Interest in acute promyelocytic leukemia (APL), the M3 subtype of the French-American-British (FAB) classification of acute myeloid leukemias, has recently grown enormously because of its paradigmatic behavior with respect to three distinct and unique features: i) accumulation in the bone marrow of tumor cells with promyelocytic features; ii) invariable association with specific translocations which always involve chromosome 17 and the retinoic acid receptor α (RARα) locus; iii) exquisite sensitivity of APL blasts to the differentiating action of retinoic acid (RA). From this point of view APL has become the paradigm for therapeutic approaches utilizing differentiating agents. The last five years have been crucial for the understanding of the molecular basis of APL. RARα translocates in 99% of cases to a gene located on chromosome 15 that we initially named myl and is now known as PML. In a few cases RARα variably translocates to chromosome 11, where it fuses to the PLZF gene or to a gene, also on 11, which has not yet been characterized. In addition, RARα is also found translocated to chromosome 5, where it fuses to the NPM gene. The cloning of variant translocations in APL and comparative analysis of their associated products is crucial for the understanding of the molecular etiopathogenesis of the disease. Functional analysis of the various fusion proteins as well as RARα partners is revealing strikingly common features beneath a misleading structural heterogeneity which unravels a possible unifying molecular mechanism towards APL leukemogenesis.

Key words: APL, chromosome translocations, gene rearrangement, RARα

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APL is, in the majority of patients, associated with a reciprocal translocation between chromosome 15 and chromosome 17 (Figure 1).3-6 At the molecular level the breakpoint on chromosome 17 lies within the retinoic acid receptor α (RARα) locus. The breakpoints on chromosome 15 cluster within a locus originally called myl and now named PML (for promyelocytic leukemia zinc finger (PLZF).12,13

In a few cases, the translocation involves chromosome 11 instead of chromosome 15 and a newly identified gene named promyelocytic leukemia zinc finger (PLZF).12,13

In only 2 cases described so far the translocation has involved chromosome 5 and the nucleophosmin gene (NPM) (ref. #14 and our unpublished results). The common feature of the 3 translocations is the involvement of chromosome 17 and the RARα locus. As a consequence of the translocation two fusion genes are produced, and, at least in the t(15;17) and t(11;17)
cases, both of these are transcribed into their respective fusion proteins (Figure 2).

Very recently a new and as yet uncharacterized case of APL was reported in which once again RARs translocates to chromosome 11 but not to the PLZF locus (Table 1).¹⁵

RARα

RARs are members of the super family of nuclear hormone receptors that are involved in fundamental biological processes such as development and differentiation.¹⁶-¹⁹ They act as ligand inducible transcriptional activators which are able to recognize and bind to retinoic acid responsive elements (RAREs) located in the promoter/enhancer regions of RA-responsive genes.²⁰ In addition to the RARs, a second class of nuclear retinoid receptors, RXRs, exist.²¹,²² These are able to heterodimerize with RARs and other nuclear receptors in order to facilitate their binding to RAREs (in the case of RARs) and the other response elements.²² RARs, RXRs, are able to recognize and bind to RAREs (in the case of RARs) and the other response elements.²² RARα isoforms are the predominant RAR expressed in hemopoietic cells.²⁶ Recently it has been shown that HL60 myeloid cells are rendered unresponsive to the differentiating activity of RA by a mutation in the RARα gene.²⁷ The resistance is overcome by transfecting the wild type RARα cDNA.²⁸ In addition, a dominant negative form of RARα introduced into multipotent murine FDCPmix A4 cells can cause a switch in their differentiation program from the neutrophil/monocyte lineage to that of basophil/mast cell.²⁹ These findings implicate RARα in the control of normal differentiation of myeloid cells. However, it has also been found that mice lacking all RARα isoforms do not show any remarkable disturbance of hemopoiesis and that other forms of RARs or RXRs can restore RA-induced differentiation of RA resistant HL60 cells.³⁰,³¹

PML, PLZF and NPM gene structure and nuclear localization

PML is a member of a family of proteins which share a novel zinc finger binding motif termed RING, and one or two additional Cys/His-rich regions (B-Boxes) followed by a predicted coiled-coil domain.³²-³⁴ This family contains genes with no apparent functional similarity, although some of them are transcription factors and two other members (T18/HPRR and RFP) are, like PML, found to be involved in oncogenesis as fusion proteins.³⁵-³⁷ Recently, a RING finger domain has been identified in the breast cancer susceptibility gene BRCA1, with one of the identified predisposing mutations resulting in a deletion of the RING finger.³⁸,³⁹

The RING and B-Boxes are located at the PML N-terminal end, followed by an α-helical domain, a coiled-coil region and, at its C-terminal end, a serine/proline-rich region where PML is phosphorylated. No clear function has yet been attributed to PML RING and B-box domains, and at present no experimental evidence that those regions have DNA binding capacities is available.⁴⁰,⁴¹ The coiled-coil region is responsible for the formation of stable PML homodimers.⁴² The RING finger, B-boxes and coiled-coil domains are retained in all 13 PML isoforms identified so far.⁴³ PML is a nuclear phosphoprotein which is detectable as part of structures associated with the nuclear matrix called nuclear bodies (NBs) or PML oncogenic domains (PODs).⁴⁴-⁴⁶ PML is not rigidly confined to the NBs, but represents a protein with the capacity to shuttle between the nucleus and the cytoplasm (ref. #48, #49 and our unpublished results).

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### Table 1. Occurrence of variant translocations in APL

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Frequency</th>
<th>Genes involved</th>
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</thead>
<tbody>
<tr>
<td>t(15;17)</td>
<td>99.9%</td>
<td>PML (15); RARα (17)</td>
</tr>
<tr>
<td>t(11;17)</td>
<td>6 cases</td>
<td>PLZF (11); RARα (17)</td>
</tr>
<tr>
<td>t(11;17)</td>
<td>1 case</td>
<td>Uncharacterized gene (11); RARα (17)</td>
</tr>
<tr>
<td>t(5;17)</td>
<td>2 cases</td>
<td>NPM (5); RARα (17)</td>
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The function of these NBs is unclear. There is evidence that a number of proteins associated with specific metabolic activities, such as pre-mRNA processing and DNA replication, show specific localization to certain subnuclear domains. The small nuclear ribonucleoprotein particles (snRNPs), which are the major subunits of spliceosomes, for example, show a punctuate pattern of distribution that also results from their association with specific subnuclear structures. PML domains are distinct from structures containing splicing factors. A number of other proteins are detectable in the NBs, among them the SP100 protein, originally identified as an autoantigen in patients with primary biliary cirrhosis and also known to be inducible by interferon.

In the promyelocytic cell line NB4, as well as in promyelocytes from APL patients, the normal localization pattern of PML is abolished and aberrant structures into which PML, RAR and the PML/RAR fusion protein co-localize become apparent. Co-localization of PML/RAR and RXR to distinct nuclear structures has also been demonstrated in NB4 cells, with RXR showing a diffuse nuclear staining pattern in control HL60 cells. Therefore PML/RAR has the ability to direct PML, RAR, RXR and presumably other nuclear antigens into these aberrant structures, thereby diverting them from their natural sites of action. If APL cells are treated with RA, the aforementioned proteins reacquire their natural nuclear localization and PML relocates to the NBs. For these reasons delocalization of PML from the NBs and delocalization of the other proteins from their natural sites of action by PML/RAR is thought to represent a crucial event in the pathogenesis of APL.

PLZF is also found in the nucleus, where it shows speckled localization similar to that of PML in the NBs, although the two proteins do not show completely overlapping nuclear distribution. PLZF is a phosphoprotein and a member of the large group of C2H2 zinc finger-containing transcription factors typified by the Drosophila gap gene Krüppel. PLZF has definite DNA binding and transcriptional effector activities and, in addition to the zinc finger motif, contains a POZ (POxvirus and Zinc finger) domain. This region was initially identified in a protein called ZID and was shown to facilitate protein-protein interactions as well as confer transcriptional repressor activity to the transcription factor that contains the domain. The PLZF POZ domain represents the most highly conserved region between avian and mammalian PLZF sequences, suggesting a crucial functional role. We named the proteins sharing the POZ and the Krüppel DNA binding domain POK proteins (POZ and Krüppel) (Figure 3). At present the family is composed of at least 9 members. Interestingly, BCL-6, another POK protein, is involved in lymphomagenesis. The PLZF protein is expressed as at least two isoforms which differ in sequences encoding its N-terminal region.

NPM (nucleophosmin, protein B23, NO38, numatrin) is a major nonribosomal nucleolar phosphoprotein which is significantly more abundant in tumor and growing cells than in normal resting cells. At its N-terminal end NPM displays a potential protein kinase C phosphorylation site and a potential metal binding motif (Figure 2). NPM shows high binding affinity to single-stranded nucleic acids. NPM is not rigidly confined to the nucleolus but shuttles, like PML, between the nucleus and the cytoplasm. Finally, NPM has the capacity to oligomerize.

NPM was previously found to be involved in a translocation between chromosome 2 and chromosome 5 associated with anaplastic large cell lymphomas (ALCL). In this translocation it is fused to the novel anaplastic lymphoma kinase (ALK) gene. In addition, NPM is fused to the MLF1 gene as a result of a translocation between chromosome 3 and chromosome 5, which is associated with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).

PML, PLZF and NPM in normal hemopoiesis

In the bone marrow PML is highly expressed in cells of the myeloid lineage, while it shows minimal or no expression in mature circulating polymorphonucleates and monocytes.
Nevertheless, in those cells as well as in tissue macrophages, PML expression is specifically induced, at the transcriptional level, by class I and II interferons. That is to say that PML expression declines in fully differentiated myeloid cells, but its expression is restored in response to interferon. Within the hemopoietic compartment and among cell lines of hemopoietic origin, PLZF expression seems to be restricted to the myeloid lineage. PLZF is also expressed in CD34+ hemopoietic progenitors. In this respect it is noteworthy that MZF-1 and Egr-1, other members of the Krüppel family, have been shown to regulate myeloid differentiation in vitro. In the myeloid cell lines NB4 and HL60, treatment with RA induces granulocytic differentiation of the cells and PLZF expression is concomitantly down-regulated. However, treatment of the human teratocarcinoma cell line NT2/D1 with RA, which also results in terminal differentiation of the cells into a wide variety of tissues of different histological origin, does not result in down-regulation of PLZF expression. This would indicate that the down-regulation observed in the myeloid cell lines reflects an event specific to the terminal differentiation of myeloid cells and not a direct effect of RA on PLZF expression per se. In this respect PLZF seems to mimic PML, which is also down-regulated and minimally or not expressed in mature circulating myeloid cells. We are at present testing whether interferon is also capable of re-inducing PLZF expression in terminally differentiated myeloid cells.

NPM is equally expressed in myeloid and lymphoid cell lines (our unpublished results). Nothing is known about the regulation of its expression in bone marrow hemopoietic precursors or during myeloid and lymphoid terminal differentiation. Because of its involvement in three different translocations associated with APL, MDS, AML and ALCL, NPM appears to be a promiscuous partner in translocations specifically associated with lymphohemopoietic tumors.
PML, PLZF and NPM genes in the control of cell growth and tumor suppression, and during the cell cycle

PML localization and expression is cell-cycle regulated. PML is in fact highly expressed in late G1 and S phases (refs. #47, #49 and our unpublished results).

PML overexpression suppresses anchorage-independent growth and tumorigenicity of NB4 cells in nude mice, blocks the oncogenic transformation of rat embryonic fibroblasts by cooperative oncogenes (Ras and a mutated p53), suppresses oncogenic transformation of neotransformed NIH/3T3 cells, and slows down growth of HeLa, CHO, A549 and NIH3T3 cells.49,77,78 PML overexpression also markedly reduces the size of tumors generated in nude mice by injection with HeLa cells.49 With this in mind, the inactivation of PML could become a critical event in the pathogenesis of APL, leading to loss of function as a growth suppressor and resulting in growth stimulation. A possible relationship is therefore emerging between the role of PML in APL transformation and its capacity to act as a growth suppressing factor whose expression is modulated by interferon.

Initial observations reveal that PLZF, like PML, shows growth suppressive behavior in cell lines such as NIH3T3 and HeLa (our unpublished results). In addition, similarly to PML, PLZF expression appears to be modulated during the cell cycle, where it peaks in late G1/S phase (our unpublished results).

NPM transcription and translation are also cell-cycle related, reaching peak levels just before entry into S phase and declining to baseline just before the onset of G2.79 Induction of mitogenesis in B-lymphocytes is characterized by significant increases in NPM synthesis, suggesting that this protein may be associated with the transduction of mitogenic signals.63,80

Molecular genetics of APL

X/RARα and RARα/X fusion products and aberrant X products

In APL, RARα variably fuses to PML, PLZF and NPM (X proteins). The various translocations are always balanced and reciprocal, and therefore two categories of products are generated: X/RARα and RARα/X. In addition, in APL blasts associated with the 15;17 translocation, a third category of transcripts encoding for aberrant PML products are coexpressed with the PML/RARα and RARα/PML transcripts:

The 15;17 translocation. As a consequence of t(15;17), two fusion transcripts can be expressed: PML/RARα and RARα/PML.13,81 PML/RARα is expressed in all t(15;17) APL cases. In leukemic blasts, it coexists with a truncated form of PML generated by alternative splicing of the PML portion of the PML/RARα fusion gene that puts the longest RARα open reading frame out of frame, which is referred to as aberrant PML.11 This truncated PML product lacks regulatory elements of the protein: the phosphorylation site and the C-terminus, but retains the RING+B-Box domains and the capacity to heterodimerize with the normal PML protein via the dimerization interface.

RARα/PML is expressed in only 70% of cases and it retains only RARα and PML regulatory domains.81 Recently, however, cases of APL characterized by the presence of RARα/PML transcripts and the absence of any detectable PML/RARα products have been described, suggesting a more important etiopathogenetic role for the former molecule.82

The PML/RARα protein retains the PML RING, B-Boxes and coiled-coil regions fused to most of the functional domains of the RARα protein (domains B to F), including its DNA and RA binding domains, and is able to heterodimerize with RXRs and wild type PML (refs. #8-10, 42, 83 and Figure 2). It is present in excess in APL cells and has the potential to interfere with both PML and RARα endogenous signaling pathways.11,42,83 This may be accomplished through sequestration of RXR from RARs and the other nuclear receptors, thereby interfering with their ability to bind to their respective response elements. The PML/RARα-RXR heterodimer is able to bind to retinoic acid responsive elements and PML/RARα as a homodimer is also able to bind, thus generating competition for specific DNA binding sites of the wildtype nuclear receptor complexes.8,83
The 11;17 translocation. As a consequence of t(11;17), two fusion transcripts can also be expressed: PLZF/RARα and RARα/PLZF.12 The PLZF/RARα chimeric protein results from fusion of the POZ domain and the first 2 or 3 zinc-fingers of PLZF to RARα B-F domains (ref. #12 and Figure 2). Similarly to PML/RARα, PLZF/RARα has the capacity to heterodimerize with PLZF via the POZ domain, and with RXR via the RARα portion.42,59 The reciprocal RARα/PLZF fusion transcript, which is found in all patients studied so far, encodes for a protein that retains one RARα transactivation domain fused to the last seven PLZF zinc fingers.12 This protein therefore retains the potential capacity to bind DNA, and it could play a significant role in APL leukemogenesis. When transfected into myeloid cell lines (HL60, U937), the PLZF/RARα fusion protein exerts a dominant negative effect on the transactivation mediated by endogenous RARα, as well as on co-transfected wild type RARα and RXR, over a large range of concentrations of ATRA.84 In this sense it behaves much the same as the PML/RARα fusion molecule in terms of antagonizing the function of RARα transcriptional activities in the absence of RA.4 In contrast, at pharmacological doses of RA, PML/RARα dominant negative effects are released, whereas this dual behavior is not reproduced by PLZF/ RARα.8,84

It is of note that, clinically, the presence of the two variant translocations involving chromosomes 15 and 11 results in two distinct syndromes. Patients harboring t(15;17) are uniquely sensitive to treatment with all-trans retinoic acid (ATRA), which yields complete remission rates of 75% to 95%.85 They are also highly responsive to conventional chemotherapy. However, APL associated with t(11;17) shows a distinctly worse prognosis, with poor response to chemotherapy and little or no response to treatment with ATRA.86

The 5;17 translocation. The NPM/RARα fusion protein retains the same RARα domains present in the chimeric products generated by the other two translocations, as well as a large portion of the NPM protein, including a putative metal binding domain. This has no structural similarities either to the PML or to the PLZF portion fused to RARα (Figure 2, and ref. #14). Two NPM/RARα fusion molecules have been described so far and found to coexist in the APL blast: the short and long forms.14 The long NPM/RARα form retains an intervening in-frame sequence, which is placed between NPM and RARα sequences and is probably of intronic origin.14

NPM/RARα can also heterodimerize with NPM and RARα/RXR since the regions that mediate NPM and RARα/RXR homodimerization are retained in the fusion moiety.14,67

The unifying hypothesis

At a first glance it therefore appears that the contribution of the three different RARα partners to the chimeric products is, at a structural level, dramatically different and that the only common feature among the X/RARα fusion proteins is the presence of the B-F RARα domains (Figure 2). Despite this diversity, preliminary characterization of the biochemical properties of the three X/RARα fusion mole-
1) X/RARα proteins have the capacity to heterodimerize with PML, PLZF or NPM, since the regions which mediate PML, PLZF and NPM homodimerization are retained in the fusion moiety. Similarly, the RARα portion is able to mediate heterodimerization with RXR. 

2) X/RARα proteins retain the RARα capacity of binding DNA and regulating transcription, albeit in an altered manner, since the transactivation domain of the RARα protein is missing and is substituted by domains from the X proteins.

3) X proteins, like RARα, are all nuclear proteins and localize within the nucleus in a discrete, punctuated/speckled/microspeckled manner. Therefore X/RARα proteins remain confined to the nucleus but acquire new localization properties due to their capacity to heterodimerize with X;

4) expression of X proteins is regulated during the cell cycle, where it peaks in late G1. Therefore expression patterns of X/RARα proteins very probably fluctuate during the cell cycle as well;

5) PML and PLZF show growth suppressive behavior when transfected into various cell lines of different histological origin. Little is still known about NPM and its possible capacity for modulating cell growth, although induction of mitogenesis in B-lymphocytes is characterized by significant increases in NPM synthesis, suggesting that this protein may also be associated with the transduction of mitogenic signals.

In conclusion, X/RARα proteins can disrupt, in a dominant negative manner and possibly by delocalization, PML, PLZF and NPM tumor/growth suppressive activity, resulting in growth advantage for APL blasts (Figure 5A and B). Concomitantly, by a similar mechanism, inactivation of, or interference with, RARα/RXR pathways would result in a differentiation block at the promyelocytic stage. Concomitantly, the X/RARα proteins could gain new DNA binding properties or protein heterodimerization specificity. The role of RARα/X proteins is presently unclear, although in some cases it could be crucial, e.g. for RARα/PLZF. In general, RARα/X proteins could cooperate with X/RARα in a multistep view of APL pathogenesis.

One of the mechanisms by which X/RARα fusion proteins exert their dominant negative action is by delocalizing the various normal proteins from their natural sites of action, as in the case of PML/RARα.

The tumor has selected for the presence of this molecule, the protein must play a crucial pathogenetic role. At the same time, X/RARα proteins could acquire totally new and aberrant DNA binding properties or protein heterodimerization specificity.
However, none of the fusion molecules have been shown to display leukemogenic or transforming activity in vivo (in animal transgenic models), and all the information that has accumulated so far on the function of APL-specific chimeric products relies on experiments carried out on leukemic cell lines, which are valuable for studying hemopoietic differentiation and maturation, but obviously are not the ideal model system to study oncogenic transformation. In addition, even in these model systems, the role of RARα/PML and RARα/PLZF fusion proteins and aberrant PML products in APL leukemogenesis is still totally unexplored. Transgenic mice carrying individual or multiple APL fusion genes, and mice in which the function of X and RARα/RXR genes has been inactivated by gene targeting will be invaluable for addressing these questions.

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