Original Paper

Journal of Biomedical Science

J Biomed Sci 1997;4:229–234

Received: June 1, 1997
Accepted: July 1, 1997

Christine Neuveut[a]
Dong-Yan Jin[a]
Oliver J. Semmes[a]
Francesca Diella[b]
Robert Callahan[b]
Kuan-Teh Jeang[a]

[a] Laboratory of Molecular Microbiology, National Institutes of Allergy and Infectious Diseases, and
[b] Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, Md.,USA

Divergent Subcellular Locations of HTLV-I Tax and Int-6: A Contrast between in vitro Protein-Protein Binding and Intracellular Protein Colocalization

Key Words
HTLV
Tax
Int-6
Transformation

Abstract
Protein-protein interactions define many important molecular and cellular processes in prokaryotic and eukaryotic biology. In trying to delineate the contact between two proteins, the yeast two-hybrid assay has emerged as a powerful technique. Complementing the yeast two-hybrid assay are in vitro techniques (e.g. GST-fusion-protein chromatography) that can also yield information on protein-protein associations. However, unambiguous functional significance to these interactions is best supported through a finding of colocalization of two proteins inside cells. In instances where two proteins interact in vitro but have divergent localizations within cells one needs to reconsider the biological importance of the former finding. Here, we present evidence for different subcellular locations of HTLV-I Tax and the Int-6 protein. We suggest a reexploration of the functional significance between Tax and Int-6 in cellular transformation.

The mechanism by which HTLV-I causes adult T-cell leukemia is incompletely understood. Hence, it was of significant interest when Desbois et al. [1] recently suggested that the exclusion of Int-6 from PML nuclear bodies by Tax might play a role in the transformation of cells. We have examined the interactions between Int-6 and Tax, and our findings are substantially different from those reported by Desbois et al. [1].

The *int-6* locus was first described in 1995 [3] from the laboratory of one of us (R.C.). Since then, six peptide-specific antibodies, each with varying degrees of affinity for Int-6, have been generated. When mouse HC11 mammary epithelial cells were stained with either of two antipeptide sera individually, Int-6-specific signal was seen in a perinuclear pattern consistent with localization into the Golgi apparatus [2]. In those experiments, we detected little nuclear presence of Int-6; thus, we were intrigued that Desbois et al. [1] localized Int-6 to nuclear PML bodies in primate cells [1]. Because the human [1] and mouse [3] Int-6 proteins are identical, our antisera should also recognize primate Int-6. We used one Int-6 specific peptide antibody (Anti-peptide 47; peptide 47 is a synthetic peptide corresponding to the YKNLYSDDIPHLREK sequence of Int-6. The peptide was conjugated to keyhole limpt hemocyanin. Antiserum against Int-6 peptide 47 was raised in rabbit by i.m. injections of the immunogen

KARGER
E-Mail karger@karger.ch
Fax + 41 61 306 12 34
http://www.karger.ch

Kuan-Teh Jeang
Building 4, Room 306
9000 Rockville Pike, NIH
Bethesda, MD 20892-0460 (USA)
Tel. + 1/301/496 66 80, Fax +1/301/402 02 26, e-mail kjeang@atlas.niaid.nih.gov

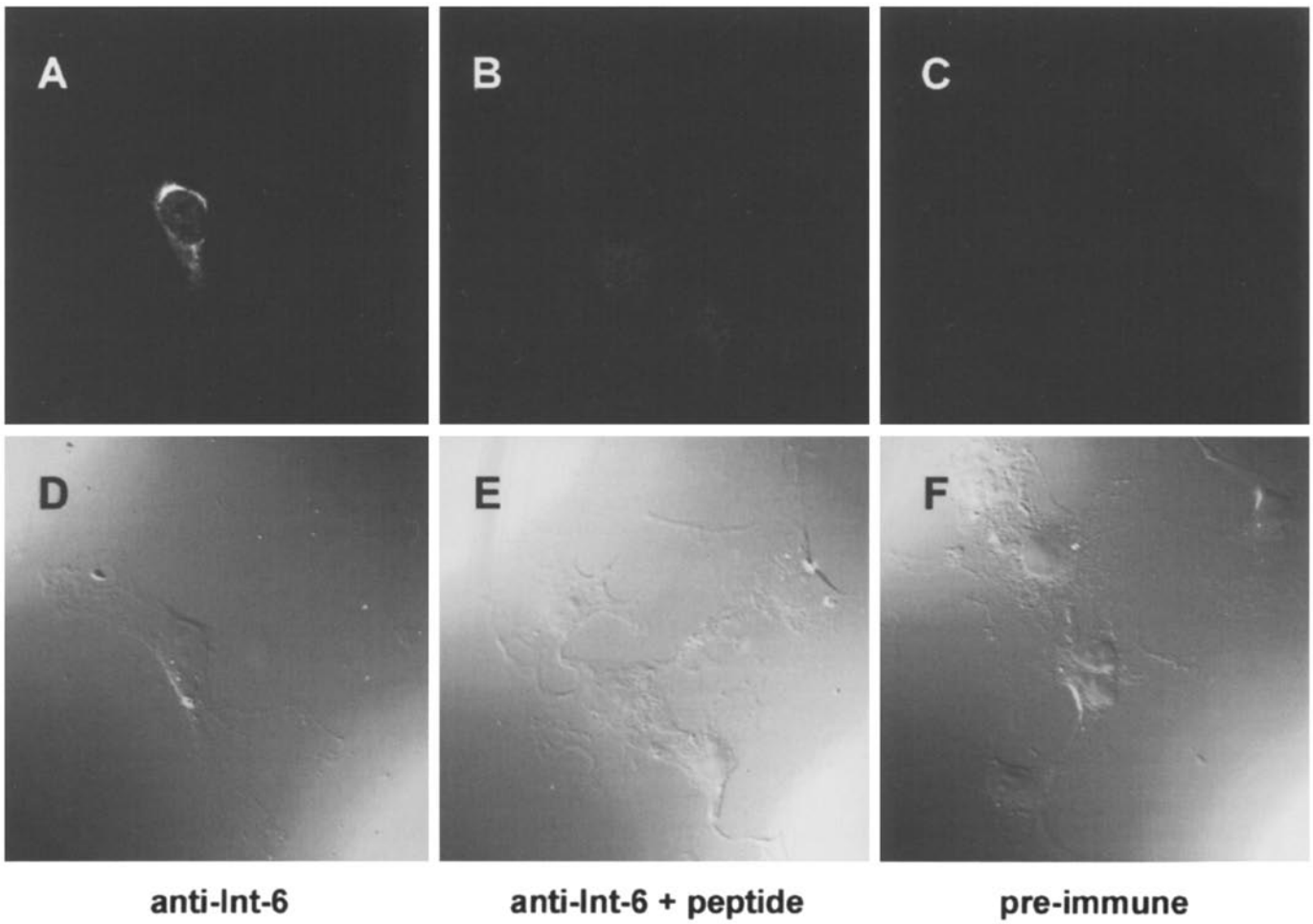


Fig. 1. Confocal fluorescent images demonstrating perinuclear cytoplasmic location of Int-6 in Cos7 cells. Cos7 cells were seeded onto coverslips for 24 h. Adherent cells were fixed for 15 min in 4% paraformaldehyde/PBS at room temperature. The cells were then washed three times in PBS and permeabilized with 100% methanol for 2 min at room temperature. Cells were washed three more times with PBS followed by preincubation in blocking solution (PBS containing 35% bovine serum albumin; BSA for 30 min at room temperature and were then incubated with primary antibody overnight in a moisturized chamber at 4 °C. As negative controls, cells were equilibrated either with primary antibody which has been preincubated for 4 h with saturating amounts of the immunizing peptide or with rabbit preimmune serum. After three more washes, cells were equilibrated with fluorochrome-conjugated secondary antibody for 1 h at room temperature. Slides were visualized by confocal optics, using a Zeiss Axiophot inverted microscope. **A** Cells stained with rabbit polyclonal anti-Int-6. **B** cells stained with anti-Int-6 which has been presaturated with immunizing peptide. **C** Cells stained with rabbit preimmune serum. **D–F** Respective light-field images of **A–C**.

in Freund's complete adjuvant according to standard protocol) to stain Cos7 (fig. 1) and HeLa (fig. 2) cells. Whereas Desbois et al. [1] found Int-6 in nuclear PODs (PML oncogenic domains) of Cos7 cells and stated (without showing data) that a similar localization was observed for HeLa cells, we found that in both cells Int-6 was nuclear-excluded and localized to perinuclear cytoplasmic regions (fig. 1A, 2A), consistent with that observed for mouse cells [2]. To verify staining specificity, we 'blocked' the antibody with the peptide used for immunization of rabbits. In such instance, Int-6 specific perinuclear staining was eliminated (fig. 1B, 2B).

Desbois et al. [1] also stained HTLV-I Tax diffusely with closely comparable intensities in both the nucleus and the cytoplasm of transfected Cos7 cells. While one cannot exclude the existence of a small amount of protein in the cytoplasm, most studies have found that Tax is expressed almost exclusively in the nuclear compartment

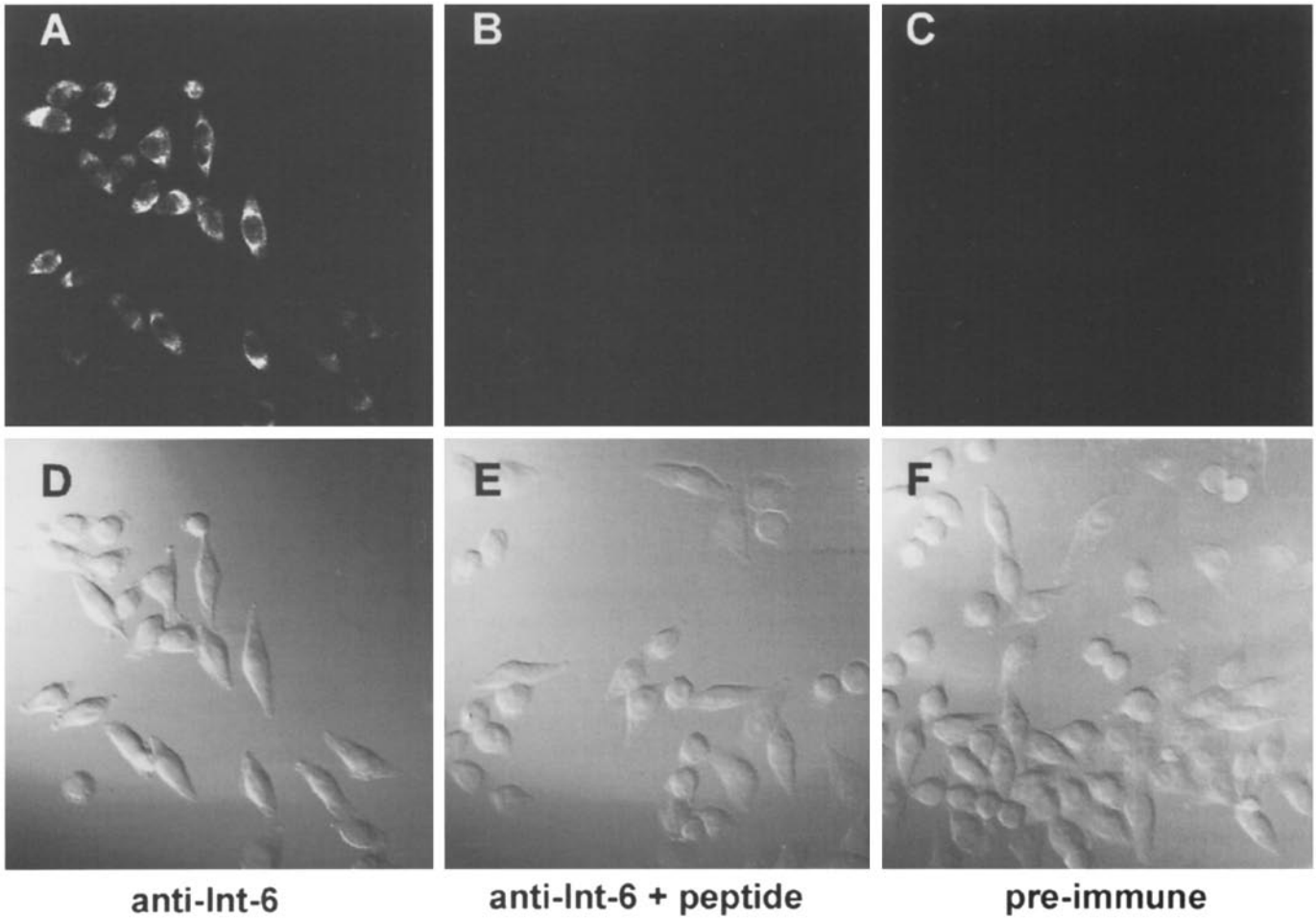


Fig. 2. Localization of Int-6 protein in the cytoplasm of HeLa cells. **A** Cells stained with anti-Int-6. **B** Cells stained with anti-Int-6 serum pre-absorbed with saturating amounts of blocking peptide. **C** Cells stained with rabbit pre-immune serum. **D–F** Corresponding light-field images.

of primate cells [5–7, 9]. We, nevertheless, checked to see if transfected Cos7 cells would show significant staining for Tax in the cytoplasm. In figures 3A and B, Cos7 cells transfected with a plasmid expressing wild-type Tax were stained with a Tax-specific antipeptide serum (antiserum was raised in rabbit against a peptide spanning amino acids 97 to 120 of HTLV-I Tax). In both fields of view, approximately 20–50% of the cells were expression-positive for Tax. All positive cells stained strongly in the nucleus with little increased fluorescence in the cytoplasm over the background intensities seen in negative cells (or cells stained with preimmune serum; not shown). This lack of cytoplasmic staining for Tax was constant with no detectable changes over a 72-hour period (data not shown). Because visual presentation of stained images is somewhat exposure-dependent, we wanted to compare directly the nuclear pattern of wild type Tax protein (fig. 3A, B) with what might be seen if Tax were localized into the cytoplasm. Hence, in panels C and D (fig. 3), Cos7 cells were transfected with plasmids expressing two Tax mutants. TxA274 and TxΔ2-58 are mutants that have been characterized previously to have aberrant subcellular localization and function [5]. Their staining patterns (diffusely cytoplasmic and nuclear for TxA274, panel C; and nuclear-excluded for TxΔ2-58, panel D) contrast significantly with the nuclear pattern for wild-type Tax (fig. 3A, B).

The above experiments suggest that Int-6 and Tax localize separately to cytoplasmic and nuclear compartments, respectively. To verify further these disparate localizations, two additional sets of experiments were performed. First, we costained Int-6 and Tax simultaneously

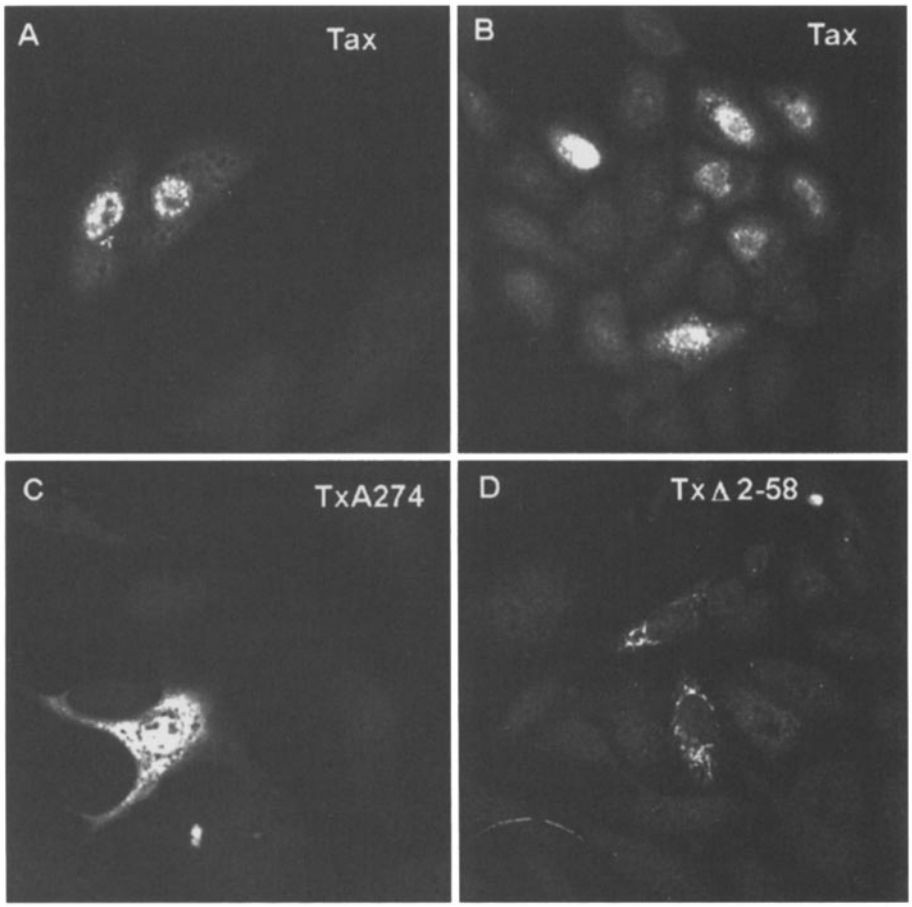
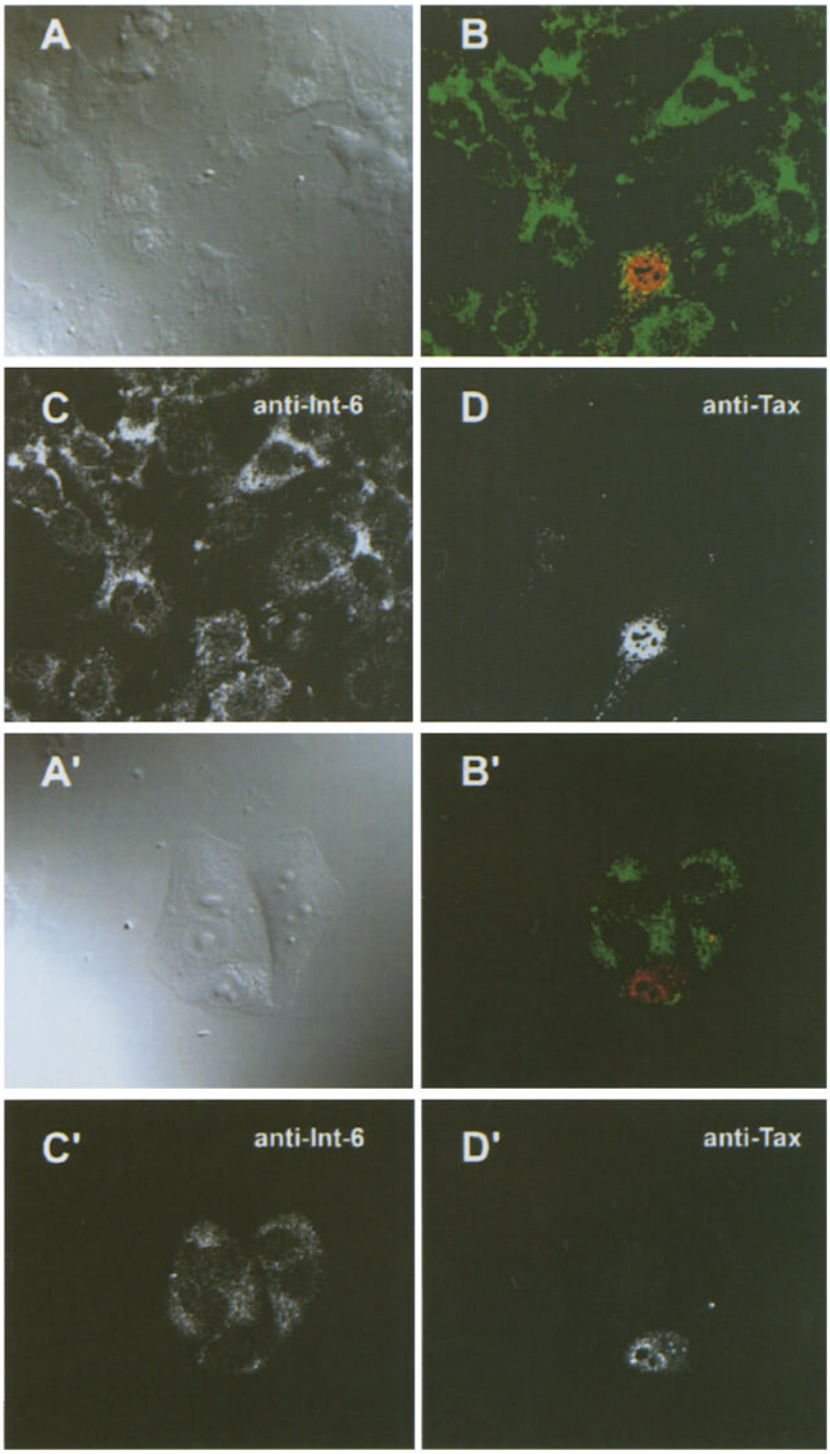


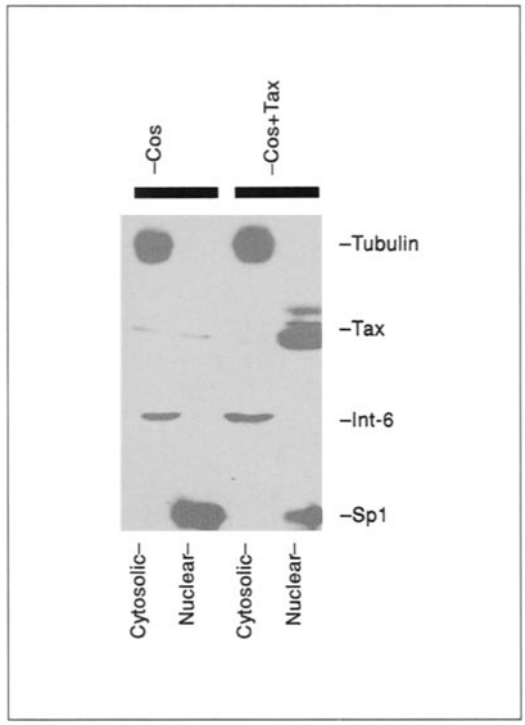
Fig. 3. HTLV-I Tax is expressed in the nucleus of Cos7 cells. **A, B** Confocal laser images of Cos7 cells transfected with a plasmid expressing wild-type Tax protein. Tax nuclear fluorescence was detected using a peptide antibody directed to amino acids 97 to 120. **C** Diffuse nuclear and cytoplasmic staining of Tax mutant, TxA274. **D** Nuclear-excluded staining pattern of Tax mutant, TxΔ2-58, in the cytoplasm. TxA274 and TxΔ2-58 have been described previously [4, 8].

in the same transfected Cos7 or HeLa cells (fig. 4). In this case, Int-6 was stained with rabbit polyclonal antibody (fig. 4C, C′), and Tax was stained with a mouse monoclonal antibody (fig. 4D, D′; Tax monoclonal 168A51-42 was obtained from the NIH AIDS Research and Reference Reagent Repository). Computer-generated 'colocalizations' of Int-6 and Tax are shown in figures 4B and 4B′. While there was slightly more signal scattering in Cos7 cells (probably due to overexpression), in neither case could one conclude on a significant colocalization between Int-6 and Tax. Second, we biochemically fractionated Cos7 or Cos7 cells transfected with a Tax-expressing plasmid (fig. 5). Cells were divided into cytosolic and nuclear portions using detergent (cytoplasmic fraction was obtained by lysis of cells on ice with 0.5% (v/v) NP-40 in 0.15 *M* NaCl/0.01 *M* Tris-HCl (pH 7.4) for 10 min. The lysed cells were pelleted, and the supernatant was kept as the cytoplasmic fraction. The 'nuclear' pellet was then washed with 50 m*M* NaCl/100 m*M* HEPES (pH 7.4)/2.5 m*M* $MgCl_2$/0.3 *M* sucrose/1% Tween40/0.5% deoxycholate for 30 s and centrifuged. Pelleted material was solubilized as the nuclear fraction). The different fractions were resolved by SDS-PAGE and transferred to the PVDF -membrane. Equivalent strips of membrane were probed individually with antiserum against tubulin (mouse monoclonal anti-tubulin antibody (clone DM 1A) was purchased from Sigma Chemical), Tax, Int-6, or Sp1 (rabbit polyclonal anti-Sp1 peptide antibody (PEP2) was purchased from Santa Cruz Biotechnology). The results showed that while tubulin and Int-6 were found in the cytosolic fractions, Tax and transcription factor Sp1 were in the nuclear fractions. At this level of resolution, little qualitative or quantitative difference was seen when comparing Int-6 from Cos cells without or with Tax expression (fig. 5).

Our findings differ from that of Desbois et al. [1] in two significant ways. First, we find that Int-6 is cytoplasmic before and after Tax is expressed in Cos7 cells. We have consistently failed to detect any semblence of Int-6 in nuclear PODs (although we have successfully stained for



4



5

Fig. 4. Simultaneous staining of Int-6 and Tax in Cos7 and HeLa cells. Cos7 (**A–D**) or HeLa (panels **A'–D'**) cells were transfected with a Tax expressing plasmid and then stained simultaneously with a rabbit polyclonal anti-Int-6 antibody and a mouse monoclonal anti-Tax antibody. Goat antirabbit-FITC and goat antimouse-Texas red conjugate were used as secondary antibodies, respectively. **A, A'** Light-field images. **B, B'** Superimposition of the two fluorescent signals. Green is Int-6, and red represents Tax. Little overlap between the two proteins was seen. **C, C'** Single fluorescence of Int-6 in the cytoplasm. **D, D'** Single fluorescence of Tax in the nucleus.

Fig. 5. Biochemical fractionation of Cos7 and Cos7-transfected with a Tax-expressing plasmid. Cells were separated into nuclear and cytosolic fractions. The individual fractions were solubilized in SDS-loading buffer and were resolved by SDS-PAGE. After transfer to PVDF membrane, equivalent strips were probed separately with anti-tubulin, anti-Tax, anti-int-6, and anti-Sp1. Panel shown is a composite of the relevant portions of the gel for the indicated antigens.

PODs in cells; data not shown). Second, we do not find any significant diffusion of Tax in Cos7 cells from the nucleus into the cytoplasm. In view of these discrepancies, we find it difficult to accord a direct physical association for Tax in redistributing Int-6 from a nuclear location into the cytoplasm [1]. While the results shown here relied on the specificity of a single Int-6 peptide antibody, the same patterns have been seen using a second anti-Int-6 peptide antibody. Detailed biochemical and subcellular characterization of Int-6 will be presented elsewhere [2].

We do not fully understand the reasons for the different findings. We do appreciate that immunostaining can present artifacts depending on stray specificities in the antiserum, methods of fixation, intactness of cell, and particular choices of stained cells for presentation. Hence, we urge that whenever possible blocking peptides be used to verify antibody specificity and that multiple views with many cells in the same field be included for illustration. Finally, since nearly one hundred different Tax point mutants [4, 8] with varying transformation phenotypes are available, one should not conclude on the biological relevance of Int-6 and Tax interactions without additional defining experiments.

Our results here underline the importance of examining in detail intracellular colocalization of proteins in order to accord functional significance to in vitro protein-protein interactions. Further experiments are needed to shed additional light on the biology of Tax-Int-6 interaction.

Acknowledgments

Work in the laboratory of K.T.J. is supported in part by the AIDS Targeted Anti-Viral Program from the Office of the Director, NIH. We are grateful to C.Z. Giam and R. Gaynor for discussions.

References

1 Desbois C, Rousset R, Bantignies F, Jalinot P. Exclusion of Int-6 from PML nuclear bodies by binding to the HTLV-I Tax oncoprotein. Science 273:951–953;1996.
2 Diella F, Levi G, Callahan R. In press.
3 Marchetti A, Buttitta F, Miyazaki S, Gallahan D, Smith HG, Callahan R. Int-6, a highly conserved, widely expressed gene, is mutated by mouse mammary tumor virus in mammary preneoplasia. J Virol 69:1932–1938;1995.
4 Semmes OJ, Jeang K-T. Mutational analysis of human T-cell leukemia virus type I Tax: regions necessary for function determined with 47 mutant proteins. J Virol 66:7183–7192; 1992.
5 Semmes OJ, Jeang K-T. Localization of human T-cell leukemia virus type 1 tax to subnuclear compartments that overlap with interchromatin speckles. J Virol 70:6347–6357;1996.
6 Siomi H, Shida H, Nam SH, Nosaka T, Maki M, Hatanaka M. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. Cell 55:197–209;1988.
7 Slamon DJ, Boyle WJ, Keith DE, Press MF, Golde DW, Souza LM. Subnuclear localization of the trans-activating protein of human T-cell leukemia virus type I. J Virol 62:680–686; 1988.
8 Smith MR, Greene WC. Indentification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes. Genes Dev 4:1875–1885;1990.
9 Smith MR, Greene WC. Characterization of a novel nuclear localization signal in the HTLV-I tax transactivator protein. Virology 187:316–320;1992.