The Localization of the HRX/ALL1 Protein to Specific Nuclear Subdomains Is Altered by Fusion with Its epsl5 Translocation Partner

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Abstract

Translocations involving the HRX/ALL1 locus at chromosomal region 11q23 are among the most frequent cytogenetic abnormalities in acute leukemias. 11q23 translocations involve different chromosomal partners and lead to the formation of HRX/ALL1 fusion proteins. The HRX/ALL1 protein is a putative transcription factor that has been implicated in developmental regulation in mammals. We report here the cellular localization of the HRX/ALL1 protein as well as that of the HRX/ALL1-epsl5 fusion protein, the result of the t(1;11) (p32-q23) translocation of acute myeloid leukemias. The HRX/ALL1 protein was localized to both the cytoplasm and the nucleus. The nuclear pattern was characterized by diffuse staining, perinuclear accumulation, and localization within nuclear bodies of variable size, morphology, and number. The HRX/ALL1-epsl5 localized exclusively to the nucleus within bodies that were smaller and more numerous than the HRX/ALL1 nuclear bodies. HRX/ALL1 fusion with an unknown partner in leukemia blasts with 11q23 abnormalities had similar morphological features. Thus, the fusion with epsl5 alters the cellular compartmentalization of HRX/ALL1, providing a putative mechanism for activation of HRX/ALL1 by 11q23 abnormalities.

Introduction

Translocations involving chromosome 11 band q23 are among the most frequent cytogenetic abnormalities in hematological malignancies and are found in 10% of acute lymphoblastic leukemias, 5% of acute myeloblastic leukemias, and 80% of treatment-related leukemias (1-3). Although more than 20 different reciprocal chromosomes have been described, the chromosomes most frequently participating in 11q23 translocations are 4q21, 9p22, and 19p13 (4). The chromosome 11q23 breakpoints have been located within the HRX/ALL1 locus, also referred to as MLL (5-7). The HRX cDNA predicts the synthesis of a 430-kDa polypeptide with structural features of a transcriptional factor. It contains two putative DNA-binding domains: (a) an amino-terminal A-T hook motif of high-mobility group proteins, which has been shown to bind to cruciform DNA; and (b) two zinc-finger regions near the midportion of the protein (5-7). Functionally, two transcriptional regulatory regions, a repression and an activation domain, have been mapped to the HRX protein (8). The carboxyl-terminal region of HRX contains a highly conserved SET domain also found in Drosophila trithorax and Polycomb group proteins, which are known to regulate a set of Hox genes during fly development (9). Targeting of the HRX locus in mice by homologous recombination provoked homeotic transformation in the axial skeleton and altered Hox gene expression, suggesting that HRX is required for proper segment identity in mammalian (10). The cellular localization and biochemical activity of the HRX protein are unknown.

The chromosome 11q23 breakpoints cluster within a narrow region of the HRX locus and consistently cause the interruption of the A-T hook motif and the repression domain on one side and the zinc fingers, the activation domain, and the Trithorax-homologous region on the other side. Many (approximately 10) of the partner chromosome breakpoints have been cloned, and the involved genes seem to be structurally distinct from one another. Rearrangements of the HRX gene have also been found in cases of leukemia with no abnormalities in 11q23, and partial duplications of the 5' end of the HRX gene have been described (11). Together, these findings suggest that alterations in the HRX gene are the relevant pathogenetic consequence of 11q23 translocations.

Like other translocations, 11q23 translocations create two reciprocal fusion genes. Molecular and cytogenetic evidence indicates that the fusion gene on the der(11) chromosome is the critical fusion in HRX-associated leukemias. The der(11) chromosome encodes a fusion protein that consists of the amino-terminal portion of HRX and the carboxyl-terminal portion of one of the various fusion partners.

To gain insight into the putative function of the HRX protein and the mechanism of activation during 11q23 translocations, we analyzed the cellular localization of the HRX protein and one of the leukemia-associated HRX fusion proteins (HRX-epsl5, Refs. 12-14). We report that the intracellular localization of the HRX protein is altered by fusion with epsl5.

Materials and Methods

Engineering of HRX, epsl5, and HRX-epsl5 Eukaryotic Expression Vectors. The HRX-epsl5 cDNA was reconstructed by recombinant PCR from wild-type HRX (5) and epsl5 (12) cDNAs. Briefly, HRX and epsl5 fragments corresponding to breakpoint sequences were isolated by PCR from HRX and epsl5 cDNAs using specific HRX (AGGTGGGTGAAGACGTTGGGA and AGGATTTCCACTTGATAACTTTTCTTTGTTTGT) and epsl5 (CAAAACCAAAAGAAGTATCTAAGTGGAACCTGTCA and AT-CACCAGACAGAAATCCTT) oligonucleotides. The HRX and epsl5 PCR-isolated sequences were then ligated with the HRX XhoI-BamHI 1375-bp fragment and the epsl5 NcoI/EcoRI 2332-bp fragment to generate a full-length HRX-epsl5 cDNA. All the DNA fragments generated by PCR were controlled by DNA sequence. The HRX-epsl5, HRX, and epsl5 cDNAs obtained in this manner were subcloned into pcDNA1/AMP (Invitrogen, NV Leek, the Netherlands) expression vector (pcDNA1/AMPHRX, pcDNA1/AMPHRX/EP5, and pcDNA1/AMPeps15).

Transient Expression of HRX, HRX-epsl5, and epsl5 cDNAs and Immunofluorescence Staining. Cos-1 and NIH-3T3 cell lines were cultured in DMEM supplemented with 10% bovine calf serum; MCF7 and HeLa cells were cultured in DMEM supplemented with 10% FCS or 5% calf serum, respectively. The pcDNA1/AMPHRX, pcDNA1/AMPHRX/EP5, and pcDNA1/AMPeps15 expression vectors were transiently transfected into the various cell lines using LipofectAMINE (Life Technologies, Inc.) according to
the manufacturer's instructions. Thirty h after transfection, exogenous protein expression was evaluated by immunofluorescence using anti-eps15 or anti-HRX polyclonal antibodies. The anti-HRX antibody was obtained by immunizing New Zealand rabbits with a glutathione S-transferase fusion protein containing the HRX amino-terminal region. Cytospins of transfected cells were fixed in methanol at room temperature for 5 min followed by acetone at -20°C for 2 min, incubated with the appropriate antibody, and stained with rhodamine-conjugated anti-rabbit Ig antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Preparations were examined on an Olympus BX-60 fluorescence microscope equipped with a chilled 3CCD digital color camera (cS10, Hamamatsu Photonics, Japan). Images were captured with a 24-bit board (Image grabber 24; Neotech, London, United Kingdom) on a 8100/80 Power Macintosh computer (Apple, Cupertino).

**RNase Protection Experiments.** Uniformly labeled antisense RNA transcripts were generated from the HRX and HRX/eps15 probes cloned in pCR2 vector using T7 polymerase and [32P]CTP. Labeled transcripts were hybridized to 50 µg of total cellular RNA in hybridization solution (80% formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM PIPES) at 50°C for 12 h. After hybridization, RNase solution [RNase A at 40 , @g/ml, RNase TI at 2 @ig/ml, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 300 mM NaCl] was added, and the digestion was allowed to proceed for 30 min at room temperature. Digestion was stopped by the addition of 0.6% SDS and 150 µg/ml proteinase K, and the mixture was incubated at 37°C for 30 min. After phenolchloroform extraction and ethanol precipitation, the samples were resuspended in loading buffer (97% formamide, 0.1% SDS, 10 mM Tris-HCl, 0.001% xylene cyanol, and 0.005% bromophenol blue) and then electrophoresed in 6% polyacrylamide-8 M urea gels. Autoradiography was performed on dried gels using intensifying screens.

**Results**

**Cellular Localization of the HRX Protein.** To analyze the cellular localization of the HRX protein, HRX cDNA was transiently expressed into different cell lines (HeLa Cos-1, C33a, NIH-3T3, and U937 cell lines). Expression of the transfected cDNA was first evaluated by RNase protection analysis using a HRX-derived DNA probe (see Fig. 1A for representative results from transiently transfected HeLa cells). A specifically protected fragment was detected in nontransfected cells. It corresponds to the endogenous HRX transcript, and its intensity increased in samples from HRX-transfected cells (Fig. 1A). The cellular localization of the HRX protein was then evaluated by indirect immunofluorescence analysis using an anti-HRX polyclonal serum raised against the amino-terminal region of the protein (see "Materials and Methods"). The specificity of the anti-HRX staining was assessed with a preimmune serum that gave no background staining in the same cell samples (data not shown). The anti-HRX antibody specifically stained both the cytoplasm and nucleus of transfected cells, although the intensity and morphology varied in the different transfected cells. Comparable results were obtained in all tested cell lines, and representative images from HeLa and Cos-1 cells are shown in Fig. 2.

All transfected cells exhibited specific nuclear staining within spherical nuclear structures that differed in number, size, and morphology. These HRX NBs ranged between 0.1 and 0.4 µm in diameter and were present at from 2–3 (Fig. 2, left cell) to several tens (Fig. 2, a–c). In some cells, they appeared as anuli (diameter, 0.3–0.4 µm), whereas in other cells they were solid (diameter, 0.1–0.15 µm). Superimposed on this NB pattern, the anti-HRX antibody also gave a nuclear diffuse pattern of staining. This staining was apparently specific, because it was not seen in the untransfected cells present on the same slides (data not shown). The majority of the transfected cells displayed specific staining at the nuclear periphery, as evidenced by reinforced nuclear membrane staining. Finally, specific cytoplasmic staining of variable intensity, mainly with a diffused pattern, was revealed in approximately 50% of transfected cells.

**Cellular Localization of the HRX-eps15 Fusion Protein.** The localization of HRX fusion proteins was investigated by analyzing mononuclear bone marrow cells from a normal volunteer by indirect immunofluorescence using the anti-HRX polyclonal antibody. Rare mononuclear cells (less than 1%) with morphological features of undifferentiated cells showed specific anti-HRX staining that was similar to that observed in the HRX-transfected cell lines, exhibiting both nuclear and cytoplasmic staining. A representative cell is shown in Fig. 3b.

In conclusion, HRX seems to be consistently localized to the nucleus and variably localized to the cytoplasm. In the nucleus, it localizes within specific subdomains (HRX NBs) and is concentrated at the periphery. The size, shape, and number of HRX NBs vary from cell to cell.

**Universal Localization of the HRX-eps15 Fusion Protein.** The localization of HRX fusion proteins was analyzed in cells transiently transfected with the HRX/eps15 cDNA and in one case of ALL with 11q23 cytogenetic abnormalities. As in the case of HRX, expression of the transfected HRX-eps15 cDNA was first evaluated by RNase protection using a probe spanning the HRX-eps15 junction (Fig. 1B). The probe yielded two fragments of partial protection (100 and 150 bp) in nontransfected HeLa cells, which had the expected size for the fragment protected by endogenous eps15 (150 bp) and HRX (100 bp)
The cellular localization of the HRX protein appeared similar in transfectants and normal human bone marrow cells. HRX protein localizes in both the nucleus and cytoplasm. However, nuclear localization was encountered in all HRX-positive cells, whereas cytoplasmic staining was detectable in only about 50% of the cells. The nuclear localization of HRX agrees with its predicted function as a transcriptional factor. The most characteristic feature of nuclear HRX was its localization to a set of heterogenous subnuclear structures, which we named HRX NBs. The size, morphology, and number of HRX NBs varied from cell to cell and ranged from a few large ring-like bodies to numerous small compact ones.

The relevance and function of the HRX NBs are not known. Many different subnuclear structures have been identified by microscopy and immunostaining with distinct morphologies, compositions, and functions. An example is the nucleolus, where synthesis and processing of rRNA and assembly of preribosomal particles takes place. Other functionally less-characterized compartments are the coiled bodies, clusters of interchromatin granules, and the PML nuclear bodies. The coiled bodies and clusters of interchromatin granules contain splicing factors and are therefore implicated in the processing of immature transcripts (15–16). The PML nuclear bodies contain the acute promyelocytic leukemia-associated PML protein and some proteins recognized by human autoantibodies, such as Sp100 (17). They are implicated in growth regulation and cellular response to DNA virus infection (18). Colocalization experiments of HRX with antigens characteristic of these nuclear subdomains should help define the function of HRX NBs.

The frequency of HRX-positive cells in normal bone marrow was very low, less than 1%. Such infrequent cells might belong to the stem cell compartment and be elective targets for the effects of 11q23 translocations. Interestingly, leukemias with the 11q23 translocations belong to either the myeloid or lymphoid lineages or present features of both (mixed leukemias), suggesting that they might derive from a stem cell (19).

The localization of the HRX fusion protein was also analyzed in HRX-eps15 transfectants and in one case of ALL with 11q23 abnormal...
ities and HRX gene rearrangements. eps15 is an ubiquitously expressed cytosolic protein that has been identified as a substrate of the epidermal growth factor receptor and has subsequently been implicated in the regulation of cellular trafficking (12–13, 20). It contains, from the amino to carboxyl terminus, three copies of a newly identified protein-protein interaction domain (eps15 homology, EH domain), a coiled coil region, and repeated aspartic acid-proline-phenylalanine motifs. The HRX-eps15 fusion protein retains the A-T hook region and repressor domain of HRX and the greater part of the eps15 protein, including the EH domains (14). The HRX-eps15 fusion protein localized exclusively to the nucleus, and its pattern of nuclear localization, although punctuated, was distinct from that of HRX. Concentrated labeling at the periphery of the nucleus was never seen, and the HRX-eps15 NBs were smaller and more numerous than the HRX NBs. The extremely heterogeneous localization of the HRX NBs suggests that the function of HRX may be regulated by its position in the cell. The fact that HRX-eps15 is localized differently from HRX argues, therefore, that the fusion with eps15 may be able to alter the function of HRX by altering its cellular localization. The situation resembles that encountered with PML/retinoic acid receptor α, the fusion protein generated by the acute promyelocytic leukemia-associated t(15;17). PML localizes to large, sporadic nuclear structures (10–20 NBs/cell with a diameter of 1–2 μm), whereas PML/retinoic acid receptor α localizes to smaller (0.1–0.2 microns) and more numerous (hundreds) nuclear subdomains (17). Functional studies on the HRX and HRX-eps15 NBs should help to clarify the function of HRX and its deregulation by 11q23 rearrangements.

References

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