Nucleolar Localization of the Nucleophosmin-Anaplastic Lymphoma Kinase Is Not Required for Malignant Transformation


Leukemia Research Fund Immunodiagnostics Unit, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom [D. Y. M., K. A. F. P., L. H. B.]; Department of Experimental Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105-2794 [D. B., M. U. K., S. W. M.]; and Unit Propre de Recherche Centre National de la Recherche Scientifique 8291 and Laboratoire Central d'Anatome Pathologique, Hospital University Center Toulouse Purpan, 31059 Toulouse Cedex, France [L. L., G. D.]

ABSTRACT

The (2;5)(p23;q35) lymphoma-associated chromosomal translocation creates a novel fusion gene that incorporates parts of the anaplastic lymphoma kinase (ALK) receptor tyrosine kinase and nucleophosmin genes. We report here that the product of this fusion gene accumulates within the nucleoli of neoplastic cells, and that previous reports of a predominantly cytoplasmic localization for the protein represent a tissue-processing artifact. However, nucleolar accumulation of nucleophosmin-ALK may not be necessary for its oncogenic action, because an ALK protein expressed in a lymphoma carrying a variant (1;2) chromosomal translocation did not accumulate in nucleoli. Furthermore, an engineered hybrid TPR-ALK protein can transform rodent fibroblasts and produce lymphomas in mice while remaining confined to the cytoplasm. We propose that the transforming action of ALK may not be reliant on its nucleolar localization, a hypothesis that may have implications for studies of other proteins involved in oncogenesis that are relocalized after the creation of fusion genes.

INTRODUCTION

Anaplastic large cell lymphoma is associated with a (2;5)(p23;q35) chromosomal translocation (1), which fuses the gene encoding the ALK receptor tyrosine kinase, found at 2p23, with the NPM gene at 5q35 (2). The resultant gene encodes a chimeric 80-kDa protein, constituting the NH2-terminal 40% of NPM linked to the entire intracellular portion of ALK (2–4). It is likely that expression of the activated ALK kinase is sufficient to induce malignant cell transformation: the kinase is potently transforming for fibroblasts in vitre (3, 4), and NPM-ALK expression in murine hematopoietic cells induces a transplantable lymphoma (5). However, the subcellular site of action of NPM-ALK has not yet been defined. We report here that NPM-ALK accumulates within the nucleoli of anaplastic large cell lymphoma cells carrying the (2;5) translocation but also provide evidence that this may not necessarily be its site of action.

MATERIALS AND METHODS

Cell Lines and Tissue Samples. The SUP-M2 and SU-DHL1 cell lines (anaplastic large cell lymphomas of T-cell phenotype, obtained from Dr. M. L. Cleary, Stanford University School of Medicine, Palo Alto, CA), the COS cell line (embryonic monkey kidney epithelium), and all transfected cells were maintained in culture in RPMI 1640 containing 10% FCS (Life Technologies, Inc., Paisley, Scotland) at 37°C in 5% CO2.

RESULTS

Tumor samples were obtained from the Department of Cellular Pathology, John Radcliffe Hospital and the Lymphoma Study Group at Purpan Hospital/University Center. RT-PCR analysis for the (2;5) translocation was performed as described previously (6). Fresh tissues were snap frozen in liquid nitrogen, and 6-μm cryostat sections were cut, fixed, and stored. Tissues for paraffin embedding were fixed in Bouin's fluid (Duboscq-Brasil) or formalin or dehydrated using the Modamex method (7).

Immunoprecipitation. Cells of the t(2;5)-containing lymphoma cell line SUP-M2 (2 x 107) were lysed under mild (1% Triton X-100, 150 mM NaCl, and 25 mM Tris, pH 7.4) or more stringent (radioimmunoprecipitation assay buffer: 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.2) detergent conditions. The lysates were clarified of insoluble material by a 2-min centrifugation at 14,000 rpm in a microfuge and then incubated for 2 h at 4°C with a 1:100 dilution of either preimmune serum or ALK1 polyclonal rabbit antisera (10). Immunoprecipitated proteins were electrophoresed on a 7.5% SDS-polyacrylamide gel under reducing conditions and then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) using a semidry blotting system (SemiPhor Transfer Unit; Hoefer Scientific Instruments, San Francisco, CA). Immunoblotting was performed with a 1:2000 dilution of an anti-NPM polyclonal antibody prepared using the recombinant full-length protein as an immunogen. The blot was detected by enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, IL).

Transformation of Rat Fibroblasts in Vitro and Lymphoma Induction in Mice with TPR-ALK. The TPR-ALK mutant was generated by using three-oligonucleotide PCR with the NPM-ALK (2) and TPR-MET (11) cDNAs as templates. Helper-free retrovirus was generated by transient hyperexpression in 293T cells and used at approximately equalized titers to infect Fischer rat 3T3 cells seeded at 2 x 105 cells per 100-mm tissue culture dish 24 h before infection. Foci were visualized by staining with Giemsa 14 days after infection. F3T3 colony formation in soft agar was assayed by plating 2 x 103 cells per 35-mm dish after infection with retroviral stock in 0.6% Noble agar in Iscove's Modified Dulbecco's medium containing 15% FCS. Colonies were photographed 14 days after plating. Transduction of murine bone marrow cells using TPR-ALK retrovirus stock, transplantation of lethally irradiated mice, and the characterization of lymphomatous tumors were performed as described previously for similar studies with NPM-ALK (5).

Immunocytochemical staining of the SU-DHL1 cell line (derived from a case of anaplastic large cell lymphoma carrying the (2;5) translocation), using monoclonal antibody ALK1 (9) and also a pre-
Nucleolar localization of NPM-ALK chimeric kinase

Previously described polyclonal antiserum (10), both of which recognize the full-length ALK receptor protein and the NPM-ALK fusion protein, showed that the most intense labeling was seen over nucleoli (Fig. 1a). All nucleoli within an individual cell were labeled, and the reactivity was diffuse within each nucleolus. Furthermore, nucleolar labeling was seen in COS cells expressing NPM-ALK after transfection with the hybrid gene. The proportion of ALK-positive cells with nucleolar reactivity, however, increased with time after transfection (Fig. 1b), suggesting that the protein was accumulating in the cytoplasm before passing into the nucleus and thence to the nucleolus. In contrast, no nuclear or nucleolar labeling was seen in cells expressing full-length ALK, namely COS cells transfected with the ALK gene (Fig. 1c) or the Rh30 rhabdomyosarcoma cell line (data not shown) (2, 10).

Nucleolar accumulation of NPM-ALK also occurs in vivo, because in four cases of anaplastic large cell lymphoma expressing NPM-ALK, the strongest labeling was associated with nucleoli (Fig. 2a). In published immunocytochemical studies of NPM-ALK expression in anaplastic large cell lymphoma samples, labeling

**Fig. 1.** Immunocytochemical staining for ALK in cells expressing NPM-ALK and full-length ALK. **a:** left, in the SU-DHL1 cell line, which carries the (2;5) translocation, all cells are labeled, and the strongest staining is associated with nucleoli (arrows). Cells in mitosis (arrowhead) show diffuse cytoplasmic labeling. **Right,** no labeling of these cells is seen using an unrelated primary antibody (MR12). **b,** COS cells transfected with an NPM-ALK construct show staining that is initially present in the cytoplasm but progressively accumulates within nucleoli. **c,** COS cells transfected with the full-length ALK construct and harvested after 72 h show no nuclear or nucleolar labeling.
Fig. 2. Immunocytochemical demonstration of ALK proteins in tissue sections from cases of anaplastic large cell lymphoma. a, in a “classical” case carrying the (2:5) translocation, neoplastic cells are strongly stained in a cryostat section using the monoclonal antibody ALK1, but nucleoli stand out as the most strongly labeled structures within the tumor cells (the arrow indicates a cell in mitosis). Essentially the same staining pattern was obtained with a polyclonal ALK antiserum (Ref. 10; data not shown). However, in a paraffin-embedded tissue section from the same case, reactivity is strongest in the cytoplasm, and there is little or no nuclear reactivity. This case (and two of the other three cases giving a positive immunocytochemical reaction) were tested by RT-PCR for the (2:5) translocation, and all were positive. Neg, results obtained with an unrelated primary antibody (MR12). b, in an anaplastic large cell lymphoma carrying the variant (1:2) translocation, stained both as routinely processed, paraffin-embedded tissue and as Modamex-processed tissue (which gives results comparable to cryostat sections; Ref. 7), nuclei and nucleoli are negative. Nucleolar labeling (arrows) in a Modamex-processed t(2;5)-positive anaplastic large cell lymphoma is shown for comparison.
NUCLEOLAR LOCALIZATION OF NPM-ALK CHIMERIC KINASE

has been described (when specified) as being mainly cytoplasmic (6, 9, 12-15), with little nuclear staining and no mention of nucleolar reactivity. However, these studies have been based on paraffin-embedded samples, whereas our immunostaining was performed on frozen tissue sections. When we immunostained paraffin-embedded samples from the four cases of NPM-ALK-positive anaplastic large cell lymphoma referred to above, malignant cell staining was also most prominent in the cytoplasm (Fig. 2a). The nuclei of some positive cells were also weakly stained, but nucleioli were not preferentially labeled. We therefore interpret the pattern of predominantly cytoplasmic labeling (and weaker diffuse nuclear staining) previously reported for NPM-ALK in paraffin-embedded biopsies (with both polyclonal and monoclonal ALK antibodies) as representing diffusion of the protein from within nuclei during tissue processing.

The NPM-ALK fusion protein lacks NPM nuclear localization signals (2), but it could be directed to the nucleus and the nucleoli via association with the normal 38-kDa NPM protein, because normal NPM protein is known to form homo-oligomers in vivo (through a yet-to-be characterized motif localized within its NH₂-terminal amino acid residues) and to function as a bidirectional shuttle protein between the cytoplasm and the nuclear compartment (16). We obtained evidence for this mechanism by showing that anti-ALK immunoprecipitates from t(2;5)-positive lymphoma cell lysates contain comparable amounts of both NPM and NPM-ALK, indicating that the proteins heterodimerize (Fig. 3). Although only a small fraction of the very abundant NPM protein is complexed with NPM-ALK, their coprecipitation, using a COOH-terminal epitope-specific anti-NPM monoclonal antibody that does not recognize the NPM residues present in NPM-ALK, was also demonstrable (data not shown). In addition, NPM and NPM-ALK were observed to co-migrate in sucrose density gradients as oligomeric complexes of greater than 150 kDa in mass (4; data not shown). Further evidence of the ability of NPM-ALK to associate with NPM was obtained using a yeast two-hybrid system; in screens of a human fetal brain cDNA library performed using the full-length chimera as bait, approximately 40% of the clones identified encoded NPM.⁴

These data suggest that the nucleolar accumulation of NPM-ALK contributes directly to the pathogenesis of anaplastic large cell lymphoma. However, we have previously reported a case that exhibited the typical clinical and histopathological features of this disease but in which a variant (1;2)(q25;p23) chromosomal translocation was present, with the breakpoint on chromosome 2 at the site of the ALK gene (6, 9). RT-PCR analysis of this case showed no evidence of NPM-ALK mRNA transcripts. No frozen tissue was available from this case, but immunocytochemical labeling of a sample processed by the Modamex method, which avoids many of the artifacts associated with routine tissue fixation and embedding (7), showed that an ALK protein was expressed, but only in the cytoplasm (Fig. 2b), with no evidence of nucleolar reactivity. This contrasted with a Modamex-processed, t(2;5)-positive anaplastic large cell lymphoma sample tested at the same time, in which the expected nucleolar labeling was clearly seen (Fig. 2b).

This "experiment of nature" suggested that nucleolar localization of ALK kinase activity is not a requisite for its oncogenic potential, and we have obtained additional evidence of this hypothesis using a constitutively active, truncated ALK mutant. In this protein, the NPM segment of NPM-ALK was replaced with the portion of TPR that is present in the transforming TPR-MET chimeric protein (11). This TPR moiety contains a coiled coil motif that mediates homodimerization (11) and thereby can activate not only the truncated MET tyrosine kinase but also the TRK-A kinase domain in human papillary thyroid tumors (17) and an oncogenic RAF serine/threonine kinase in rat tumors (18). In each instance these TPR fusion proteins remain confined to the cytoplasm.

The TPR-ALK mutant protein caused transformation of Fischer rat 3T3 fibroblasts in vitro with nearly the same efficiency as NPM-ALK (Fig. 4a). Furthermore, retroviral transduction of murine bone marrow with TPR-ALK resulted in the development of large cell immunoblastic B-cell lymphomas in transplanted recipient mice that were identical in appearance and immunophenotype to those described previously with NPM-ALK (5) (Fig. 4b). Immunolocalization confirmed that the TPR-ALK protein was confined to the cytoplasm (Fig. 4c). Interestingly, an engineered NPM-MET chimeric protein also transformed 3T3 fibroblasts, albeit with lower efficiency than NPM-ALK or TPR-MET, and was localized to the nucleolus by immunostaining (data not shown).

DISCUSSION

There are now several recognized examples of neoplasia-associated proteins that relocate within the cell when gene fusion leads to their involvement as part of a novel chimeric protein. For example, the BCR-ABL fusion protein created in chronic myeloid leukemia by the (9;22) chromosomal translocation is found in the cytoplasm, whereas wild-type ABL is located within the nucleus (19). Furthermore, in cases of acute promyelocytic leukemia carrying the (15;17) chromosomal translocation, the PML transcription factor is relocated within the nucleus (after its fusion to retinoic acid receptor α) to multiple small nuclear bodies (20, 21).

Fig. 3. NPM-ALK is physically associated with normal NPM in a t(2;5)-positive lymphoma cell line (SUP-M2), as shown by Western blotting for NPM in anti-ALK immunoprecipitations. This association was seen under both mild (Lane 3) and stringent (Lane 7) lysis conditions and showed high stoichiometry, approaching a 1:1 ratio, suggesting that most of the cellular NPM-ALK is bound to NPM. The sample in Lane 4 is a positive immunoblotting control and represents 25 µg of a total cell lysate of the SUP-M2 line prepared in boiling Laemmli sample buffer and loaded directly on the SDS-polyacrylamide gel. Negative controls constitute an immunoprecipitation from the same cell line with preimmune anti-ALK (PI) and an immunoprecipitation from the t(2;5)-negative chronic myelogenous leukemia cell line (K562) with immune anti-ALK (I).

⁴ S. W. Morris, unpublished data.
The assumption is commonly made in such instances that the new sites to which the fusion proteins are located are also the sites of their oncogenic action. However, the data reported above question this assumption for NPM-ALK, because ALK appears able to transform cells even when its fusion partner shows no ability to locate to the nucleoplasm and nucleolus. The role of the NPM gene in lymphomas carrying the (2;5) translocation is therefore probably to provide a promoter that drives expression in lymphoid cells of the otherwise transcriptionally silent ALK gene, which is normally expressed only within the nervous system (10, 22), and also to activate the ALK kinase domain (by providing a homodimerization motif that mimics ligand-induced cross-linking) (3, 4). The accumulation of the chimeric protein in the nucleolus may not be relevant to its oncogenic action, although, in the absence of more detailed experiments (e.g., targeting of ALK to the nucleus through partners other than NPM), this remains to be formally proved.

By the same token, there is no a priori reason to assume that the chimeric NPM-myelodysplasia/myeloid leukemia factor 1 nucleolar protein produced by the acute myeloid leukemia-associated (3;5) chromosomal translocation that contains NPM (the portion present in NPM-ALK plus an additional 58 more COOH-terminal amino acids) fused to the novel normally cytoplasmic protein myelodysplasia/myeloid leukemia factor 1 (23) must exert its effect within the nucleolus. The same point is valid for the NPM-retinoic acid receptor α chimeric protein [produced by the (5;17) translocation in rare cases of acute promyelocytic leukemia], which presumably also accumulates within the nucleolus (24).

It may be commented that the assumption that the sites at which chimeric products of fusion genes most obviously accumulate are also their probable sites of action is understandable one, because in normal cells the force of evolutionary pressure means that proteins are rarely found at sites irrelevant to their function. However, to make this assumption is to forget that oncogenic events are not shaped by the honing process of evolution; the only selection pressure to which they are subject is the requirement that they should favor cellular malignant change. The striking, although pathologically unimportant, nucleolar accumulation of NPM-ALK represents such an instance.

ACKNOWLEDGMENTS

We thank Dr. P. K. Chan (Baylor University School of Medicine, Houston, TX) for generously providing anti-NPM antibody. The excellent technical assistance of Dr. Xiaoli Cui is also gratefully acknowledged.
REFERENCES


Nucleolar Localization of the Nucleophosmin-Anaplastic Lymphoma Kinase Is Not Required for Malignant Transformation


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/5/1057