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Interaction of the stress protein p8 with Jab1 is required for Jab1-dependent p27 nuclear-to-cytoplasm translocation

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Abstract

p8 is an 80 amino-acid polypeptide identified because of its remarkable over-expression in the stressed pancreas. This protein, apparently devoid of enzymatic activity, is a powerful regulator of several intracellular pathways, suggesting that it has to interact with several molecular partners to modulate their activity. We used two-hybrid screening of a pre-transformed human testes cDNA library to identify some of these partners. One of them was the multifunctional protein Jab1, its interaction with p8 being confirmed by His_6-pull down and co-immunoprecipitation assays. In addition, we could show that the two proteins co-localized in the cell. Our functional data demonstrate that Jab1 requires direct interaction with p8 to induce the translocation of p27 from nucleus to cytoplasm and its subsequent degradation. Experiments showing that the knock-down of p8 expression results in a strong inhibition of Jab1 activity confirmed that the mechanism by which Jab1 promotes cell growth by decreasing p27 level is p8-dependent.

Keywords: p8; Jab1; p27; Two hybrid; Cell cycle

We have previously characterized a new gene, called p8, as showing one of the strongest over-expressions in pancreas during the acute phase of pancreatitis [1]. Further experiments showed that p8 mRNA was in fact activated in almost all cells in response to several stresses [2], including minimal stresses [3], indicating that p8 was a ubiquitous stress protein. Several, apparently unrelated functions have already been described for p8 in different cell types. For instance, p8 is generally involved in cell-cycle regulation as a growth promoter [1,4,5] but can also be an inhibitor [6–8] or as a mediator of apoptosis [6]. Also, p8 is required for endothelin-induced mesangial cell hypertrophy in diabetic kidneys [9], for initiation of LHβ gene expression [10], and in *Drosophila melanogaster* to stop cell growth in response to starvation [11]. More recently, we found that over-expression of p8 is an important component of the defence program against LPS challenge [12] and improves the pancreatic response to acute pancreatitis by enhancing the expression of the anti-inflammatory protein PAP I [13]. Finally, a particularly attractive role in the control of tumour progression was proposed for p8 [14–16].

If p8 is involved in several functions in the cell, the molecular mechanisms by which such regulation occurs remain largely unknown. The variety of pathways potentially controlled by p8, the small size of the protein, and its lack of specific tri-dimensional structure suggest that it could enroll different partners to target different pathways. In an effort to identify such partners, we screened a human testes cDNA library by the two-hybrid system and found that p8 binds the Jun-activating binding protein 1 (Jab1). Jab1 is a multifunctional protein associated with the signaling pathway, cell-cycle regulation, and

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development, and acts as a key subunit of COP9 signalosome. One of its most important functions is to control the level of p27/Kip1, a key cell-cycle regulator. In the present work, we were able to demonstrate that p8 expression is required for Jab1-dependent p27 nuclear to cytoplasm translocation and degradation.

Materials and methods

Antibodies. Anti-Jab1 mouse monoclonal (catalogue number B-7) and rabbit polyclonal (catalogue number FL-334) antibodies were purchased from Santa Cruz Biotechnology. Anti-β-actin mouse monoclonal antibody (catalogue number A5441) was from Sigma. Anti-p27 rabbit polyclonal antibody (C-19) and rabbit polyclonal anti-Lamin (catalogue number sc-20680) were from Santa Cruz Biotechnology. The mouse monoclonal anti-HA (catalogue number 1666606) was from Roche Diagnostics and the rabbit polyclonal anti-Flag (catalogue number F7425) was from Sigma.

Yeast two-hybrid screen. The Matchmaker two-hybrid system (Clontech Laboratories) was used according to the protocols provided by the manufacturer. Using polymerase chain reaction-based strategies, we subcloned the complete coding sequence of the human p8 [4] into the *Bam*HI/*Sal*I site of the pGBT9 vector to generate a fusion protein with the GAL4 DNA binding domain (BDhp8). That protein was used as bait to screen a pre-transformed human testes cDNA library (catalogue number HY4035AH) constructed in the pACT2 vector into the *Xho*I/*Eco*RI restriction site to generate fusion products with the GAL4 activation domain. Yeast cells were initially selected for growth on -His/-adenine plates. Of the approximately 1.5×10^6 transformants screened, 9 grew well on -His/-adenine plates (colony diameter >2 mm). Plasmid DNAs were isolated from each one. To identify false positives and self-activators, the plasmids were reintroduced into the original yeast strain expressing no BDhp8.

Co-precipitation assays. The full-length sequence of Jab1 (nucleotides −11 to 1020) cloned into the *Eco*RI/*Bam*HI restriction site of the mammalian expression vector pSG5 (Stratagene) to create the pSG5-Jab1 construct [17] was obtained from Dr. H. Loosfelt (INSERM U.135, Paris). Jab1 protein was synthesized with a TnT-coupled reticulocyte lysate system (Promega) and the pSG5-Jab1 construct in the presence of L-[^{35}S]methionine according to the manufacturer's instructions. Labeled protein was incubated with 1 μg hp8-His_6-tagged protein [18] bound to nickel–agarose resin (Qiagen) at 4 °C for 2 h with in vitro interaction buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, and a mix of protease inhibitors) and washed four times with 500 μl of in vitro interaction buffer and two times with 500 μl PBS. Bound proteins were eluted with 1× SDS sample buffer, separated by denaturing SDS–PAGE, and analyzed by autoradiography.

pcDNA3p8 [4] and pSG5-Jab1 expression plasmids were transfected into 293T cells using the Fugene reagent following manufacturer's recommendations (Roche Diagnostics) and allowed to express for 24 h. After lysis in 50 mM Tris–HCl, pH 8.0, 0.5% Nonidet P-40 with protease inhibitors, p8 was immunoprecipitated by adding antibodies against the ERKLVTKLQNSERKKRGARR p8 epitope [4] followed by rocking 2 h at 4 °C. Then, 20 μl of protein A–Sepharose conjugate (Zymed Laboratories) was added and incubated for an additional 2 h at 4 °C. The Sepharose beads in each tube were washed three times followed by SDS–PAGE and Western blotting using the anti-Jab1 monoclonal antibody (Santa Cruz Biotechnology).

Immunofluorescence. 293T cells were grown on coverslips in 6-well plates and transfected with 1 μg p8 and Jab1 expression plasmids. Twenty-four hours later they were washed once with 1× PBS, fixed for 15 min at room temperature in PBS–3% paraformaldehyde, washed twice with PBS, and blocked in PBS, 50 mM NH_4Cl for 10 min at room temperature. Cells were then incubated for 60 min with the primary antibodies at room temperature and washed four times (5 min each) in PBS before incubation for 60 min with the appropriate fluorochrome-conjugated secondary antibody.

Semi-quantitative reverse transcription-PCR analyses. Expressions of Jab1 and RL3 mRNAs were determined by semi-quantitative reverse transcription (RT)-PCR on RNA extracted with Trizol (Gibco Lifetechnologies) from HeLa cells transfected with increasing amounts of p8 expression plasmid [4] using the forward primer 5′-GACGAC AACTTCTCCGCTTC-3′ and the reverse primer 5′-GCCAACCTGTT TTGCATTTT-3′ for amplification of Jab1, and the forward primer 5′-GAAAGAAGTCGTGGAGGCTG-3′ and the reverse primer 5′-ATCTCATCCTGCCCAAACAC-3′ for amplification of RL3. Jab1 was amplified for 30 cycles and RL3 for 22 cycles. Amplification products were submitted to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

SDS–PAGE and Western blot analysis. HeLa cells were transfected with increasing amounts of p8 expression plasmid [4] alone or together with the pSG5-Jab1 construct and/or the p27 expression vector (pCMV-p27 tag HA kindly provided by Dr. P. O'Hare, Marie Curie Research Institute, UK) as described above. After 24 h, cells were lysed and 100 μg of total cell protein was separated on 12.5% SDS–PAGE using the Mini Protean System (Bio-Rad) and transferred to a nitrocellulose membrane (Sigma). The intracellular levels of Jab1, p27, and β-actin were estimated by Western blot using specific antibodies. The second antibody was a peroxidase-labeled anti-rabbit-IgG antibody provided with the ECL-kit (Amersham).

Fractionation of cellular proteins. Fractionation of cellular proteins was performed basically as described [19]. Briefly, HeLa cells were trypsinized, washed with PBS, and lysed in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and fresh proteinase inhibitors (Sigma)) for 15 min. Then, NP40 was added to 0.5% and the samples were centrifuged for 5 min at 3000 rpm. Resulting supernatants were used as cytoplasmic extracts. Pelleted nuclei were directly recovered in 1× SDS sample buffer.

siRNA design and transfection. We tested four sequences and established that although all four worked (data not shown), p8-2 (sense 5′-CCCUGCAAAAGUCCAAGAd(TT)-3′) and p8-4 (sense 5′-GGAGG ACCCAGGACAGGAUd(TT)-3′) siRNA were the most efficient. p8-2 and p8-4 siRNA were used to carry on the functional studies. The siRNA was obtained from Qiagen annealed and ready to use after rehydration. The day before transfection, cells were plated in 6-well plates to give 30–50% confluence. After removal of the medium, cells were washed once with serum-free medium and transfection was done in serum-free medium by addition of a mix composed of 10 μl Oligofectamine reagent (Invitrogen), 0, 0.5, 1.0, 1.5 or 2.0 μg siRNA diluted in 240 μl serum-free medium. After an incubation of 4 h at 37 °C, the transfection medium was replaced by fresh medium. After 24 h, cells were lysed and 100 μg of total cell protein was separated on 12.5% SDS–PAGE using the Mini Protean System (Bio-Rad), transferred to a nitrocellulose membrane, and p27 and β-actin were estimated by Western blot using specific antibodies as described above.

Results

Identification of Jab1 as a partner of p8

Five specifically interacting cDNA clones were identified that were positive for the expression of the selection markers (histidine and adenine) upon reconstitution analysis. Subsequent sequence analysis showed that three of these clones had identical inserts, the two others being different. All three sequences showed 100% identity with that of human Jab1.

Direct interaction of p8 and Jab1 was examined in vitro by His_6-pull down assays. Fig. 1 shows that L-[^{35}S]methionine-labeled Jab1 prepared by in vitro transcription/translation can bind to hp8-His_6 but not to the nickel–agarose resin. In order to determine whether p8 and Jab1 associate

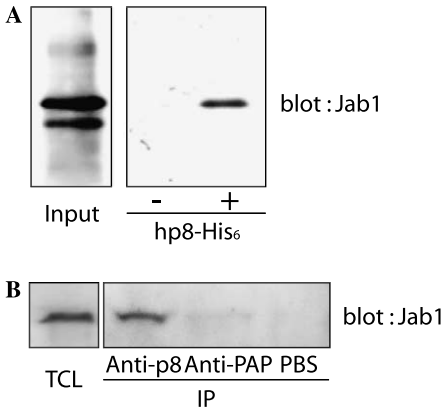

Fig. 1. p8 and Jab1 interact in vitro and in vivo. (A) [^{35}S] Jab1 synthesized in vitro (10 μl) was incubated with hp8-His_6-tagged protein immobilized on nickel–agarose resin or free nickel–agarose resin as described in Materials and methods. Bound proteins (pellet) were analyzed by SDS–PAGE and autoradiography. Input: total amount of [^{35}S] Jab1 (2 μl). Two bands were systematically observed. The minor, faster migrating species, which also binds p8 (not shown) is probably a truncated form of Jab1 generated by the in vitro system. (B) 293T cells were transfected with 2 μg of pcDNA3p8 together with 2 μg pSG5-Jab1 expression plasmids. Immunoprecipitations of cell lysates were performed using either an anti-ERKLVTKLQNSERKKRGARR p8 epitope (2 μl), an irrelevant antibody (rabbit polyclonal antibody against rat pancreatitis associated protein) (2 μl) or PBS (2 μl) for 2 h at 4 °C. Then, 20 μl of protein A–Sepharose conjugate was added and incubated for an additional 2 h period. The immunocomplexes were detected by Western blotting using the anti-Jab1 monoclonal antibody. TCL, total cell lysate.

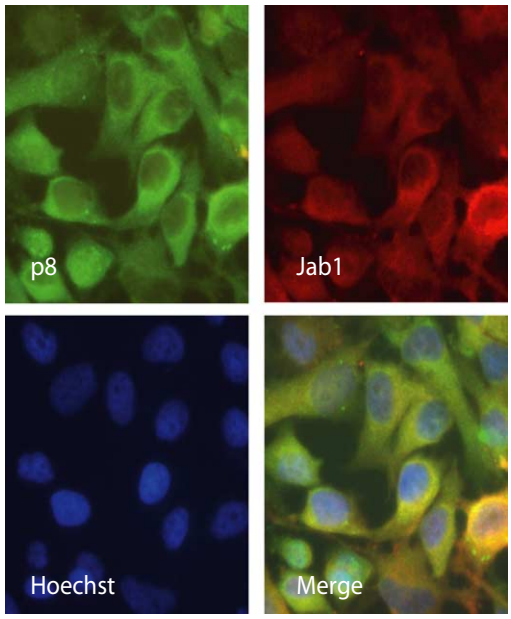

Fig. 2. p8 co-localizes with Jab1. 293T cells were transfected with pcDNA3p8 and pSG5-Jab1 expression plasmids. p8 (green) and Jab1 (red) were detected by indirect immunofluorescence using polyclonal anti-ERKLVTKLQNSERKKRGARR p8 epitope and monoclonal anti-Jab1 antibodies. Nuclei were stained with Hoechst 33258. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

in vivo, we prepared total lysates from 293T cells co-transfected with pcDNA3p8 and pSG5-Jab1 and putative p8 + Jab1 complexes were immunoprecipitated with an anti-p8 antibody attached to protein A–Sepharose beads. The presence of Jab1 in the complex was detected with an anti-Jab1 antibody as shown in Fig. 1. By contrast, irrelevant antibody (rabbit polyclonal antibody against rat pancreatitis associated protein) or protein A–Sepharose beads alone failed to precipitate Jab1, indicating that p8 + Jab1 interaction was specific.

Co-localization of p8 and Jab1 in vivo

We next examined whether the association of p8 with Jab1 observed in vitro would be confirmed by evidence of co-localization in vivo. We used double immunofluorescence staining of 293T cells transfected with p8 and Jab1 expression plasmids. As shown in Fig. 2, p8 and Jab1 were present throughout the cytosol of 293T cells but predominantly in the perinuclear space. p8 and Jab1 proteins were also found in nuclei but to a lesser extent.

p8 enhances the Jab1 activity on p27

Since Jab1 is known to be involved in the translocation of p27 to the cytoplasm and its eventual degradation by the 26S proteasome, we tested whether p8 could influence this activity. To this end, 1 μg of pSG5-Jab1 plasmid was transfected together with 0.5 μg of the p8 expression plasmid pcDNA3p8 in the presence or not of the p27 expression plasmid (0.5 μg). Endogenous and transfected p27 were then assessed by Western blot in nuclear and cytoplasmic extracts. Fig. 3 shows that as previously reported, over-expression of Jab1 decreased p27 expression. Unexpectedly, over-expression of p8 also decreased expression of endogenous as well as transfected p27 proteins. More importantly, when Jab1 was transfected together with p8, p27 remained almost undetectable in the nucleus and gave a very faint signal in the cytoplasm. Experiments were repeated three times with similar results.

Silencing p8 increases p27 protein amounts

We intended to knock down p8 by using specific siRNA in HeLa cells. After transfecting increasing amounts of p8 siRNA, expression of p27 was measured by Western blotting. As shown in Fig. 4, p27 levels increased proportionally to the p8 siRNA amounts utilized. These results strongly support a role of p8 in p27 degradation.

p8 is necessary for Jab1-dependent p27 nuclear to cytoplasm translocation

Since Jab1 expression induces p27 translocation to the cytoplasm, we studied the role of p8 in this process by immunocytochemistry. Fig. 5 shows that as previously reported p27 is located into the nucleus of HeLa and

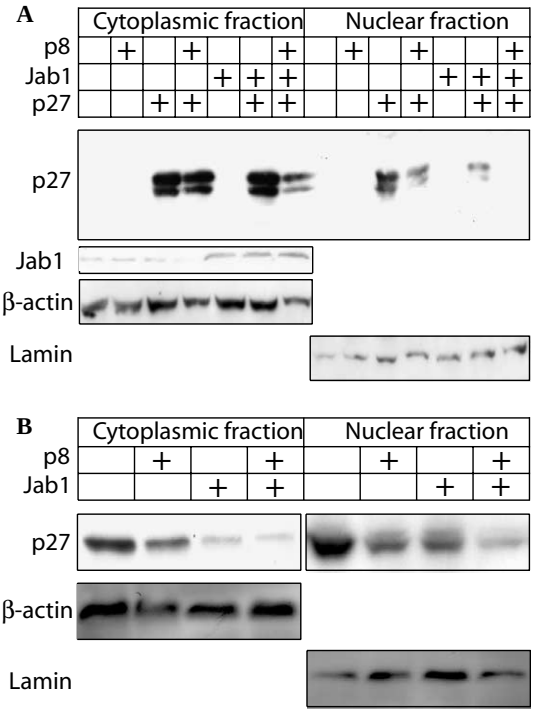

Fig. 3. p8 enhances the Jab1 activity on degradation of transfected and endogenous p27. (A) HeLa cells were transfected with combinations of pcDNA3p8, pSG5-Jab1, and pCMV-p27 tag HA expression plasmids and 24 h later cell lysates were separated into nuclear and cytoplasmic fractions and immunoblotted with anti-HA monoclonal antibody. The doublet obtained for p27 is probably due to phosphorylation [24]. (B) HeLa cells were transfected with combinations of p8 and Jab1 constructs and endogenous p27 level was measured in nuclear and cytoplasmic fractions with an anti-p27 rabbit polyclonal antibody. β-Actin and lamin were used as control.

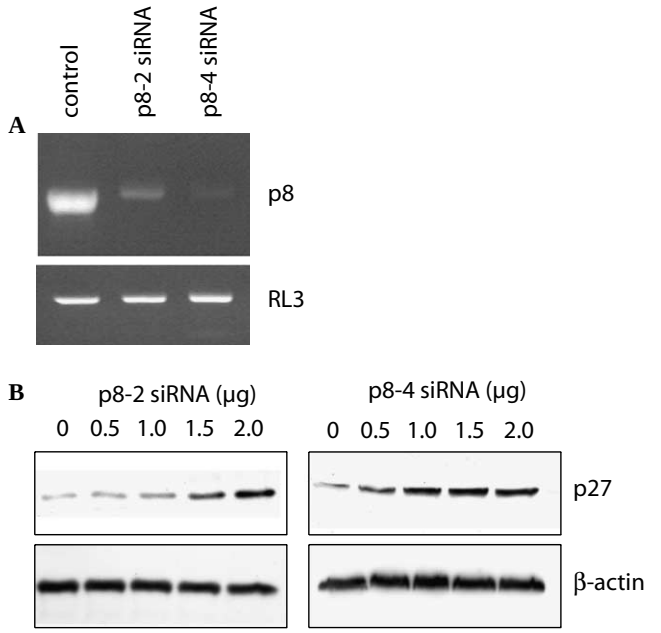

Fig. 4. siRNA-mediated inhibition of p8 increases p27 expression. (A) p8-2 and p8-4 siRNA efficiently block expression of p8 mRNA. HeLa cells were seeded at 30–50% density the day before transfection. Transfection of p8-2, p8-4 or control siRNA was performed using the Oligofectamine reagent. Twenty-four hours later, RNA was purified and p8 mRNA was measured by RT-PCR as described under Materials and methods. (B) p8 siRNA increases p27 expression. HeLa cells were transfected with increasing amounts of p8-2 or p8-4 siRNA and p27 expression was monitored by Western blot using an anti-p27 rabbit polyclonal antibody as described under Materials and methods. β-Actin was used as control.

translocated to the cytoplasm upon Jab1 over-expression [20]. Over-expression of p8 induced a further increase in p27 translocation to the cytoplasm (Fig. 5). However, unexpectedly, when Jab1 was over-expressed but p8 expression was concomitantly knocked down by siRNA, p27 was unable to translocate to the cytoplasm. Altogether, these results confirm that a minimal level of p8 expression is required to allow induction of p27 translocation by Jab1 over-expression.

Increasing expression of p8 does not modify Jab1 expression

Alteration by p8 of Jab1 activity might result in part from regulation by p8 of Jab1 gene expression. To check that possibility, HeLa cells were transfected with increasing amounts of pcDNA3p8 (0.25, 0.5, 1.0, and 2.0 μg) and Jab1 mRNA expression was monitored. We found that Jab1 mRNA concentration did not change significantly as judged by semi-quantitative RT-PCR analysis as shown in Fig. 6. Then, we evaluated the Jab1 protein content by Western blot analysis of total extract from cells transfected with increasing amounts of pcDNA3p8. Fig. 6 shows that the intracellular levels of Jab1 protein were not altered by the forced expression of p8.

Discussion

Strongly over-expressed shortly after triggering a stress, p8 appears as one of the key regulators of the cell stress response. It may be surprising that a protein rather small in size (~8 kDa), with no stable tri-dimensional structure [18] is involved in so many regulatory pathways. One possible explanation is that p8 takes advantage of the plasticity of its structure to engage into associations with specific molecular partners to exert its various functions. The resulting protein complex could acquire new functions through p8 binding or, alternatively, complex formation could alter the function of one of the partners. We used a two-hybrid screening to identify partners of p8, with the hope of finding proteins actually involved in known regulatory pathways. The screening revealed that Jab1 interacted with p8, which was confirmed by His_6-pull down and co-immunoprecipitation assays (Fig. 1). Jab1 turned out to be a quite interesting partner. This multifunctional protein was first identified by yeast two-hybrid screen through its ability to associate with the N-terminal c-Jun activation domain, the interaction potentiating c-Jun transactivation activity [21]. Jab1 was also shown to interact with p27 in the nucleus, then evoke its translocation to the cytoplasm and facilitate its degradation by the

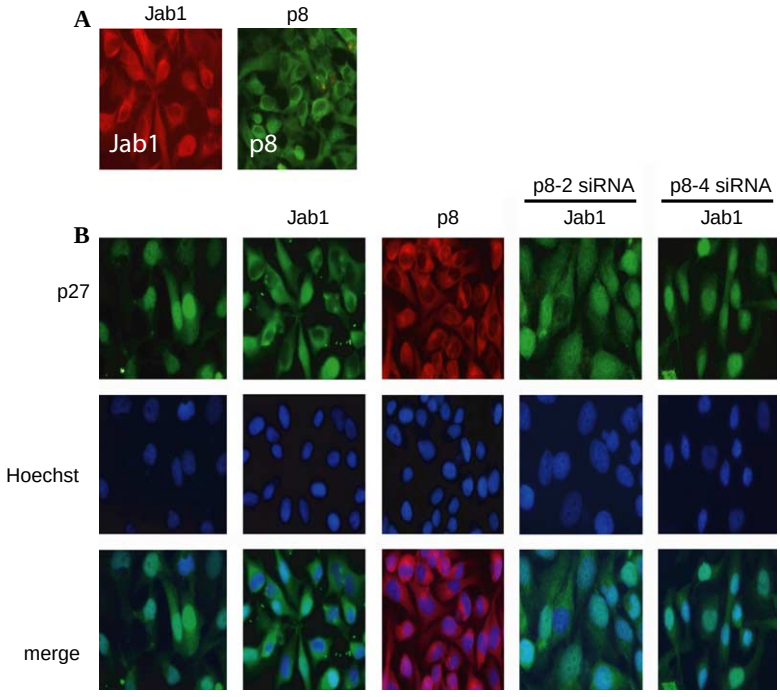

Fig. 5. p8 is required for Jab1-dependent nuclear to cytoplasmic translocation of p27. Hela cells were transfected with pcDNA3p8 and pSG5-Jab1 expression plasmids, alone or together with p8-2 or p8-4 siRNA. p8 and Jab1 (A) and p27 (B) were detected by indirect immunofluorescence using polyclonal anti-ERKLVTKLQNSERKKRGARR p8 epitope, monoclonal anti-Jab1 antibody, and the polyclonal antibody anti-p27 (C-19), respectively. Nuclei were stained with Hoechst 33258.

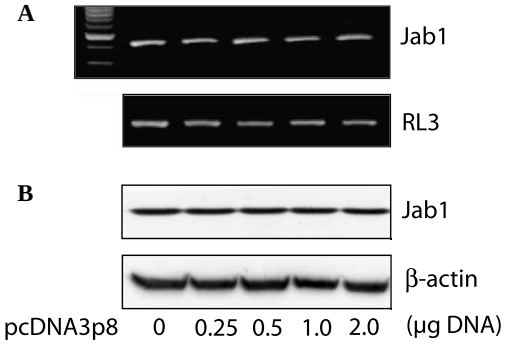

Fig. 6. p8 does not influence Jab1 expression. HeLa cells were transfected with increasing amounts of pcDNA3p8 and Jab1 expression was studied by semi-quantitative RT-PCR analysis (A) or Western blot (B) as described under Materials and methods. RL3 mRNA and β-actin were used as controls.

26S proteasome [20]. Jab1 was also identified as part of a large multiprotein complex named signalosome [22]. Finding so many different functions linked to a single protein strongly suggests that each function results from the binding of that protein with a specific set of interactors. Knowing that p8 was one of them, we started investigating the functional consequences of its binding to Jab1 on the activities listed above.

If the activity and intracellular level of the Cdk inhibitor p27 are regulated through several mechanisms [23], Jab1 controls the most important one by triggering p27 exportation to the cytoplasm and subsequent ubiquitin-mediated degradation by the 26S proteasome [22]. Jab1-mediated exportation of p27 to the cytoplasm can be modulated by p8. In fact, we observed that p8 binding enhanced Jab1 activity on p27 degradation. Apparently, p8 binding increases the specific activity of Jab1 because increased activity after p8 forced expression was not associated with increased Jab1 mRNA expression or intracellular protein level (Fig. 6). Interestingly p8 over-expression also induced p27 degradation in the absence of transfected Jab1, as evidenced by a drastic decrease of p27 in the nuclear fraction (Fig. 3). These data suggest that endogenous Jab1 expression is sufficient to accommodate additional p8 expression and, consequently, that p8 over-expression following a stress will result in the promotion of cell growth through increased p27 degradation. Endogenous p8 expression also displays significant activity as p27 level increases upon p8 knock down by specific siRNAs (Fig. 4), Jab1 expression remaining unaltered (data not shown). However, neither the constitutive expression of Jab1 nor that of p8 allows maximal activity since over-expression of one of them decreases significantly p27 intranuclear level, whereas over-expressing both leads to almost complete depletion (Fig. 3). The mechanism by which p8 regulates Jab1 activity was analyzed by immunocytochemistry (Fig. 5). We confirmed that Jab1 over-expression induces the nuclear to cytoplasm translocation of p27 but, to our surprise, treating cells with a p8 siRNA blocked that effect almost completely, indicating that Jab1 inhibition of p27 activity is p8-dependent.

Acknowledgments

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