Association of the mammalian proto-oncoprotein Int-6 with the three protein complexes eIF3, COP9 signalosome and 26S proteasome

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Abstract The mammalian Int-6 protein has been characterized as a subunit of the eIF3 translation initiation factor and also as a transforming protein when its C-terminal part is deleted. It includes a protein domain, which also exists in various subunits of eIF3, of the 26S proteasome and of the COP9 signalosome (CSN). By performing a two-hybrid screen with Int-6 as bait, we have isolated subunits belonging to all three complexes, namely eIF3-p110, Rpt4, CSN3 and CSN6. The results of transient expression experiments in COS7 cells confirmed the interaction of Int-6 with Rpt4, CSN3 and CSN6, but also showed that Int-6 is able to bind another subunit of the CSN: CSN7a. Immunoprecipitation experiments performed with the endogenous proteins showed that Int-6 binds the entire CSN, but in low amount, and also that Int-6 is associated with the 26S proteasome. Taken together these results show that the Int-6 protein can bind the three complexes with various efficiencies, possibly exerting a regulatory activity in both protein translation and degradation.

Key words: Int-6; COP9 signalosome; 26S Proteasome; eIF3; Two-hybrid

1. Introduction

The mouse *int-6* gene was originally characterized as a common site of integration of the mouse mammary tumor virus (MMTV) in preneoplastic and neoplastic mammary lesions [1]. The human cDNA encoding the 52 kDa Int-6 protein was identified independently from the results of a two-hybrid screen performed with the human T-cell leukemia virus type 1 transforming protein Tax as bait, as well as from the sequence of peptides of a subunit of the purified eukaryotic translation initiation factor eIF3 [2,3]. In mouse, insertion of MMTV occurred approximately in the middle of the *int-6* gene, putatively leading to expression of C-terminally truncated forms of the protein. It has been shown recently that a shortened form of Int-6 can transform NIH3T3 cells [4], as well as mouse and mammary epithelial cell lines, injection of such transformed cells in nude mice leading to growth of mammary tumors [5]. Hence, these results support the notion that transformation by MMTV results from alteration of the Int-6 protein that gains transforming properties and not from a reduction in the amount of the protein as a consequence of the loss of an allele. The Int-6 protein is well conserved among eukaryotes, with so far the only exception of the yeast *Saccharomyces cerevisiae*. In this organism Pci8p has been identified as a homologue of human Int-6 [6], but it is much more distantly related as compared with *Schizosaccharomyces pombe* Int-6 (36.8% identity between human and *S. pombe* Int-6s, 12.4% identity between Pci8p and human Int-6). In *S. pombe*, *int-6* has been shown to be a non-essential gene, but to contribute to several aspects of cell physiology, including growth in minimal medium, resistance to various drugs via activation of several Pap-1-regulated genes, along with microtubule network functioning at mitosis [7–10]. This latter aspect also involves Moe-1, the *S. pombe* homologue of eIF3-p66, and both proteins act in a Ras-1 pathway [9]. Our previous immunofluorescence observations of the endogenous protein have shown that Int-6 is present within both nucleus and cytoplasm of primary blood lymphocytes, as well as in different cell lines [11]. Guo et al. [12] have shown that Int-6 includes both a nuclear export signal (NES), corresponding to the N-terminal end, and a nuclear localization signal (NLS). This is in agreement with the presence of the protein in both cellular compartments and suggests that Int-6 can travel between both of them. Another remarkable protein domain of Int-6 is the proteasome–COP9 signalosome–initiation factor 3 (PCI) of translation domain that is present in two other subunits of eIF3, as well as in a cellular protein, HSPC021, that is associated with this translation initiation factor [13,14]. It also exists in five subunits of the lid of the 26S proteasome and in six subunits of the constitutive photomorphogenesis 9 (COP9) signalosome (CSN). Another protein domain is similarly shared by subunits of these three protein complexes: the Mpr1–Pad1–N-terminal domain (MPN) that exists in two subunits of each of them. These PCI and MPN domains are thought to play an important role in the complex assembly, but also to allow interactions with proteins that transiently associate with it. Interestingly, the homology between some subunits of eIF3, CSN and 26S proteasome extends beyond these domains. So far it is not clearly known whether these homologies underlie some common functional properties. eIF3 is an important translation initiation factor that interacts with the mRNA, the 40S ribosome subunit, as well as with

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Abbreviations: eIF, eukaryotic translation initiation factor; CSN, COP9 (constitutive photomorphogenesis 9) signalosome; MMTV, mouse mammary tumor virus; PCI, proteasome–COP9 signalosome–initiation factor 3; MPN, Mpr1–Pad1–N-terminal domain; SCF, Skip–Cullin–F box; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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other eIFs [15,16]. The 26S proteasome degrades poly-ubiquitinylated cellular proteins [17]. CSN exhibits a kinase activity and is involved in regulation of the degradation of cellular proteins [18]. Recently, it has been shown that it interacts with the Skip–Cullin–F box (SCF) complexes and promotes denedyllation of cullins [19,20]. Alteration of CSN subunit expression causes accumulation of both neddylated proteins and poly-ubiquitinylated forms of cellular proteins [19,21,22].

It has been shown previously that Int-6 interacts with different proteins that are either subunits of eIF3 or tightly associated with this complex: eIF3-p110, eIF3-p66/Moe-1 and HSPC021 [9,11,14,23]. It has been observed that the *Arabidopsis thaliana* homologues of both Int-6 and eIF3-p110 associate with the CSN [23,24]. In this report we present evidence showing that human Int-6 also binds to three CSN subunits, as well as to a subunit of the 26S proteasome. It is also shown by immunoprecipitation experiments that association of Int-6 with these two protein complexes naturally occurs within cells. This suggests that Int-6 might regulate in a coordinate manner the activity of all three complexes.

2. Materials and methods

2.1. Two-hybrid screening

The two-hybrid screen of a cDNA library of human lymphocytes immortalized by Epstein–Barr virus [25] with Int-6 fused to the DNA binding domain of GAL4 was performed as previously described [11]. The cDNA clones encoding CSN3, CSN6 and Rpt4 were isolated and characterized by sequencing.

2.2. Cell culture and transfection

Jurkat cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in a 5% CO_2-humidified atmosphere. COS7 cells were cultured under the same conditions, except that the medium was Dulbecco's modified Eagle's medium. COS7 cells were transfected by the calcium phosphate precipitation method with 3 μg of each expression plasmid in 100 mm diameter Petri dishes.

2.3. Constructs

Plasmids pSGF-CSN3, pSGF-CSN6 and pSGF-Rpt4 were generated by inserting the *Xho*I restriction fragments of clones THI 45, THI 78 and THI 8, respectively, in the *Xho*I restriction site of pSGF [2]. The eight subunits of CSN were cloned in the vector pSGM, which is a derivative of pSG5 including the sequence encoding the Myc epitope. The sequences encoding CSN1, CNS2, CSN3 and CSN5 were obtained by polymerase chain reaction (PCR) amplification from expressed sequence tags (ESTs) (obtained from the MRC UK HGMP Resource Centre) using primers that create *Xho*I and *Bgl*II restriction sites. The EST image numbers and primer sequences were as follows: CSN1: EST 2822616, sense primer: 5′-GGGAATTCCCTCGAGGGCGGGGTGGGTGCAAGATG, antisense primer: 5′-AAGGTAGATCTCTCACATGTTGGTGCTCATCC; CSN2: EST 4278113, sense primer: 5′-CCAGGATCCCCTCGAGTGGAGGATGATTTCATGTGCG, antisense primer: 5′-CTTGAGATCTGTTAAGCCAGTTTACTGAC; CSN3: EST 4403030, sense primer: 5′-GGGGAATTCCCTCGAGTTTGTGAACTGATCAACAAG, antisense primer: 5′-GGATGTAGATCTCAAGAATAACTGGATGG; CSN5: EST 4054896, sense primer: 5′-GCTGAATTCGCTCGAGCGGCGTCCGGGAGCGG, antisense primer: 5′-CTTCTCAGATCTTGTTTAAGAGATGTTAATTTG. The amplified sequences were subcloned into the pCRScript vector (Stratagene), digested with *Xho*I and *Bgl*II restriction enzymes, and inserted between the same sites in pSGM. The CSN4 and CSN6 cDNAs were obtained by PCR amplification from EST 4339678 and from clone THI78, respectively, using primers creating *Xho*I and *Sal*I restriction sites. The sequences of primers were as follows: CSN4: sense primer: 5′-GGCGGTCGACCCTCGAGATTTGGCCCAGCTCATG, antisense primer: 5′-CTGCGTCGACTCACTGAGCCATCTGGG; CSN6: sense primer: 5′-GAGGGGACTCGAGCGGCGGCGG, antisense primer: 5′-CCTTCAAGTCGACTCATCAGAAAAAGAGCCCT. The amplified fragments were digested with *Xho*I and *Sal*I restriction enzymes and cloned into the *Xho*I site of pSGM. The CSN7a and CSN8 cDNAs were generated by PCR amplification from ESTs 4362956 and 3833419, respectively, using primers that create *Bam*HI and *Bgl*II sites. The sequences of primers were as follows: CSN7a: sense primer: 5′-ACAGAATTCGCGGATCCGAGTGCGGAAGTGAAGGTG, antisense primer 5′-CGACAGATCTTTCAATTCGACTTGGACC, CSN8: sense primer 5′-GGCCGGGATCCAAAGATCTAGTGGCGGTGATGGCGG, antisense primer 5′-CTCAGAGGGATCCATCAGTTTTCAAGGAAAGCCAC. The amplified sequences were subcloned into the pCRScript vector, digested with *Bam*HI and *Bgl*II enzymes, and inserted into the *Bgl*II site in pSGM.

2.4. Immunoprecipitation and immunoblot

Transfected COS7 cells were lysed in RIPA buffer (600 μl) for 20 min. Lysates were centrifuged for 10 min at 12 000 rpm and supernatants were used for immunoprecipitation. They were first cleared by a 1 h incubation with protein A beads equilibrated in RIPA buffer. The beads were spun down by centrifugation for 2 min at 2000 rpm and supernatants were then incubated with antibodies. Half of each supernatant was incubated with the antibody to Int-6, and the other half with either preimmune serum or a control antibody. Antibodies were diluted 1:200. After 2 h, protein A beads were added to the mixes which were further incubated for an additional hour. Protein A beads were collected and washed three times in RIPA buffer. All incubations were done at 4°C. Proteins were eluted in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer at 80°C for 10 min.

For endogenous proteins, Jurkat cells were either lysed in RIPA buffer or resuspended in buffer A (50 mM Tris, 5 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ATP, 1 mM dithiothreitol (DTT), 10% glycerol) as indicated. To test the interaction between Int-6 and CSN5, 13×10^6 cells were resuspended in 1 ml of buffer A. To test the interaction of Int-6 with Rpt4 and HC3, 25×10^6 and 50×10^6 cells respectively were resuspended in 1 ml of buffer A or RIPA buffer. In the case of buffer A, cells were lysed by two cycles of freezing/thawing in liquid nitrogen. After centrifugation for 10 min at 12 000 rpm, immunoprecipitation was performed as described above except that the protein A beads were equilibrated in buffer A and that they were then washed in 20 mM Tris, 20 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM ATP, 0.1% SDS, 0.5% Nonidet P-40, 17% glycerol. Proteins were separated by SDS–PAGE, and transferred to polyvinylidene difluoride membrane. For immunoblot, antibodies to either FLAG, HC3 or Rpt4 were used diluted 1:500, antibody to CSN5 diluted 1:1000, and antibody to MYC diluted 1:200. Secondary antibodies were revealed by chemiluminescence using the ECL reagent (Amersham Pharmacia Biotech). 10 μl of lysates were used in the various gels as control for the position of the various proteins.

2.5. Gel filtration column

Jurkat cells (100×10^6) were lysed by freeze-thaw in 400 μl of buffer L (50 mM HEPES pH 8, 300 mM NaCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 10% glycerol, protease inhibitor cocktail (complete EDTA-free, Roche)). Lysate was cleared by centrifugation and subjected to nuclease digestion for 90 min (benzonase, Sigma). The whole cell extract was loaded at a flow rate of 0.3 ml/min on a Superpose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris–HCl pH 7.4, 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT. Fractions of 0.6 ml each were collected and concentrated with 20 μl StrataClean resin (Stratagene). Gels for immunoblots were done with one third of each fraction.

3. Results

3.1. Binding of Int-6 to subunits of the CSN and of the 26S proteasome

Int-6 has been shown to be present in purified human eIF3 [3]. In a previous publication we reported on the results of a two-hybrid screen performed with Int-6 as bait and eIF3-p110 was one of the cDNAs corresponding to several clones identified by this method [3]. This screen of a library of cDNA

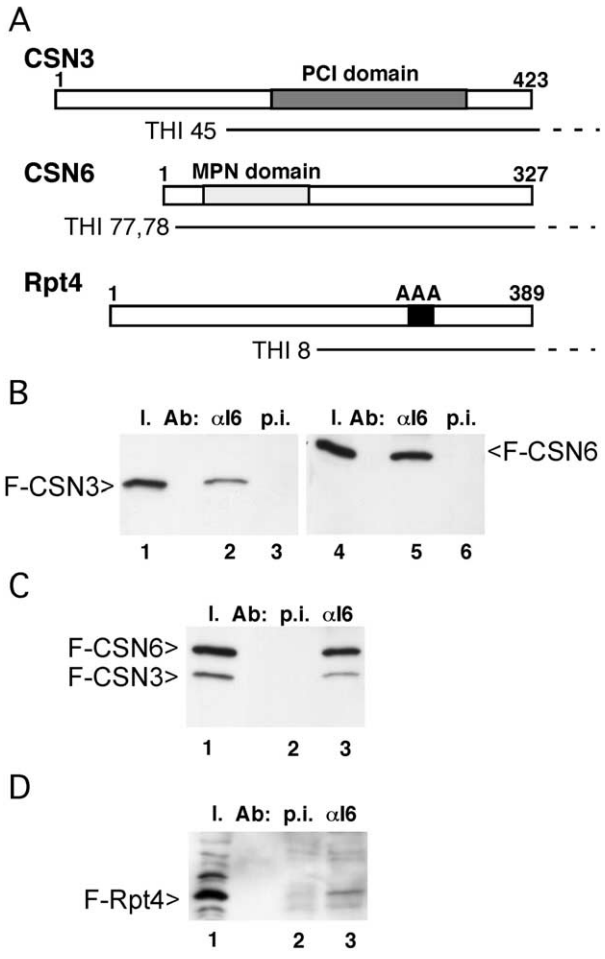

Fig. 1. A: Schematic representation of the clones encoding CSN3, CSN6 and Rpt4 isolated by two-hybrid screen with Int-6 as bait. The PCI domain (dark gray box), MPN domain (light gray box) and AAA domain (black box) are indicated. THI 45 includes the sequence encoding CSN3 between amino acids 155 and 423. THI 77 and THI 78 are identical clones including the sequence encoding CSN6 between amino acids 5 and 327. THI 8 includes the sequence encoding Rpt4 between amino acids 184 and 389. B–D: Interaction between the products of the isolated clones and Int-6 in mammalian cells. COS7 cells were co-transfected with vectors expressing Int-6 (all lanes) and either FLAG-tagged CSN3-155/423 (B, lanes 1–3; C, lanes 1–3), FLAG-tagged clone CSN6-5/327 (B, lanes 4–6; C, lanes 1–3) or FLAG-tagged Rpt4-184/389 (D, lanes 1–3). An aliquot of the lysates of the corresponding transfected cells was loaded in lanes 1 and 4. Immunoprecipitations were performed with either the antibody to Int-6 (B, lanes 2, 5; C and D, lane 3) or with the rabbit preimmune serum (B, lanes 3 and 6; C and D, lane 2). Immunoblots were performed with the M2 monoclonal antibody to FLAG.

from human B lymphocytes also led to identification of several clones corresponding to two CSN subunits: CSN3 and CSN6, as well as to one subunit of the 26S proteasome: Rpt4 (Fig. 1A). CSN3 contains a PCI domain between positions 175 and 342. The clone obtained was partial, but included the entirety of this protein domain (Fig. 1A). CSN6, like CSN5/Jab1, contains a MPN domain in the N-terminal region. The isolated clones comprise almost the complete coding sequence, including the MPN domain (Fig. 1A). The subunit of the 26S proteasome obtained in this screen was Rpt4, which is one of the six ATPases associated with various cellular activities (AAA) constituting the base of the 19S proteasome that associates with the core 20S proteasome [26]. This subunit is not one of the 12 proteins constituting the lid of the 19S regulatory particle, of which some are homologous to subunits of both eIF3 and CSN. The clone corresponding to Rpt4 included approximately the C-terminal moiety of the protein (Fig. 1A).

It was further verified whether these interactions also occurred in the context of mammalian cells. The various cDNAs were inserted in the pSGF expression vector in frame with the sequence encoding the FLAG epitope. By transfection of COS7 cells, the resulting fusion proteins were co-expressed with Int-6. Immunoprecipitation experiments, performed using RIPA buffer along with a rabbit polyclonal antibody directed against the C-terminal 20 amino acids of Int-6, clearly showed that Int-6 binds to these FLAG-tagged forms of CSN3 (Fig. 1B, lane 2) as well as of CSN6 (Fig. 1B, lane 5). This experiment was also performed by co-expressing both CSN subunits. Int-6 precipitated both proteins, the ratio between them being similar to that observed in the extract (Fig. 1C, compare lanes 3 and 1). This indicated that the affinities of Int-6 for both proteins were comparable. Int-6 was also able to precipitate the FLAG-tagged Rpt4 (Fig. 1D, lane 3). These results show that Int-6 can bind to these CSN and 26S proteasome subunits in mammalian cells and raise the interesting possibility that Int-6 physiologically associates with these two protein complexes. However, at least in the case of the CSN, the similarity existing between CSN3 and CSN6 and subunits of eIF3 raised the possibility that this binding was due to abnormally elevated concentrations obtained in these transient expression experiments. Another concern was also to determine whether Int-6 was able to interact with these subunits in the context of the whole complex. Hence, we analyzed further the interaction of Int-6 with the endogenous CSN and 26S proteasome complexes.

3.2. Interaction of Int-6 with the CSN

The CSN is composed of eight subunits, six of them including a PCI domain (CSN1, 2, 3, 4, 7 and 8) and two a MPN domain (CSN5 and 6). To determine whether the interaction of Int-6 with CSN3 and CSN6 was specific or not, the eight subunits were systematically tested for binding to Int-6. The coding sequence of the eight subunits was amplified from ESTs and inserted in the pSGM expression vector, in frame with the sequence encoding the Myc epitope. This was also done for CSN3 and CSN6 to test complete forms of the proteins. These expression vectors together with that expressing Int-6 were co-transfected in COS7 cells. Immunoprecipitation experiments using the antibody directed against Int-6, followed by immunoblot with the anti-Myc antibody, showed no binding of this protein to CSN2, CSN5, and CSN4 (Fig. 2A, lanes 6, 8 and 11). A weak binding was observed with CSN1 and CSN8 (Fig. 2A, lanes 4 and 14). Finally, a clear binding was observed with CSN3 and CSN6, but also with CSN7a (Fig. 2A, lanes 18, 23 and 20), in agreement with previous results obtained with the *A. thaliana* homologues of these proteins. These results showed that binding of Int-6 to both CSN3 and CSN6 was not dependent on the nature of the tagging epitope.

To examine the association of Int-6 with the entire COP9 signalosome, the endogenous Int-6 present in an extract of Jurkat cells prepared in RIPA buffer was immunoprecipitated using the C-20 antibody and the precipitated proteins were analyzed by immunoblot with an antibody directed against

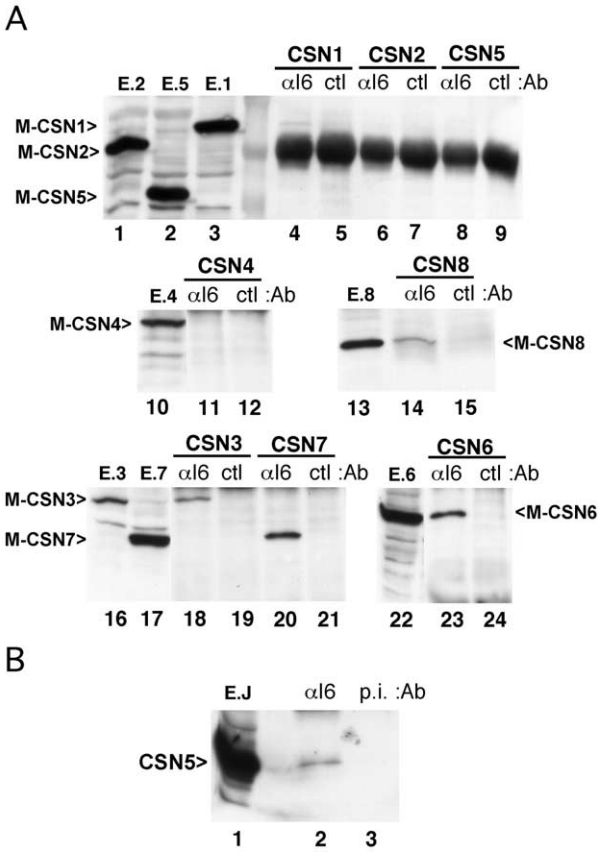

Fig. 2. A: Interaction between Int-6 and the various CSN subunits. COS7 cells were transfected with a vector expressing Int-6 (all lanes), together with a vector expressing one of the eight MYC-tagged CSN subunit: pSGM-CSN1, which encodes amino acids 13–500 of CSN1 (lanes 3–5); pSGM-CSN2, which encodes amino acids 5–443 of CSN2 (lanes 1, 6 and 7); pSGM-CSN3, which encodes amino acids 25–423 of CSN3 (lanes 16, 18 and 19); pSGM-CSN4, which encodes amino acids 8–406 of CSN4 (lanes 10–12); pSGM-CSN5, which encodes amino acids 4–334 of CSN5 (lanes 2, 8 and 9); pSGM-CSN6, which encodes amino acids 2–327 of CSN6 (lanes 22–24); pSGM-CSN7a, which encodes amino acids 2–275 of CSN7a (lanes 17, 20 and 21); pSGM-CSN8, which encodes amino acids 3–209 of CSN8 (lanes 13–15). An aliquot of the extracts of the transfected cells was loaded in lanes 1–3, 10, 13, 16, 17 and 22. Immunoprecipitations were performed with the anti-Int-6 antibody (lanes 4, 6, 8, 11, 14, 1, 20 and 23) or with a control antibody raised against a peptide of the TIP-1 protein (lanes 5, 7, 9, 12, 15, 19, 21 and 24). Immunoblots were done with the 9E10 monoclonal antibody directed against the Myc epitope. B: Interaction between endogenous Int-6 and CSN5. Jurkat cell extract (lane 1) was subjected to immunoprecipitation using either the antibody directed against Int-6 (lane 2) or the preimmune serum (lane 3). Immunoblot was done with the anti-CSN5 monoclonal antibody (Abcam).

CSN5. A signal at the position of CSN5 was observed specifically (Fig. 2B, lane 2), since it was not present when preimmune serum was used in the immunoprecipitation experiment (Fig. 2B, lane 3). This observation supports the notion that endogenous Int-6 interacts with the CSN complex. However, the amount of CSN5 precipitated together with Int-6 was weak as compared to that present in the extract (Fig. 2B, compare lanes 1 and 2). Therefore, although Int-6 strongly binds to three different CSN subunits, only a small amount of the protein appears to be associated with the endogenous complex in Jurkat cells.

3.3. Interaction of Int-6 with the 26S proteasome

The 26S proteasome is composed of the core 20S proteasome, which includes the proteolytic subunits, as well as of the 19S regulatory particle, which contains the base formed of the six AAAs and of the lid. It is known that preservation of the whole structure requires specific biochemical conditions. Hence, to analyze the association of endogenous Int-6 with the 26S proteasome, the immunoprecipitation experiments were performed in a buffer satisfying these requirements (buffer A, see Section 2). As control, the experiments were also done in RIPA buffer. First, it was analyzed whether the association of Int-6 with Rpt4 was observed with the endogenous proteins. Under RIPA buffer conditions, Rpt4 was clearly co-precipitated with Int-6 (Fig. 3A, lane 1). In buffer A a signal was seen also, but of lower intensity (Fig. 3A, lane 2). When the same experiment was done with a control antibody in the immunoprecipitation reaction, no signal was detected (data not shown). It was further tested whether a subunit of the 20S proteasome was also present in the proteins immunoprecipitated with Int-6. By using a monoclonal antibody reacting against HC3 for the immunoblot analysis, a signal was observed under buffer A conditions, but not when RIPA buffer was used (Fig. 3B, lane 2 and 4). When the immunoprecipitation experiment was performed with a control antibody, no signal at the position corresponding to HC3 was detected under both buffer conditions (Fig. 3B, lanes 3 and 5). It was further evaluated whether this association of Int-6 was specific and not seen with various cellular proteins. To this end different proteins were immunoprecipitated and the pre-

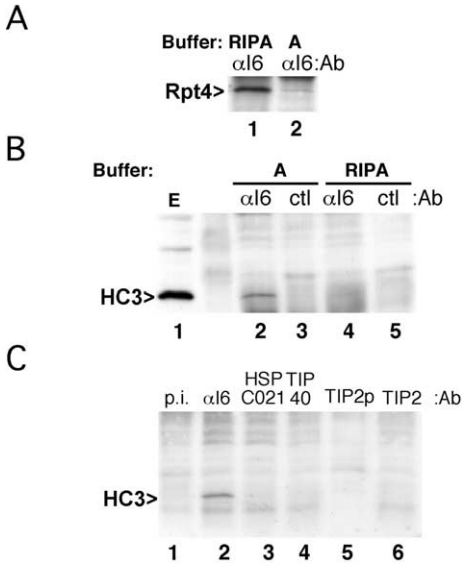

Fig. 3. A,B: Association of Int-6 with subunits of the 19S and 20S proteasome. Jurkat cells were lysed in RIPA buffer (A, lane 1; B, lanes 4 and 5) or in buffer A (A, lane 2; B, lanes 2 and 3). The corresponding lysates were used to perform immunoprecipitations using either the anti-Int-6 antibody (A, lanes 1 and 2; B, lanes 2 and 4) or the preimmune serum (B, lanes 3 and 5). For B, lane 1 was loaded with an aliquot of the extract in buffer A. Immunoblot was done with the monoclonal antibody directed against Rpt4 (Affiniti) (A), or with the MCP21 monoclonal antibody that recognizes HC3 (Affiniti) (B). C: Jurkat cells were lysed in buffer A and subjected to immunoprecipitations with various antibodies: preimmune serum (lane 1), anti-Int-6 (lane 2), anti-HSPC021 (lane 3), anti-TIP-40 (lane 4), purified anti-TIP2 (lane 5), anti-TIP2 (lane 6). The membrane was immunoblotted with the MCP21 monoclonal antibody.

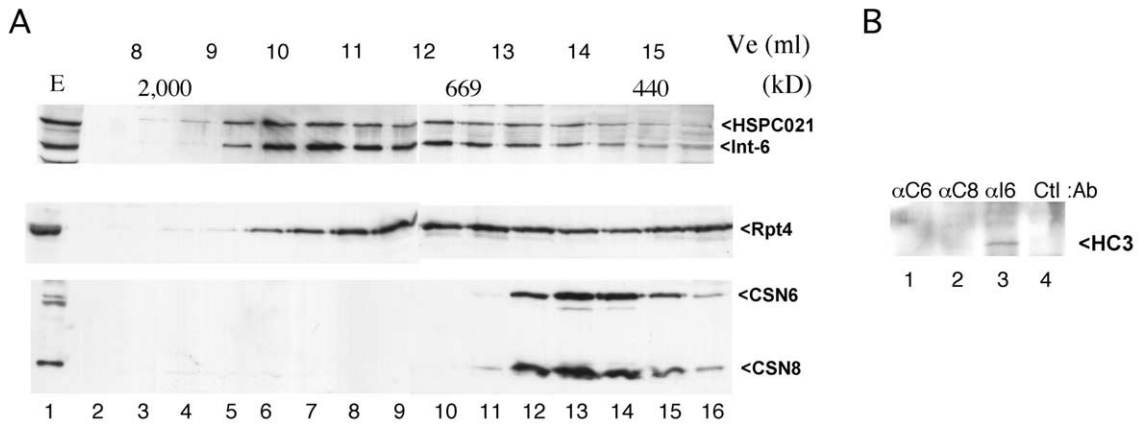

Fig. 4. A: Gel filtration analysis of Int-6 and of proteins associated with eIF3, CSN and proteasome. A whole cell extract of Jurkat cells was loaded on a Superose 6 gel filtration column. Fractions were analyzed by immunoblot with antibodies to the eIF3-associated protein HSPC021 and Int-6 (upper panel), proteasomal subunit Rpt4 (middle panel) and signalosome constitutive subunits CSN6 (Affiniti) and CSN8 (Affiniti) (lower panel). Lane 1 corresponds to the extract loaded onto the column. The elution volume corresponding to the different fractions in ml is indicated at the top of the figure. The column was calibrated with protein standards of which molecular weights is represented in kDa below the elution volume. B: Analysis of the interaction of CSN with the 20S proteasome by immunoprecipitation experiment. Jurkat cell extracts prepared in buffer A were used to perform immunoprecipitations with different antibodies: anti-CSN6 (lane 1), anti-CSN8 (lane 2), anti-Int-6 (lane 3) and control antibody (lane 4). Immunoblot was done with the MCP21 monoclonal antibody to HC3.

cipitates analyzed with the antibody directed against HC3. All the antibodies used in this experiment were rabbit polyclonal antibodies raised against a peptide coupled to a carrier. Only the antibody directed against Int-6 was able to precipitate HC3 (Fig. 3C, lane 2). These observations showed that endogenous Int-6 forms a complex with subunits of both the 19S and 20S proteasome, strongly supporting the notion that Int-6 associates with the complete 26S proteasome.

3.4. Distribution of Int-6 among the three protein complexes

To evaluate how Int-6 was distributed among the three protein complexes, i.e. eIF3, CSN and 26S proteasome, a cellular extract prepared in buffer L was fractionated through a gel filtration column. The fractions were analyzed by immunoblot using antibodies directed against Int-6, HSPC021, Rpt4, CSN6 and CSN8. As reported before [14], Int-6 was present in several fractions ranging from 1.5×10^3 kDa to less than 400 kDa (Fig. 4A, upper panel). Other eIF3 subunits have been observed previously to coelute with Int-6, as well as with HSPC021, which is also associated with eIF3 [14]. Rpt4 also was observed to be present in a large set of fractions (Fig. 4A, middle panel). This observation did not allow us to evaluate the relative amount of Int-6 present in both eIF3 and 26S proteasome complexes, as they are present in common fractions. CSN6 and CSN8 were observed to coelute in fractions of lower molecular weights at 400–600 kDa (Fig. 4A, lower panel). Int-6 was also present in these fractions, but in relatively low amounts as compared with the fractions which also contained eIF3 and the 26S proteasome. Immunoprecipitation of Int-6 present in eluates containing the CSN showed that it was associated with a fraction of CSN5 (data not shown). In agreement with the results obtained with the cellular extracts, these data indicated that only a limited amount of Int-6 was associated with the CSN. It is noteworthy that in the high molecular weight fractions, the presence of both proteasome and signalosome subunits was not observed. Indeed from the similarity between the lid of the proteasome and CSN, it was proposed that this latter complex might associate with the proteasome [27]. The results obtained by fractionation through this gel filtration column do not support this hypothesis and show that these three complexes are apparently independent. To further test this point, it was analyzed whether precipitation of subunits of the CSN was able to pull down a 20S core proteasome subunit. Whereas Int-6 clearly co-precipitated with HC3 (Fig. 4, lane 3) as already shown, this 20S proteasome subunit was not co-precipitated with either CSN6 or CSN8 (Fig. 4B, lanes 1 and 2). It was observed in the same experiment that the precipitation of these two CSN subunits was efficient and also that another subunit, CSN5, was strongly co-precipitated with both CSN6 and CSN8 (data not shown). Taken together, these data do not support, at least in the case of Jurkat cells, a model where CSN binds to the 20S proteasome, either by substituting the lid of the 19S regulatory particle or by interacting with it.

4. Discussion

Previous studies have characterized the association of Int-6 with the translation initiation factor eIF3 in several organisms: mammalian, *A. thaliana* and *S. pombe* [3,7,8,10,23]. The results presented in this report show that human Int-6 is also associated with the CSN and 26S proteasome complexes. For this latter protein complex it has been observed that Int-6 binds to the Rpt4 protein that is one of the six AAAs forming the base of the 19S regulatory particle. These ATPases are thought to play an important role by unfolding the protein before degradation by the 20S core proteasome [28]. Recent data have also shown that Rpt4 plays a role in the association of the 19S regulatory particle with the 20S core [29]. In agreement with this aspect a systematic study of the interactions of the *Caenorhabditis elegans* proteasome subunits has shown that Rpt4 binds to subunits α2 and α7 [30]. It will be interesting to determine whether the association of Int-6 with Rpt4 merely allows binding of the former protein to the proteasome or has some functional effect on the activity of this proteolytic complex.

In the case of the CSN it has been observed that Int-6 binds to three different subunits: CSN3, CSN6 and CSN7a. In the

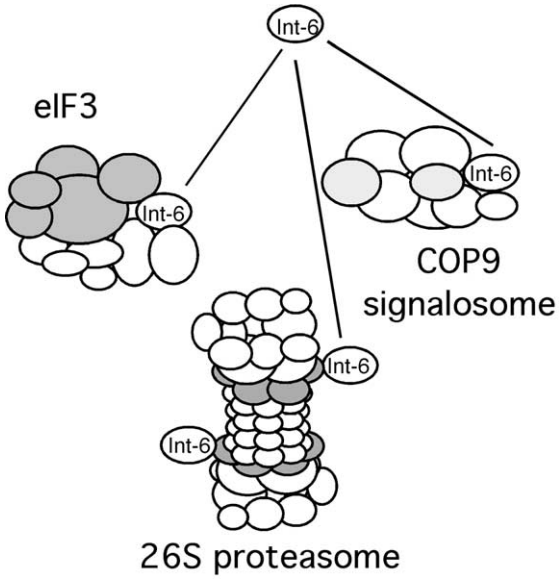

Fig. 5. Schematic representation of the association of Int-6 with the three protein complexes eIF3, CSN and 26S proteasome. The translation initiation factor eIF3 consists of a core of five subunits (represented in gray) and additional non-core subunits. The COP9 signalosome contains eight subunits, six of which possess the PCI domain, and two subunits that include the MPN domain (represented in light gray). The 26S proteasome is composed of the 20S proteolytic core particle, as well as of two 19S regulatory particles at both ends. The 19S complex consists of the base (represented in gray) and of the lid of which subunits share similarities with the eIF3 and CSN subunits. Int-6, which is one of the non-core subunits of eIF3, can also associate with Rpt4 of the base of the 26S proteasome, along with subunits 3, 6 and 7 of the CSN.

A. thaliana model it has previously been shown that Int-6 binds to CSN7a and copurifies with CSN. In this organism the association of Int-6 with the signalosome was observed in cells of the root, but not in the cells of the leaf, Int-6 in this tissue being mainly associated with eIF3 [23]. In human cells also, our data support an interaction of Int-6 with the CSN, but such a complex appears to exist in a low amount. By analogy with what has been observed in *A. thaliana*, it is possible that this interaction depends on the cell type. The role of the association of Int-6 with the CSN is unclear, but on the model of CSN5 that like Int-6 includes both a NES and a NLS, it is tempting to speculate that Int-6 might mediate association of specific proteins with this complex. This might cause their phosphorylation by the CSN-associated kinase activity. It might also favor their ubiquitinylation by SCF complexes associated with the COP9 signalosome. In this regard it is interesting to recall the interaction of Int-6 with the ret finger protein that might be part of an E3 ubiquitin ligase complex [11], like many of the known ring finger proteins [31].

The association of Int-6 with the three protein complexes eIF3, CSN and 26S proteasome suggests that their activities might be coordinately regulated (Fig. 5). Distribution of Int-6 between them is likely to vary depending on cellular conditions. It has been proposed that in *A. thaliana* binding of Int-6 to the CSN triggers its absence from eIF3 [23]. From the similarity between subunits of CSN and 26S proteasome, it has been proposed that the former might represent an alternative lid of the regulatory particle for the proteasome [27]. It is also possible to consider such a possibility for eIF3. Our results do not support this model. Using Jurkat cell extracts we obtained no evidence of an association of the CSN with the 20S core proteasome. This is in agreement with results previously obtained in *A. thaliana*. In this organism Kwok et al. [32] observed that FUS6 (the CSN1 homologue) can interact with AtS9 (the homologue of Rpn6) in a two-hybrid assay, but that immunoprecipitation of the former did not trigger precipitation of AtS9, nor of AtS6A (the homologue of Rpt5) which is a base subunit interacting with AtS9. Considering these negative results, an alternative possibility might be that Int-6 helps protein complexes to shuttle between the CSN and the 26 proteasome. Proteins bound to Int-6 might associate with CSN, being then phosphorylated by the associated kinase and also possibly ubiquitinylated by the associated SCF complexes. Once these modifications are accomplished Int-6 might transport the protein to the 26S proteasome for degradation. In such a model the association of Int-6 with CSN would be transient, this being a possible explanation of the low amount of Int-6 observed in association with the CSN that contrasts with its strong binding to three different subunits. Another possible explanation for this latter aspect would be that in cells in culture Int-6 drives active protein translation in association with eIF3, but not protein degradation in association with the CSN and the 26S proteasome.

In conclusion, the Int-6 protein is associated with the three protein complexes eIF3, CSN and 26S proteasome, possibly exerting some regulatory roles in both protein translation and degradation. An important question that remains to be answered is whether this effect of Int-6 on these aspects of protein metabolism is general or specific of particular protein targets.

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