001. Regulation of nuclear localization during signaling. J Biol Chem 276:20805–20808.

Damelin M, Silver PA, Corbett AH. 2002. Nuclear protein transport. Methods Enzymol 351:587–607.

Dietrich C, Scherwat J, Faust D, Oesch F. 2002. Subcellular localization of beta-catenin is regulated by cell density. Biochem Biophys Res Commun 292:195–199.

Encinar JL, Mallo GV, Mizyrycki C, Giono L, González-Ros JM, Rico M, Cánepa E, Moreno S, Neira JL, Iovanna JL. 2001. Human p8 is a HMG-I/Y-like protein with DNA binding activity enhanced by phosphorylation. J Biol Chem 276:2742–2751.

Fagman H, Larsson F, Arvidsson Y, Meuller J, Nordling M, Martinsson T, Helmbrecht K, Brabant G, Nilsson M. 2003. Nuclear accumulation of full-length and truncated adenomatous polyposis coli protein in tumor cells depends on proliferation. Oncogene 22:6013–6022.

Fornerod M, Ohno M, Yoshida M, Mattaj IW. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell 90:1051–1060.

García-Montero A, Vasseur S, Mallo GV, Soubeyran P, Dagorn JC, Iovanna JL. 2001. Expression of the stress-induced p8 mRNA is transiently activated after culture medium change. Eur J Cell Biol 80:720–725.

Goruppi S, Bonventre JV, Kyriakis JM. 2002. Signaling pathways and late-onset gene induction associated with renal mesangial cell hypertrophy. EMBO J 21:5427–5436.

Hoffmeister A, Ropolo A, Vasseur S, Mallo GV, Bodeker H, Ritz-Laser B, Dressler GR, Vaccaro MI, Dagorn JC, Moreno S, Iovanna JL. 2002. The HMG-I/Y-related protein p8 binds to p300 and Pax2 transactivation domain-interacting protein to regulate the transactivation activity of the Pax2A and Pax2B transcription factors on the glucagon gene promoter. J Biol Chem 277:22314–22319.

Hoshikawa Y, Kwon HJ, Yoshida M, Horinnouchi S, Beppu T. 1994. Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylases in human carcinoma cell lines. Exp Cell Res 214: 189–197.

Igarashi T, Kuroda H, Takahashi S, Asashima M. 2001. Cloning and characterization of the *Xenopus laevis* p8 gene. Develop Growth Differ 43:693–698.

Ito Y, Yoshida H, Motoo Y, Miyoshi E, Iovanna JL, Tomoda C, Uruno T, Takamura Y, Miya A, Kobayashi K, Matsuzuka F, Matsuura N, Kuma K, Miyauchi A. 2003.

Expression and cellular localization of p8 protein in thyroid neoplasms. Cancer Lett 201:237–244.

Jiang YF, Vaccaro MI, Fiedler F, Calvo EL, Iovanna JL. 1999. Lipopolysaccharides induce p8 mRNA expression in vivo and in vitro. Biochem Biophys Res Commun 260: 686–690.

Lee S, Chen DY, Humphrey JS, Gnarra JR, Linehan WM, Klausner RD. 1996. Nuclear/cytoplasmic localization of the von Hippel-Lindau tumor suppressor gene product is determined by cell density. Proc Natl Acad Sci USA 93: 1770–1775.

Macara IG. 2001. Transport into and out of the nucleus. Microbiol Mol Biol Rev 65:570–594.

Malicet C, Lesavre N, Vasseur S, Iovanna JL. 2003. p8 inhibits the growth of human pancreatic cancer cells and its expression is induced through pathways involved in growth inhibition and repressed by factors promoting cell growth. Mol Cancer 2:37–49.

Mallo GV, Fiedler F, Calvo EL, Ortiz EM, Vasseur S, Keim V, Morisset J, Iovanna JL. 1997. Cloning and expression of the rat p8 cDNA, a new gene activated in pancreas during the acute phase of pancreatitis, pancreatitic development, and regeneration, and which promotes cellular growth. J Biol Chem 272:32360–32369.

Mohammad HP, Seachrist DD, Quirk CC, Nilson JH. 2004. Re-expression of p8 contributes to tumorigenic properties of pituitary cells and appears in a subset of prolactinomas in transgenic mice that hypersecrete LH. Mol Endocrin 18:2583–2593.

Päth G, Opel A, Knoll A, Seufert J. 2004. Nuclear protein p8 is associated with glucose-induced pancreatic beta-cell growth. Diabetes 53(Suppl 1):S82–S85.

Petridou S, Maltseva O, Spanakis S, Masur SK. 2000. TGF-beta receptor expression and smad2 localization are cell density dependent in fibroblasts. Invest Ophthalmol Vis Sci 41:89–95.

Ree AH, Tvermyr M, Engebraaten O, Rooman M, Rosok O, Hovig E, Meza-Zepeda LA, Bruland OS, Fodstad O. 1999. Expression of a novel factor in human breast cancer cells with metastatic potential. Cancer Res 59:4675–4680.

Richardson WD, Mills AD, Dilworth SM, Laskey RA, Dingwall C. 1988. Nuclear protein migration involves two steps: Rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell 52: 655–664.

Sierra-Honigmann MR, Bradley JR, Pober JS. 1996. Cytosolic phospholipase A2 is in the nucleus of subconfluent endothelial cells but confined to the cytoplasm of confluent endothelial cells and redistributes to the nuclear envelope and cell junctions upon histamine stimulation. Lab Invest 74:684–695.

Sussman J, Stokoe D, Ossina N, Shtivelman E. 2001. Protein kinase B phosphorylates AHNAK and regulates its subcellular localization. J Cell Biol 154:1019–1030.

Taieb D, Malicet C, Garcia S, Rocchi P, Arnaud C, Dagorn JC, Iovanna JL, Vasseur S. 2005. Inactivation of stress protein p8 increases murine carbon tetrachloride hepatotoxicity via preserved CYP2E1 activity. Hepatology 42: 176–182.

Tajima Y, Goto K, Yoshida M, Shinomiya K, Sekimoto T, Yoneda Y, Miyazono K, Imamura T. 2003. Chromosomal region maintenance 1 (CRM1)-dependent nuclear export of Smad ubiquitin regulatory factor 1 (Smurf1) is essential for negative regulation of transforming growth factor-beta signaling by Smad7. J Biol Chem 278:10716–10721.

Thompson PR, Kurooka H, Nakatani Y, Cole PA. 2001. Transcriptional coactivator protein p300. Kinetic characterization of its histone acetyltransferase activity. J Biol Chem 276:33721–33729.

Vasseur S, Mallo GV, Fiedler F, Bodeker H, Cánepa E, Moreno S, Iovanna JL. 1999a. Cloning and expression of human p8, a nuclear protein with mitogenic activity. Eur J Biochem 259:670–675.

Vasseur S, Mallo GV, García-Montero A, Ortiz EM, Fiedler F, Cánepa E, Moreno S, Iovanna JL. 1999b. Structural and functional characterization of the mouse p8 gene: Promotion of transcription by the CAAT-enhancer binding protein alpha (C/EBPalpha) and C/EBPbeta transacting factors involves a C/EBP cis-acting element and other regions of the promoter. Biochem J 343:377–383.

Vasseur S, Hoffmeister A, García S, Bagnis C, Dagorn JC, Iovanna JL. 2002a. p8 is critical for tumour development induced by rasV12 mutated protein and E1A oncogene. EMBO Rep 3:165–170.

Vasseur S, Hoffmeister A, Garcia-Montero A, Mallo GV, Feil R, Kuhbandner S, Dagorn JC, Iovanna JL. 2002b. p8-deficient fibroblasts grow more rapidly and are more resistant to adriamycin-induced apoptosis. Oncogene 21: 1685–1694.

Vasseur S, Hoffmeister A, Garcia-Montero A, Barthet M, Saint-Michel L, Berthezene P, Fiedler F, Closa D, Dagorn JC, Iovanna JL. 2003. Mice with targeted disruption of p8 gene show increased sensitivity to lipopolysaccharide and DNA microarray analysis of livers reveals an aberrant gene expression response. BMC Gastroenterol 3:25.

Wu P, Meng L, Wang H, Zhou J, Xu G, Wang S, Xi L, Chen G, Wang B, Zhu T, Lu Y, Ma D. 2005. Role of hTERT in apoptosis of cervical cancer induced by histone deacetylase inhibitor. Biochem Biophys Res Commun 335:36–44.

Yoshida M, Kijima M, Akita M, Beppu T. 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265:17174–17179.

Zhang F, White RL, Neufeld KL. 2001. Cell density and phosphorylation control the subcellular localization of adenomatous polyposis coli protein. Mol Cell Biol 21: 8143–8156.