Interaction between the Ret finger protein and the int-6 gene product and co-localisation into nuclear bodies

Christelle Morris-Desbois, Valérie Bochard, Caroline Reynaud and Pierre Jalinot*

Laboratoire de Biologie Moléculaire et Cellulaire, UMR 5665 CNRS-ENSL, 46, Allée d’Italie, 69364 Lyon Cedex 07, France

*Author for correspondence (e-mail: pjalinot@ens-lyon.fr)

Accepted 16 July; published on WWW 22 September 1999

SUMMARY

The mouse int-6 gene was identified as an integration site for the mouse mammary tumor virus. Its human counterpart encodes a product that interacts with the Tax viral oncoprotein of the human T cell leukemia virus type 1. This interaction impedes the localisation of over-expressed Int-6 in nuclear bodies containing the promyelocytic leukemia gene product (PML). In this study, Int-6 is characterised as a 52 kDa protein that is localised within nuclear bodies in primary lymphocytes. Screening of a human B cell cDNA library for proteins that interact with Int-6 led to isolation of four clones coding for the p110 subunit of eIF3, in accordance with previous detection of Int-6 in purified forms of this translation initiation factor. Another clone was interesting with respect to the subcellular localisation of Int-6. It encodes the Ret finger protein (Rfp) which interacts with PML and localises within a subset of PML nuclear bodies. The interaction of Rfp with Int-6 is mediated through a region in Rfp designated ‘Rfp domain’, distinct from that involved in the interaction with PML. Int-6 and Rfp are co-localised in certain PML nuclear bodies in lymphocytes and transfection studies in HeLa cells strongly suggest that Rfp triggers translocation of Int-6 to nuclear bodies.

Key words: Int-6, Rfp, Rfp-like domain, Nuclear body, Two-hybrid

INTRODUCTION

The mouse int-6 gene was identified as an integration site of the mouse mammary tumor virus (MMTV) in a mammary hyperplastic outgrowth line and two independent mammary tumors (Marchetti et al., 1995). Insertion of the provirus led to expression of a chimaeric RNA expressing a truncated gene product which is likely to interfere with the control of cell proliferation, either as a dominant negative mutant or as an activated form of the normal protein. The human cDNA coding for Int-6 was independently isolated in a two-hybrid screen for proteins interacting with the Tax viral oncoprotein of the human T cell leukemia virus type 1 (HTLV-1) (Desbois et al., 1996). Immunofluorescence analysis of transfected COS7 cells showed that Int-6 co-localised with PML in nuclear structures referred variously to as nuclear domain 10 (ND10) (Ascoli and Maul, 1991), Kr bodies (Lamond and Carmo-Fonseca, 1993), PML oncogenic domains (PODs) (Dyck et al., 1994), or PML nuclear bodies (NBs) (Koken et al., 1994; Weis et al., 1994). These NBs appear to be dynamic structures whose number, size, and composition may vary. They contain constitutive components as PML and Sp100 (Szostecki et al., 1990), along with several other proteins including NDP55 (Ascoli and Maul, 1991), Sp140 (Bloch et al., 1996), the small ubiquitin-like protein SUMO-1 (formerly PIC-1) (Boddy et al., 1996), the ubiquitin-specific protease HAUSP (Everett et al., 1997), ISG20 (Gongora et al., 1997), Rfp (Cao et al., 1998), the CREB binding protein (CBP) (LaMorte et al., 1998), the retinoblastoma gene product Rb (Alcalay et al., 1998), and members of the heterochromatin protein 1 family (Lehming et al., 1998; Seeler et al., 1998). PML NBs have gained considerable interest since it was discovered that they were disrupted in acute promyelocytic leukemia (APL) cells and during the process of DNA virus infection (reviewed by Maul, 1998; Sternsdorf et al., 1997). The binding of Int-6 to Tax was found to exclude Int-6 from PML NBs (Desbois et al., 1996). Independently, the Int-6 protein was also characterised as a subunit of the eIF3 translation initiation factor (Asano et al., 1997). eIF3 promotes the binding of the initiator methionyl tRNA to the 4OS ribosomal particle and also intervenes in the binding of this complex to the mRNA. As discussed by Asano et al. (1997), Int-6 may correspond to a negative regulator of eIF-3. Alteration by MMTV insertion or by binding to Tax would result in an increased rate of translation initiation, allowing an increased cell proliferation. Alternatively, one can not exclude that Int-6 controls cell growth by mechanisms unrelated to translation. This latter possibility is supported by the localisation of Int-6 in the nucleus (Desbois et al., 1996).

To progress in the elucidation of the function of Int-6, a two-hybrid screen with this protein as bait was performed. This led to isolation of clones coding for another subunit of eIF3, but also for nuclear proteins including Rfp. Since Rfp has been described to be a component of PML NBs (Cao et al., 1998),
its putative function in localising Int-6 to these structures was further evaluated.

MATERIALS AND METHODS

Plasmids

The pTL1-88 plasmid consists of the int-6 cDNA (clone 88) (Desbois et al., 1996) cloned into the XhoI site of pTL1, which is a pSG5 derivative including multiple cloning sites. This int-6 cDNA starts at nucleotide 23 with respect to the first nucleotide of the translation initiation codon. The pTL1-Int-6 plasmid that contains the full-length Int-6 coding sequence was constructed in two steps: first, the S’ end of the cDNA was obtained by polymerase chain reaction (PCR) amplification from the Marathon Ready cDNA kit (Clontech) and cloned into the pGEM-T Easy vector (Promega). Second, the cDNA fragment coding for amino acids 1 to 300 of Int-6 was amplified from pGEM using the Pfu polymerase, digested with EcoRI and HpaI restriction enzymes, and cloned between the EcoRI and HpaI sites of pTL1-88. The human full-length rfp cDNA was also constructed in two steps: the sequence corresponding to the N-terminal moiety was obtained by digestion with the HindIII and SacI restriction enzymes, the pSV2retT plasmid (kindly provided by M. Takahashi through L. D. Etkin; Takahashi et al., 1988a). The sequence coding for the C-terminal moiety was obtained by digestion with SacI and BglII restriction enzymes of clone 9 which was isolated by two-hybrid screening with Int-6 as bait. The two cDNA fragments were inserted into pTL1 between the HindIII and BglII restriction sites, giving pTL1-Rfp. The pSGF-Rfp plasmid contains the rfp cDNA fused at its S’ end to the FLAG epitope into pSG5. The rfp cDNA was generated by PCR amplification using pTL1-Rfp as matrix and appropriate primers creating by PCR amplification using pSGF-Rfp as matrix and appropriate primers creating a PCR product. The resulting plasmid (pSGF-Int-6) was introduced into the HF7c yeast strain (Clontech). These cells were further transformed with a cDNA library of EBV-transformed human lymphocytes (Durfee et al., 1993). Transformation, screening, and β-galactosidase assays on filters were performed as previously described (Rousset et al., 1998). From an initial screen of 1×10^6 transformants, 81 clones were found to grow on a minimal medium lacking His and were positive for β-galactosidase expression. Plasmids of these colonies were recovered as previously reported (Hoffman and Winston, 1987) and transformed into Escherichia coli XL1-blue strain by electroporation. Liquid culture β-galactosidase assays for clones of interest into the SF5Y56 yeast strain (Clontech) transformed either with pGBT9 or pGB-Int-6, were performed as previously described (Rousset et al., 1998).

Immunofluorescence and confocal microscopy

HeLa cells were plated on SonicSeal slide wells (NUNC) and transfections were performed by the calcium phosphate precipitation method. Primary lymphocytes were isolated from human blood samples by successive centrifugations on Ficoll and Percoll gradients. Activation was performed by treatment with 10 U/ml of interleukin-2 (IL-2) and 2 μg/ml of phytohemagglutinin (PHA) for 3 to 5 days as indicated in the legends to figures. Lymphocytes were adsorbed on microscope slides treated with polylysine. HeLa cells and primary lymphocytes were fixed for 10 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS), incubated twice for 10 minutes in 0.1 M glycine in PBS, and permeabilised for 5 minutes in PBS with 0.5% Triton X-100. Cells were blocked with 1% BSA in PBS. For indirect immunofluorescences, cells were incubated with primary antibodies diluted in PBS: the αFLAG, αRfp, and αC-20 Int-6 antibodies were diluted 1:1000 and the 5E10 antibody to PML was diluted 1:20. Cells were then incubated with secondary antibodies conjugated to cyanine 2 (Cy2) or rhodamine. In order to perform double-labeling of Rfp and Int-6 with two rabbit antibody polyclonal antibodies, the αC-169 Int-6 antibody was conjugated to Cy5 using the Cy5 Ab labeling kit (Amersham Pharmacia Biotech). Prior to conjugation, the crude serum was purified on a Hi-Trap Protein A column. For Int-6 and Rfp double-immunostaining, the polyclonal antibody against Rfp was first incubated and revealed with an anti-rabbit IgG coupled to Cy2. A normal rabbit serum was then added in order to block any non-occupied binding site of the anti-rabbit secondary antibody. Finally, the anti-Int-6 antibody coupled to Cy5 was added. Following immunostaining, slides were mounted in...
medium containing Mowiol (Calbiochem) and 2.5% DABCO (1,4 diazabicyclo 2,2,2 octane, Sigma) and observed with a LSM 510 confocal microscope (Zeiss) using the 488, 543, and 633 nm lines of an Ar-Kr and two He-Ne lasers, respectively. The Cy2 and Cy5 fluoroscences were collected simultaneously and the rhodamine signal was recorded separately because of possible cross-talk with the other channels in the case of intense labeling.

RESULTS

Characterisation of the int-6 gene product

The int-6 cDNA has been cloned in mouse and human (Desbois et al., 1996; Marchetti et al., 1995). It appears that both mouse and human Int-6 proteins have exactly the same sequence (Desbois et al., 1996). To evaluate whether Int-6 was also highly conserved in more distant species, the int-6 cDNA was amplified from a Xenopus laevis library. A databank search indicated that this gene also exists in Caenorhabditis elegans and sequence comparison with the int-6 cDNA from other species led to the prediction that the gene in C. elegans would contain three introns. The positions of the introns have been confirmed by sequencing an RT-PCR product and by analysing one expressed sequence tag (EST) for the first intron. Alignment of Int-6 deduced amino acid sequences from human, Xenopus and the nematode (Fig. 1) clearly shows a high degree of conservation (45% identity and 31% similarity between human and the nematode and also between Xenopus and the nematode). This conservation among multicellular organisms contrasts with the absence of a homologue in yeasts.

To characterise the Int-6 protein, several antibodies were generated. Two polyclonal antibodies were directed against peptides corresponding to the N-terminal 19 and to the C-terminal 20 amino acids of human Int-6. A third polyclonal antibody was raised against a protein corresponding to the C-terminal 169 amino acids. These three antibodies recognised a unique protein migrating at 52 kDa in extracts from different cell lines (Fig. 2A, lane 3, Fig. 2B, lane 1, Fig. 2C, lanes 4 and...
5). This is in agreement with the calculated molecular mass of 52.2 kDa. The pre-immune sera did not recognize any band at this size (C. Morris-Desbois, data not shown). A single 52 kDa protein was also evident in extracts from normal PBLs (Fig. 2A, lane 2 and Fig. 2C, lane 3). In vitro translation of the int-6 cDNA also gave a 52 kDa band (data not shown). When COS7 cells were transfected with an expression vector containing the full-length int-6 cDNA, the 52 kDa band was increased (Fig. 2B, lane 2 and Fig. 2C, lane 1). By contrast, when COS7 cells were transfected with a cDNA lacking the first AUG, a major band was observed at 46 kDa, which is in agreement with a translation initiation at the second methionine codon in the sequence (Fig. 2A, lane 1 and Fig. 2C, lane 2). Another weaker band which migrates at 43 kDa may correspond to a product initiated at the third methionine. These two protein species were not detected with the antibody raised against the N-terminal 19 amino acids of Int-6 (Fig. 2B, lane 2). Taken together, these data clearly support that the int-6 gene product is a 52 kDa protein initiated at the first methionine codon in the cDNA. These results are in disagreement with previous observations showing that in various cell types, including NIH 3T3 cells, the Int-6 protein migrates with an apparent molecular mass of 43 kDa (Diella et al., 1997). The authors concluded from their results that the third methionine codon is the major start for Int-6 translation. The exact reason for the discrepancy between their results and ours is not known. Independently, Int-6 present in purified eIF3 was estimated to migrate with an apparent molecular mass of 48 kDa on polyacrylamide gels, in accordance with a translation initiation at the first methionine codon (Asano et al., 1997).

To investigate whether the size or the amount of Int-6 was modified when cells are induced to proliferate, PBLs were activated with PHA. The apparent molecular mass remained unchanged. However, a moderate increase in the amount of Int-6 was consistently observed with the three antibodies after activation for two days (Fig. 3D). The three antibodies to Int-6 that were used in this study allow us to conclude unambiguously that the int-6 gene encodes a protein with an apparent molecular mass of 52 kDa which is expressed in all cell types tested so far.

Subcellular localisation of the Int-6 protein

In a previous study, immunostaining showed that a FLAG-tagged form of Int-6, in which the eight first amino acids were replaced by the FLAG epitope, clearly co-localised with endogenous PML within PML NBs (Desbois et al., 1996). In order to determine the subcellular localisation of endogenous Int-6, indirect immunofluorescence was performed on primary lymphocytes using the antibody raised against the recombinant Int-6 protein and a secondary antibody coupled to Cy5. Int-6 was detected in discrete nuclear foci regardless of whether the

---

Fig. 2. Characterisation of the int-6 gene product as a 52 kDa protein. (A) Immunoblot of COS7, PBLs and NIH3T3 cell lysates using a rabbit antiserum raised against the C-terminal 20 amino acids of Int-6 (αC-20). COS7 cells (lane 1) were transfected with pTL1-88. The arrow on the left marks endogenous Int-6 which migrates at 52 kDa. The asterisk marks the major form of the N-terminal truncated Int-6 produced by pTL1-88. The lower band marked by a thin line probably corresponds to a minor form resulting from translation being initiated from an internal initiation codon. (B) Immunoblot of COS7 cell lysates using a rabbit antiserum raised against the N-terminal 19 amino acids of Int-6 (αN-19). Cells were transfected with either pTL1-88 (lane 2) or pTL1-Int-6 (lane 3). (C) Immunoblot of COS7, PBLs, C8166, and HeLa cell extracts using a rabbit antiserum raised against a protein consisting of the FLAG epitope fused to amino acids 276 to 445 of Int-6 (αC-169). COS7 cells were transfected with pTL1-Int-6 (lane 1) and with pTL1-88 (lane 2). Full-length Int-6 and truncated forms are labeled as in A. (D) Expression of Int-6 was analysed in resting PBLs (lanes 1, 6, 11) and in PBLs stimulated with 5μg/ml of PHA for one day (lanes 2, 7, 12), two days (lanes 3, 8, 13), three days (lanes 4, 9, 14), and five days (lanes 5, 10, 15). Immunoblots were probed with the three anti-Int-6 antibodies, previously described, as indicated below each blot image.
Interaction of Int-6 with Rfp

In order to further elucidate the function of Int-6, a two-hybrid screen was performed with Int-6 as a bait. A cDNA library of B lymphocytes immortalised by EBV was used (kindly provided by S. Elledge; Durfee et al., 1993). The screen led to isolation of several clones encoding proteins that may interact with Int-6 in normal cells. Among these clones, five were found to correspond to proteins relevant to established properties of Int-6. Thus, Int-6 has previously been identified as a subunit of the purified eIF3 translation initiation factor (Asano et al., 1997). Four clones encoding the p110 subunit of eIF3 were isolated in the screen (Fig. 4A). These clones did not include the entire coding sequence, but only the C-terminal third. Future studies will determine whether this interaction between Int-6 and a C-terminal domain of eIF3 p110 is causative of the presence of Int-6 in the eIF3 translation initiation factor.

Another clone was interesting with respect to the ability of Int-6 to localise within PML NBs. This clone was found to code for the Rfp protein (Fig. 4A). The rfp gene was first identified as part of the ret transforming gene which results from recombination between the rfp gene and the c-ret tyrosine kinase proto-oncogene (Takahashi et al., 1985). In several cell lines, Rfp was found to localise within NBs by interacting with PML (Cao et al., 1998). In a cell line in which Rfp is
cytoplasmic, over-expression of PML triggered Rfp translocation to the NBs (Cao et al., 1998). To evaluate the relative strength of the interaction, the two-hybrid assay was used. The \( \beta \)-galactosidase activity induced by the interactions between Int-6 and Rfp and between Int-6 and eIF3 p110 was measured in a liquid culture assay (Fig. 4B). As control, the \( \beta \)-galactosidase activity induced by the interaction of Int-6 with the Tax viral protein was also measured. For all three proteins showing an interaction with Int-6, a clear difference was observed between the vector expressing the GAL4 DNA binding domain alone or fused to Int-6, showing that the interaction of Rfp with Int-6 is specific.

The possible binding of Int-6 to Rfp was next investigated in the context of mammalian cells by performing immunoprecipitation experiments using COS7 cells transfected with expression vectors for both proteins. For Rfp expression, FLAG-tagged forms corresponding to either the entire protein or the fragment encoded by the clone isolated in the two-hybrid screen were used. Both complete and truncated Rfp proteins were able to precipitate Int-6 (Fig. 5A). Reciprocally, precipitation of Int-6 carried both forms of Rfp (Fig. 5B). These results show that Int-6 forms a complex with Rfp when both proteins are co-expressed in mammalian cells.

Domains of Rfp and Int-6 mediating the association of both proteins

The regions of Rfp involved in the interaction with Int-6 were mapped. Rfp contains several specific motifs: a RING finger, a B box zinc finger, a coiled-coil domain with three defined helices C1, C2, and C3, and a C-terminal region called the Rfp domain (Cao et al., 1996, 1997; Takahashi et al., 1988b).
The B box and the C2 and C3 helices of the coiled-coil domain were found to be involved in the interaction with PML (Cao et al., 1998). The clone obtained from the two hybrid-screen consisted only of the C2 and C3 helices of the coiled-coil domain together with the B box. This indicates that the B box, the C2, and the C3 helices are not required for interaction with Int-6. To further define the Rfp C-terminal region that binds to Int-6, several Rfp deletion mutants were cloned into an expression plasmid containing a FLAG epitope and were then co-transfected with the Int-6 expression vector into COS7 cells. Immunoprecipitation experiments were performed using the FLAG M2 antibody and immunoblots were probed with an antibody to Int-6. Two N-terminal deletion mutants that lacked the C2 and C3 regions (constructs Rfp275-513 and Rfp317-513, respectively) were first tested (Fig. 6A). The corresponding truncated proteins precipitated Int-6 as efficiently as the entire Rfp (Fig. 6B, lanes 2, 10, and 12). Three C-terminal deletion mutants were further constructed to remove one third, two thirds, and the entire Rfp domain (constructs Rfp1-445, Rfp1-379, and Rfp1-316, respectively) (Fig. 6A). The first two deletions did not affect the ability of Rfp to interact with Int-6 (Fig. 6B, lanes 4 and 6). In contrast, the Rfp1-316 construct completely lost the ability to precipitate Int-6 (Fig. 6B, lane 8). These experiments clearly map the region mediating interaction with Int-6 to the N-terminal third of the Rfp domain. The other regions of Rfp are apparently not involved in this interaction.

Reciprocally, Int-6 regions involved in the interaction with Rfp were examined. A particular homology domain has been identified in the Int-6 protein: the PCI domain (for Proteasome, COP9, Initiation factor 3). This domain was also found in the Int-6 protein: the PCI domain (for PML). The clone obtained from the two hybrid-screen consisted only of the C2 and C3 helices of the coiled-coil domain; Rfp domain. All mutants were fused at their 5′ end to the sequence coding for the FLAG epitope (black box). (B) COS7 cells were transfected with pTL1-Int-6 and with a plasmid expressing a Rfp mutant construct as indicated on the figure. Immunoprecipitations were performed with the anti-FLAG antibody (even-numbered lanes) and immunoblot was done with the αC-20 Int-6 antiserum. Odd-numbered lanes are negative controls, in which the antibody to FLAG was omitted during the immunoprecipitation step. As described in legend to Fig. 5, asterisk on the left of the blot marks a non-specific band.

The B box and the C2 and C3 helices of the coiled-coil domain were found to be involved in the interaction with PML (Cao et al., 1998). The clone obtained from the two hybrid-screen consisted only of the C2 and C3 helices of the coiled-coil domain together with the B box. This indicates that the B box, the C2, and the C3 helices are not required for interaction with Int-6. To further define the Rfp C-terminal region that binds to Int-6, several Rfp deletion mutants were cloned into an expression plasmid containing a FLAG epitope and were then co-transfected with the Int-6 expression vector into COS7 cells. Immunoprecipitation experiments were performed using the FLAG M2 antibody and immunoblots were probed with an antibody to Int-6. Two N-terminal deletion mutants that lacked the C2 and C3 regions (constructs Rfp275-513 and Rfp317-513, respectively) were first tested (Fig. 6A). The corresponding truncated proteins precipitated Int-6 as efficiently as the entire Rfp (Fig. 6B, lanes 2, 10, and 12). Three C-terminal deletion mutants were further constructed to remove one third, two thirds, and the entire Rfp domain (constructs Rfp1-445, Rfp1-379, and Rfp1-316, respectively) (Fig. 6A). The first two deletions did not affect the ability of Rfp to interact with Int-6 (Fig. 6B, lanes 4 and 6). In contrast, the Rfp1-316 construct completely lost the ability to precipitate Int-6 (Fig. 6B, lane 8). These experiments clearly map the region mediating interaction with Int-6 to the N-terminal third of the Rfp domain. The other regions of Rfp are apparently not involved in this interaction.

Reciprocally, Int-6 regions involved in the interaction with Rfp were examined. A particular homology domain has been identified in the Int-6 protein: the PCI domain (for Proteasome, COP9, Initiation factor 3). This domain was also found in the p110 subunit of eIF3, five proteasome subunits, two subunits of the COP9 complex, and subunits of other multiprotein complexes (Glickman et al., 1998; Hofmann and Bucher, 1998; Seeger et al., 1998; Wei et al., 1998). The PCI domain is localised between amino acids 304 to 395 of Int-6. Several deletions were introduced at the N-terminal and C-terminal sides of Int-6 (Fig. 7A). The FLAG-tagged Int-6 deletion mutants were co-transfected with the Rfp expression vector into COS7 cells. Immunoprecipitations were performed using the FLAG M2 antibody and immunoblots were revealed with an antibody to Rfp (kindly provided by L. D. Etkin; Cao et al., 1996). Deletion of the N-terminal 143 amino acids reduced Rfp binding as compared to the FLAG-tagged Int-6 (Fig. 7B, lanes 2 and 4). Larger deletion to amino acid 245 did not further decrease the amount of Rfp that was co-precipitated (Fig. 7B, lane 6). Rfp binding was increased again when the mutant lacking the N-terminal 306 amino acids of Int-6 was used (Fig. 7B, lane 8). This mutant corresponds roughly to the PCI domain. At the C-terminal end, deletion to amino acid 395 or 330 did not modify the interaction with Rfp (Fig. 7B, lanes 10 and 12). Longer deletion to amino acid 294 clearly reduced Rfp binding (Fig. 7B, lane 14). However, the region of Int-6 from amino acids 9 to 195 was found to interact with Rfp as efficiently as the entire protein (Fig. 7B, lanes 16 and 2). These results indicate that the interaction of Int-6 with Rfp probably involves two regions, one at the N-terminal end between amino acids 9 to 144, and the other in the N-terminal third of the PCI domain between amino acids 307 to 330. Further analyses with point mutants as well as more data on the structure of the protein should help to determine the Int-6 motifs involved in the interaction with Rfp.

Co-localisation of Int-6 and Rfp in PML NBs

Since Rfp and Int-6 were independently found to be present within a subpopulation of PML NBs (Cao et al., 1998; Desbois et al., 1996; Fig. 3), it was further assessed whether the two proteins may be co-localised within these nuclear structures. Immunofluorescence analysis was performed on primary lymphocytes using antibodies recognising Int-6, Rfp, and PML. Fig. 8A,B,C shows an optical section of Int-6, Rfp, and PML stainings respectively. Nuclear dots were observed for the three stainings. In addition, a diffuse nuclear staining was detected for Int-6 and Rfp. However, the diffuse labeling obtained for Int-6 is likely the consequence of a poorer sensitivity of the antibody coupled to Cy5 as compared to the staining obtained by using a secondary antibody coupled to the
fluorochrome (Fig. 3 and data not shown). For Rfp, the diffuse nuclear labeling has been observed by others (Cao et al., 1997, 1998). The superimposition of the three confocal images clearly showed co-localisation between Int-6, Rfp, and PML in some but not all nuclear dots (Fig. 8D). Certain dots were only labeled by anti-Rfp (Fig. 8D, arrow). Conversely, other dots were found to contain only Int-6 and PML, but not Rfp (Fig. 8D, arrowhead). Dots containing only Int-6 were not observed with this low sensitivity staining procedure, however they are likely to exist as suggested by results in Fig. 3. The co-localisation of Rfp and PML within only a subset of NBs has already been reported (Cao et al., 1998). These observations clearly show that a portion of PML NBs contain both Int-6 and Rfp.

To further investigate the role of the interaction between Int-6 and Rfp in the targeting of Int-6 to PML NBs, transfection studies were carried out in HeLa cells and immunostaining was performed according to the procedure described above. Overexpression of Int-6 led to a diffuse staining in both the cytoplasm and nucleus (Fig. 9A, panel 1). Co-expression of PML, which localised within NBs (Fig. 9A, panel 3), did not modify the diffuse staining observed for Int-6 (Fig. 9A, panel 2). In contrast, when Int-6 and Rfp were co-expressed, a punctate nuclear staining was observed for Rfp and also for a fraction of Int-6 (Fig. 9B, panels 1 and 2). Superimposition of the two confocal sections clearly showed a co-localisation of Int-6 and Rfp into nuclear dots (Fig. 9B, panel 3). To determine whether PML would interfere with the co-localisation of Int-6 and Rfp into nuclear dots, HeLa cells were transfected with expression vectors for Int-6, Rfp, and PML. A punctate nuclear staining pattern was still observed for Int-6 (Fig. 9C, panel 1) as for Rfp and PML (Fig. 9C, panels 2 and 3).
Fig. 9. Exogenous Int-6 and Rfp co-localise in a subset of PML NBs of HeLa cells. (A) Cells were transfected with the pTL1-Int-6 plasmid alone (panel 1) or with the pTL2-Myl vector (kindly provided by P. Chambon) which codes for PML (panels 2 and 3). Int-6 was detected by direct immunofluorescence with the antibody coupled with Cy5 (panels 1 and 2). PML was labeled by indirect immunofluorescence with the 5E10 mouse monoclonal antibody revealed with a Cy2-conjugated secondary antibody (panel 3). (B) HeLa cells were transfected with pTL1-Int-6 and pTL1-Rfp. Int-6 was detected as above (panel 1) and Rfp was labeled by indirect immunofluorescence with the rabbit polyclonal antibody to Rfp revealed with a Cy2-conjugated secondary antibody (panel 2). Superimposition of the two confocal images is shown in panel 3 and co-localisation of Int-6 and Rfp is visualised as a yellow color. (C) HeLa cells transfected with pTL1-Int-6, pTL1-Rfp, and pTL2-Myl were subjected to triple staining. Int-6 and Rfp were visualised as above (panels 1 and 2, respectively). PML was labeled with the 5E10 antibody revealed with a rhodamine-conjugated anti-mouse IgG (panel 3). The area delineated by the square on the three optical sections was superimposed for co-localisation analysis. A ×2 magnification corresponding to Int-6 plus Rfp staining is shown in the inset at the top right of panel 1. The one corresponding to Rfp plus PML staining is at the top right of panel 2. Co-localisation of staining is seen as a yellow colour. Arrowheads in the insets indicate red staining that represents NBs containing Int-6 and PML but not Rfp. Arrows point to a green NB that contains only Rfp.

respectively. Although stainings do not completely overlap, as already mentioned for the endogenous proteins in lymphocytes, it is clear that most of nuclear dots contain the three proteins. Taken together these observations suggest that
the presence of Int-6 in PML NBs is probably due to its interaction with Rfp. Moreover, interactions of Rfp with PML and with Int-6 do not seem to be exclusive.

DISCUSSION

The data presented here establish that Int-6 is a 52 kDa protein that is highly conserved. In agreement with previous observations (Asano et al., 1997; Marchetti et al., 1995), Int-6 appears to be ubiquitously expressed. Its amount is increased in activated PBLs as compared to non-activated ones. Examination of the intracellular localisation of endogenous Int-6 in resting and proliferating primary lymphocytes showed a clear staining in the nucleus. This nuclear localisation suggests that Int-6 is likely to exert functions in this cellular compartment which remain to be determined. The results of the two-hybrid screen identified Rfp and eIF3 p110 as possible partners of Int-6. That Int-6 is not probably a core component of eIF3, but rather a regulatory subunit, is supported by a recent work showing the existence of a yeast eIF3 core complex consisting of only five proteins, namely YBR079c, Prt1p, Nip1p, Tif34p, and YDR429c (Phan et al., 1998). Several pairwise interactions have been defined between these five proteins (Asano et al., 1998). By analogy, their mammalian counterparts, which correspond to p170, p116, p110, p36, and p44, respectively, might constitute the core complex to which other subunits would bind to form the complete eIF3 complex. These additional subunits may regulate the activity of the core complex. In this model, Int-6 might correspond to a regulatory subunit that associates with the p110 core subunit. Therefore, it will be important to determine whether Int-6 can modify the activity of eIF3 on translation and in which sense, i.e. positively or negatively.

As it was a possible explanation for the localisation of Int-6 within PML NBs, its interaction with Rfp was further studied. Rfp possesses a tripartite motif including a RING finger, a B-box zinc finger, and an α-helical coiled-coil domain (Cao et al., 1996, 1997; Takahashi et al., 1988b). This tripartite motif is present in several proteins that constitute the so-called RBCC family, of which PML is a member (Saurin et al., 1996). At its C-terminal end, Rfp contains a domain also found in a subset of the RBCC proteins and in other proteins with diverse functions, such as butyrophilin and stonustoxins. This region has been designated the Rfp-like domain or B30.2-like domain (Henry et al., 1997). Our results demonstrate an interaction between two cellular proteins which are implicated in oncogenesis when altered. This raises the possibility that they participate in a common regulatory process. It is possible that Int-6 and Rfp, in the PML NBs, act on a nuclear function important for cell growth. Alternatively, binding of Int-6 to Rfp in the nucleus could alter its activity on eIF3. If Int-6 is a negative regulator of eIF3, this would cause increased protein translation which might cause cell transformation.

Our observations show that in normal cells such as lymphocytes, Int-6 is present in NBs together with PML and Rfp. In HeLa cells, over-expressed Int-6 was present diffusely in cytoplasm and nucleus. In similar transfection studies the FLAG-tagged form of Int-6, which lacks the N-terminal 8 amino acids, was exclusively localised in PML NBs (Desbois et al., 1996). These findings indicate that the N terminus of Int-6 is likely to play a role in the localisation of the protein. In HeLa cells, detection of the full-length Int-6 molecules within PML NBs correlates with over-expression of Rfp. This study indicates that Rfp triggers translocation of Int-6 to nuclear dots. In lymphocytes, some NBs were found to contain both PML and Int-6, but not Rfp. When tested by co-immunoprecipitation experiments, Int-6 was not observed to bind to Rfp in PML (C. Morris-Desbois, unpublished results). It is possible that other proteins similar to Rfp intervene in the localisation of Int-6 in PML NBs in which Rfp is absent. In this respect, it is interesting to mention that several RBCC family members have an Rfp-like domain, such as SS-A/Ro52, Staf50 and efp (Henry et al., 1997; Itoh et al., 1991; Tissot and Mechti, 1995; Orimo et al., 1995). Future studies should elucidate if these factors are also able to interact with Int-6 and mediate its localisation to NBs. Despite the growing group of proteins characterised as being present in PML NBs, little is known as to the exact function of these nuclear structures. They have been hypothesised to constitute sites of protein stocking, either for reuse or for further degradation (Maul, 1998). Alternatively, these structures could be important for replicative or transcriptional processes. Interestingly, LaMorte et al. (1998) have shown by using a novel nucleic acid labeling method that the majority of PML NBs correspond to sites of incorporation of fluorescein-conjugated uridine triphosphate in nascent RNA polymerase II transcripts. Moreover, the CBP transcriptional co-activator is present in these nuclear structures (LaMorte et al., 1998). These data together with other observations (Vallian et al., 1998; Wang et al., 1998; Zheng et al., 1998), strongly support the notion that PML is a transcription factor. This protein is probably an essential component of the PML NBs. Differently, Rfp and Int-6 are likely to be less tightly associated with these structures via interaction with PML for Rfp, and via interaction with Rfp for Int-6. Future studies will help to determine whether Rfp and Int-6 are present in these structures only for stocking or whether they also participate actively in specific nuclear processes such as transcription or replication.

Our results demonstrate an interaction between two cellular proteins which are implicated in oncogenesis when altered. This raises the possibility that they participate in a common regulatory process. It is possible that Int-6 and Rfp, in the PML NBs, act on a nuclear function important for cell growth. Alternatively, binding of Int-6 to Rfp in the nucleus could alter its activity on eIF3. If Int-6 is a negative regulator of eIF3, this would cause increased protein translation which might cause cell transformation. Future studies should allow to discriminate...
between these different possibilities and to better understand the involvement of Int-6 and Rfp in oncogenesis.

We thank D. L. D. Etkin, R. van Driel, M. Takahashi, P. Chambon, and S. Elledge for the generous gifts of reagents. We are indebted to A. Roisin for the production of the recombinant Int-6 protein and E. Derrington for critical reading of the manuscript. This work was supported by the Agence Nationale de Recherches sur le Sida, the Association pour la Recherche contre le Cancer, and the Ligue Nationale Contre le Cancer.

REFERENCES


Asano, K., Merrick, W. C. and Hershey, J. W. (1993). Association for the Recherche contre le Cancer, and the Ligue Roisin for the production of the recombinant Int-6 protein and E. S. Elledge for the generous gifts of reagents. We are indebted to A.


