Exclusion of Int-6 from PML Nuclear Bodies by Binding to the HTLV-I Tax Oncoprotein

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The Tax transactivator of the human T cell leukemia virus type I (HTLV-I) exhibits oncogenic properties. A screen for proteins interacting with Tax yielded a complementary DNA (cDNA) encoding the human Int-6 protein. In mice, the *Int-6* gene can be converted into a putative dominant negative oncogene after retroviral insertion. Here, Int-6 was localized in the cell nucleus to give a speckled staining pattern superposed to that of the promyelocytic leukemia (PML) protein. The binding of Tax to Int-6 caused its redistribution from the nuclear domains to the cytoplasm. Thus, Int-6 is a component of the PML nuclear bodies and Tax disrupts its normal cellular localization by binding to it.

The HTLV-I retrovirus causes adult T cell leukemia and tropical spastic paraparesis (*1*). Because of its well-established oncogenic properties (*2*), the viral Tax protein is thought to play an important role in proliferative diseases caused by HTLV-I. However, the precise molecular basis of Tax-mediated transformation remains unclear. It is possible that Tax could deregulate cell proliferation by interacting with cellular proteins normally involved in growth control, similar to the mechanism of oncoproteins of DNA tumor viruses (*3*). To identify such cellular targets of Tax, a yeast two-hybrid screen was carried out (*4*). A cDNA library from Epstein-Barr virus (EBV)–transformed human peripheral lymphocytes (*5*) was screened with the use of the entire Tax protein as bait (*6*). Two clones isolated in this screen (numbers 70 and 88) encode the human counterpart of the mouse *Int-6* gene product. The *Int-6* locus was discovered recently as a common integration site for the mouse mammary tumor virus (MMTV) (*7*). Because it results in the expression of truncated Int-6 proteins, it is possible that the MMTV insertion generates dominant negative oncoproteins. The deduced amino acid sequence of the human protein was identical to that of the mouse form, with no significant homology with other known proteins.

To verify the specificity of the two-hybrid interaction, we further tested clone 70 against three protein baits unrelated to Tax: the TATA box binding protein, the adenosine 3',5'-monophosphate (cAMP) responsive element binding protein, and the p53 protein with its NH_2-terminal transcriptional activation domain deleted. Int-6 was found to interact only with Tax (Fig. 1A). The strength of this association was comparable to that of the large T SV40 antigen with p53 (Fig. 1A). Thus, the interaction between Tax and Int-6 was strong and specific. To substantiate the interaction observed in yeast, we carried out a two-hybrid assay in human cells. HeLa cells were transfected with a reporter plasmid containing the gene encoding chloramphenical acetyltransferase (CAT) under the control of GAL4 sites, together with two expression vectors: Tax fused to the GAL4 DNA binding domain (GAL4-Tax) and Int-6 fused to the VP16 activation domain (VP16–Int-6). The fusion protein GAL4-Tax activated CAT expression to some extent (Fig. 1B) (*8*). However, greater CAT production was measured when GAL4-Tax and VP16–Int-6 were coexpressed (Fig. 1B). Finally, a direct protein-protein interaction was demonstrated by a coimmunoprecipitation assay. COS7 cells were transiently transfected with expression vectors encoding Tax and Int-6 fused to the FLAG epitope at its NH_2-terminus. Immunoprecipitations followed by immunoblotting with antibodies specific for the FLAG epitope or Tax showed that the two proteins coprecipitate (Fig. 1, C and D).

We evaluated the expression profile of the *Int-6* gene in human tissues by probing Northern (RNA) blots with human Int-6 cDNA. In all tissues tested, a transcript of about 1.6 kb was detected, although at variable levels (Fig. 2A). The highest levels were found in pancreas, muscles, and lymphoid tissues.

Next, we examined the subcellular localization of the Int-6 protein to determine whether it colocalizes with Tax inside cells. COS7 cells were transfected with a plasmid expressing Int-6 tagged with the FLAG epitope, either alone or with a Tax expression vector. Cells were then analyzed by immunofluorescence (*9*). Int-6 was predominantly nuclear and gave a speckled staining pattern (Fig. 3A). The punctate nuclear localization appeared to be a distinctive property of Int-6 because a FLAG-tagged unre-

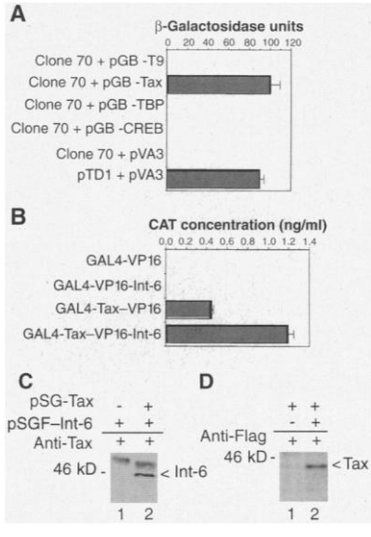

Fig. 1. Specific association of Tax with Int-6. (**A**) Analysis of the interaction between Int-6 and Tax or unrelated proteins by a liquid culture β-galactosidase assay. SFY526 cells were transformed with Int-6 clone 70 together with plasmids expressing the GAL4 DNA binding domain (GB) alone (pGB-T9) or fused to Tax (pGB-Tax). (The Int-6 cDNA nucleotide sequence has been deposited with GenBank under the accession number M62962). The Int-6 clone was also cotransformed with plasmids encoding fusion proteins associating GB with the TATA box binding protein (pGB-TBP), the cAMP-responsive element binding protein (pGB-CREB), and the p53 protein consisting of amino acids 72 to 390 (pVA3). As a positive control, yeast cells were cotransformed with pVA3 and pTD1, which encodes a protein consisting of GB fused to the SV40 large T antigen (amino acids 84 to 708). The β-galactosidase assay with *O*-nitrophenyl-β-D-galactoside (ONPG) as substrate was carried out on two independent colonies per transformation. The mean values expressed in Miller units are shown. (**B**) Interaction of Tax and Int-6 in a HeLa cell two-hybrid system. Cells were transfected with a CAT reporter construct containing GAL4 sites together with two expression vectors: one encodes GB alone (GAL4) or fused to Tax (GAL4-Tax) and the other encodes the VP16 trans-activation domain alone (VP16) or fused to the clone 70 Int-6 cDNA (VP16–Int-6) (*21*). Mean values are represented. (**C** and **D**) Coimmunoprecipitation of Int-6 and Tax in COS7 cells. In (C), cells were overexpressing the FLAG-tagged Int-6 protein alone (lane 1) or with Tax (lane 2). Immunoprecipitation was performed with a polyclonal Tax antibody (*22*) and immunoblotting with the M2 monoclonal antibody (Kodak) directed against the FLAG epitope. In (D), cells were overexpressing Tax alone (lane 1) or with Int-6 (lane 2). Immunoprecipitation was done with the FLAG antibody and immunoblotting with the antiserum to Tax (*23*).

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lated transcription factor (*10*) gave a uniform nuclear staining pattern under the same experimental conditions. Tax displayed a diffuse pattern of labeling in both the cytoplasm and the nucleus (Fig. 3C). When Int-6 and Tax were coexpressed, the characteristic speckled staining pattern of Int-6 was not observed. Under these conditions, Int-6 was mainly absent from the nucleus and formed a diffuse localized area of staining in the cytoplasm (Fig. 3, E and G). Tax was also more concentrated in this cytoplasmic area (Fig. 3, F and H). Similar results were observed in HeLa cells (*11*). This overlap in distribution suggests an association between Tax and Int-6 in vivo.

In both HeLa and COS7 cells, nuclear dots containing Int-6 were dispersed throughout the nucleoplasm, excluding nucleoli (Fig. 3A). They were often unequal in size, and their number per nucleus ranged from 10 to 40. This nuclear staining pattern is reminiscent of the labeling observed for other cellular proteins, in particular the PML protein, which concentrates within macromolecular multiprotein complexes known as nuclear bodies or PODs (for PML oncogenic domains) (*12–15*). Hence, we examined whether Int-6 and PML are localized within the same nuclear structures. Localization of the transiently expressed Int-6 protein was compared with that of endogenous PML by confocal microscopy immunofluorescence analyses (*16*), and Int-6 and PML were indeed present in the same nuclear macromolecular structures (Fig. 4, A through C).

To eliminate any artifact that was the result of Int-6 overexpression, we examined the localization of the endogenous protein with an antibody to the COOH-terminal 20 amino acids. Endogenous Int-6 was found also to colocalize with PML in nuclear bodies (Fig. 4, D through F). We next asked whether Tax redistributed specifically Int-6 or had a similar effect on another POD component, like PML itself. COS7 cells were transfected with vectors expressing PML and Tax. No overlap in the localization of the two proteins was observed, and Tax did not alter the speckled staining pattern of PML (Fig. 4, G through I).

Thus, the Int-6 protein, whose truncation in mice has been observed in different stages of mammary tumors, was identified as a cellular target of Tax. We found that Int-6 was localized with PML in PODs and that Tax induced a redistribution of Int-6 from these nuclear structures into the cytoplasm. Such a delocalization has been observed for other proteins within cancer cells. In particular, PML was first identified as part of a fusion protein with the retinoic acid receptor α (RARα) in patients suffering from acute promyelocytic leukemia (APL) (*13, 17*). In APL cells, the PML-RARα fusion protein is predominantly localized in the cytoplasm, whereas a fraction remains nuclear within multiple smaller clusters (*18*). This abnormal localization is reversed by retinoic acid treatment (*14, 15, 18*). A cytoplasmic redistribution of PML has also been observed in liver carcinomas (*19*), and the p53 tumor suppressor protein is sequestered into the cytoplasm of breast cancer cells (*20*).

From these observations and our results, we propose that Tax alters Int-6 function by diverting this cellular protein from its nat-

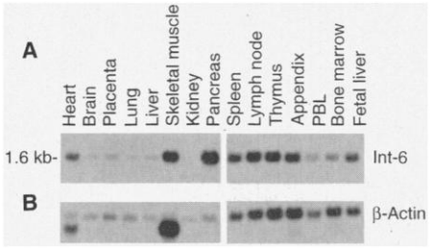

Fig. 2. Northern blot analysis of human Int-6 mRNA. (**A**) Northern blots (Clontech) of multiple human tissues containing 2 μg of polyadenylated RNA per lane were probed with the radioactively labeled human Int-6 cDNA of clone 70. Blots were exposed for 6 hours. (**B**) The same blots were stripped and rehybridized with the control β-actin cDNA provided by the manufacturer. Blots were exposed for 40 min.

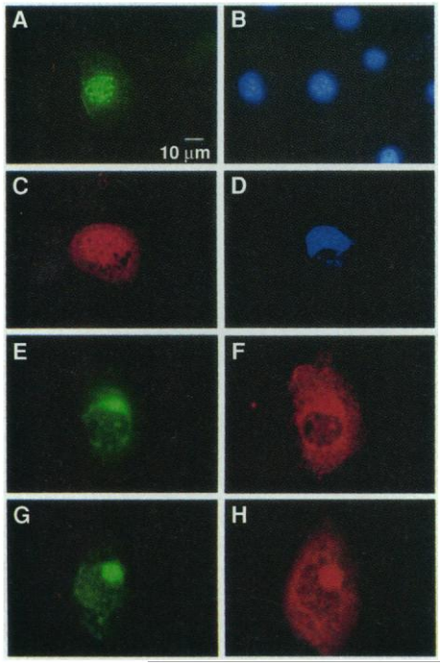

Fig. 3. Subcellular localization of the Int-6 protein in the absence or presence of Tax. (**A**) COS7 cells were transfected with pSGF-Int-6, and immunofluorescence was performed with the FLAG antibody. The specificity of the immunofluorescent staining is indicated by the absence of signal in flanking untransfected cells. (**B**) Hoechst staining. (**C**) pSG-Tax was transfected in COS7 cells, and immunofluorescence was done with the Tax antiserum. (**D**) Hoechst staining. (**E** through **H**) Cells were transfected with pSGF-Int-6 together with pSG-Tax. (E) and (G) show visualization of Int-6, whereas (F) and (H) show staining of Tax in the same cells.

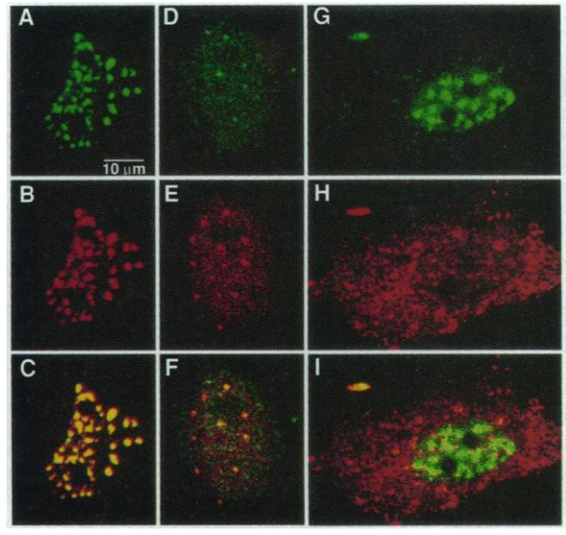

Fig. 4. Analysis by laser confocal microscopy of the colocalization of Int-6 and PML within nuclear bodies and the separate stainings of Tax and PML. (**A** through **C**) COS7 cells were transfected with pSGF-Int-6 and subjected to double immunofluorescence labeling: the FLAG antibody was used to detect the transiently expressed Int-6 protein (A), and a polyclonal antibody to PML (*15*) was used to visualize the endogenous PML protein (B). The single confocal images were superimposed, and the colocalization between the two stainings is visualized as yellow (C). (**D** through **F**) Nontransfected COS7 cells were double-labeled with a rabbit polyclonal antibody to a COOH-terminal peptide of Int-6 (*24*) to detect the endogenous protein (D) and with the 5E10 monoclonal antibody (*25*), which recognizes endogenous PML (E). An overlay of the single confocal images is shown in (F). (**G** through **I**) Confocal microscopy analysis showing the separate stainings of Tax and PML. COS7 cells were transfected with pSG-Tax and with pTL2-Myl that encodes PML (*13*). Immunofluorescence was performed with the 5E10 antibody (G) and Tax antiserum (H). (I) A superimposition of the single confocal images. In our experimental conditions, the 5E10 antibody reacted strongly against overexpressed PML but weakly against the endogenous protein, compared with the PML polyclonal antibody.

ural site of action. This activity is specific to Int-6 because Tax does not alter the POD structure, as shown by examination of PML localization in the presence of Tax. Further analyses will determine not only the importance of the interaction in the onset of adult T cell leukemia in patients infected with HTLV-I but also a possible role of Int-6 in human nonviral cancers.

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23. Complementary DNA from the Int-6 clone 88 was inserted in-frame with the FLAG epitope into pSG5, giving pSGF–Int-6. COS7 cells (0.7×10^6) were transfected with 1 μg of pSGF–Int-6 or 1 μg of pSG-Tax, or both. Cells were lysed in radioimmunoprecipitation assay buffer. The immunoprecipitates were separated on a 10% polyacrylamide–SDS gel and blotted onto nitrocellulose. The enhanced chemiluminescent detection system (ECL, Amersham) was used to visualize bound antibodies.
24. We generated a polyclonal rabbit antiserum to a peptide corresponding to the COOH-terminal 20 amino acids of Int-6 coupled to ovalbumin. To purify this serum, we produced a FLAG–Int-6 fusion protein in bacteria and coupled it to a FLAG M2 antibody affinity gel (Kodak). Covalent linkage between the protein and the M2 antibody was performed by treatment with glutaraldehyde. The antiserum to Int-6 was incubated with this matrix, and specific antibodies were eluted with 100 mM glycine (pH 2.5).
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Activation of the Budding Yeast Spindle Assembly Checkpoint Without Mitotic Spindle Disruption

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The spindle assembly checkpoint keeps cells with defective spindles from initiating chromosome segregation. The protein kinase Mps1 phosphorylates the yeast protein Mad1p when this checkpoint is activated, and the overexpression of Mps1p induces modification of Mad1p and arrests wild-type yeast cells in mitosis with morphologically normal spindles. Spindle assembly checkpoint mutants overexpressing Mps1p pass through mitosis without delay and can produce viable progeny, which demonstrates that the arrest of wild-type cells results from inappropriate activation of the checkpoint in cells whose spindle is fully functional. Ectopic activation of cell-cycle checkpoints might be used to exploit the differences in checkpoint status between normal and tumor cells and thus improve the selectivity of chemotherapy.

The spindle assembly checkpoint keeps cells with spindle defects from segregating their chromosomes. The defects the checkpoint detects include the absence of spindle microtubules (*1*, *2*), monopolar spindles (*3*), and the misalignment of a single chromosome on the spindle (*4*). Mutations in the budding yeast *MAD* (mitotic arrest defective) (*2*) or *BUB* (budding uninhibited by benzimidazole) (*1*) genes inactivate the checkpoint and allow cells with defective spindles to proceed through mitosis. Another checkpoint component, the essential protein kinase Mps1p (*5*, *6*), has two roles in the cell cycle; it is required for spindle pole body duplication in G_1 (*7*) and for the spindle assembly checkpoint in mitosis (*3*). We found that Mps1p directly phosphorylated Mad1p and that overexpression of Mps1p constitutively activated the spindle assembly checkpoint in wild-type cells.

Spindle depolymerization causes hyperphosphorylation of Mad1p that leads to a decrease in the protein's gel mobility during SDS–polyacrylamide gel electrophoresis (PAGE) (*8*). To determine whether spindle pole body defects also induced this modification, we examined the phosphorylation of Mad1p at 37°C in *cdc31*, *mps2*, and *mps1* mutants, which are all defective in spindle pole body duplication (*7*, *9*). The *cdc31* and *mps2* mutants, which induce mitotic arrest,

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