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Cloning, Chromosomal Localization, and Tissue Expression of Autotaxin from Human Teratocarcinoma Cells[1]

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Autotaxin, a potent human tumor cell motility-stimulating exophosphodiesterase, was isolated and cloned from the human teratocarcinoma cell line NTera2D1. The deduced amino acid sequence for the teratocarcinoma autotaxin has 94% identity to the melanoma-derived protein, 90% identity to rat brain phosphodiesterase I/nucleotide pyrophosphatase (PD-Iα), and 44% identity to the plasma cell membrane marker PC-1. Utilizing polymerase chain reaction screening of the CEPH YAC library, we localized the autotaxin gene to human chromosome 8q23-24. Northern blot analysis of relative mRNA from multiple human tissues revealed that autotaxin mRNA steady state expression is most abundant in brain, placenta, ovary, and small intestine.

Autotaxin (ATX) is a 125 kDa glycoprotein that was originally purified to homogeneity from the conditioned medium of the human melanoma cell line, A2058 (1). It stimulates both random and directed motility in the producing cells at high pM to low nM concentrations. A full-length cDNA clone of this melanoma-derived ATX has recently been isolated and sequenced (2). Database analysis of the ATX sequence revealed a homology with PC-1, a type I phosphodiesterase/nucleotide pyrophosphatase expressed on the surface of activated B cells and plasma cells (3, 4). PC-1 and melanoma ATX are 45% identical at the protein level and 56% at the DNA level. ATX, like PC-1, exhibits type I phosphodiesterase activity (2).

We have utilized the cDNA sequence from the melanoma ATX to clone and sequence ATX cDNA from a human teratocarcinoma cell line, Ntera2D1. Since both ATX's sequenced to date have been isolated from tumor cell lines, we also determined its relative expression levels in normal tissues.

MATERIALS AND METHODS

Reagents. Restriction endonucleases and the 5′RACE and 3′RACE kits were obtained from Gibco-BRL Life Technologies, Inc. The GeneAmp PCR Reagent kit with AmpliTaq was purchased from Perkin-Elmer. The total RNA isolation kit was from Promega Corporation. The sequenase II sequencing kit was obtained from Amersham Life Science Inc.

Cell culture. The human teratocarcinoma cell line Ntera2D1 (5, 6) was provided by Maxine F. Singer (National Institutes of Health) and was maintained as described (7).

cDNA cloning. A human teratocarcinoma (Ntera2D1) cDNA library cloned into λgt10 was kindly provided by Dr. Maxine F. Singer. Bulk cDNA was isolated from this library using a Qiagen Lambda kit (Qiagen, Inc). Total RNA was isolated from same Ntera2D1 cells using RNAgents (Promega Corporation) following the manufacturer's suggested protocols. Overlapping cDNA clones were isolated by a combination of PCR and 5′ and 3′ RACE reactions utilizing bulk isolated cDNA as template and known cDNA sequences from melanoma ATX (2) as primers (Fig 1). The 5′ and 3′ RACE methods were utilized to obtain sequence information in the 5′ and 3′ untranslated regions, respectively (Gibco/BRL Life Technologies, Inc).

DNA sequencing. DNA sequencing was performed by the dideoxynucleotide method (8) with [^{35}S]dATP (Du Pont NEN).

Chromosomal localization of the ATX gene. The chromosomal localization of the ATX gene was initially assessed by utilizing human-hamster somatic cell hybrids (BIOS Laboratories). Four restriction enzyme (EcoR1, Hind III, Pst I, or Taq

[1] GenBank Accession No. L46720.

Abbreviations: Autotaxin (ATX), polymerase chain reaction (PCR), yeast artificial chromosome (YAC).

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I) digests of hybrid DNA preparations were fractionated by agarose electrophoresis and transferred to a Biodyne B positively charged nylon membrane (Pall BioSupport Division). The membranes were probed with a 776 bp fragment from the 5′ end of the teratocarcinoma ATX cDNA (9). The pattern of hybridization was compared to known chromosomal patterns for each hybrid cell line and the % discordance was calculated.

In order to localize further the ATX gene, primers from the 3′ untranslated region of the teratocarcinoma ATX cDNA, were used to screen the CEPH YAC library by PCR. Positive YAC addresses were used to compare the gene to microsatellite markers already typed in the YAC library. The computer program 'yacsr' (unpublished data provided by M. Polymeropoulis, National Institutes of Health) was utilized to perform the searches and to locate microsatellite markers on or near the obtained YAC addresses.

Human tissue distribution of ATX mRNA. Human multiple tissue northern blots were purchased from Clontech Laboratories, Inc. and processed as suggested in the manufacturer's protocol. A 776 bp DNA fragment from the 5′ end of the teratocarcinoma ATX, was labeled by means of [α-^{32}P]dCTP and the Radprime DNA labeling system (Gibco-BRL Life Technologies, Inc.) to be used as probe.

RESULTS

Isolation of characterization of human teratocarcinoma ATX cDNA. Utilizing primers based on the known cDNA sequence of melanoma ATX (Figure 1), PCR amplification was performed on bulk isolated cDNA from the human teratocarcinoma cell line, Ntera2D1. This strategy resulted in cDNA sequence from overlapping fragments that lacked both the extreme 5′ and 3′ ends. These ends were then isolated by utilizing 5′ and 3′ RACE methodologies. The resulting full length cDNA sequence (Figure 1) was 3108 base pairs long, including an open reading frame of 2778 nucleotides encoding 863 amino acids. The teratocarcinoma cDNA sequence was confirmed from a nearly full-length clone, independently isolated by reverse transcriptase of Ntera2D1 total RNA followed by PCR amplification. The consensus cDNA sequence was 95% identical to that of the melanoma ATX.

The predicted amino acid sequence of teratocarcinoma ATX is 94% identical to melanoma ATX (Figure 2) (2). Both predicted proteins contain a transmembrane/signal peptide region with a short

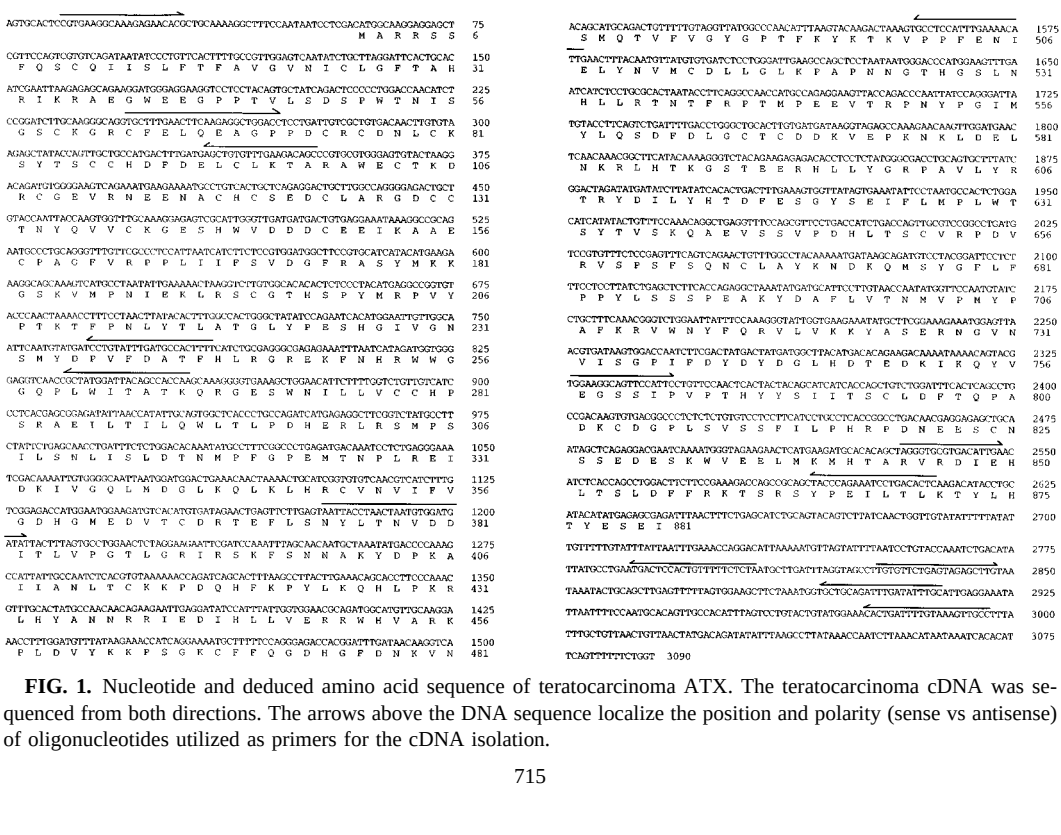

FIG. 1. Nucleotide and deduced amino acid sequence of teratocarcinoma ATX. The teratocarcinoma cDNA was sequenced from both directions. The arrows above the DNA sequence localize the position and polarity (sense vs antisense) of oligonucleotides utilized as primers for the cDNA isolation.

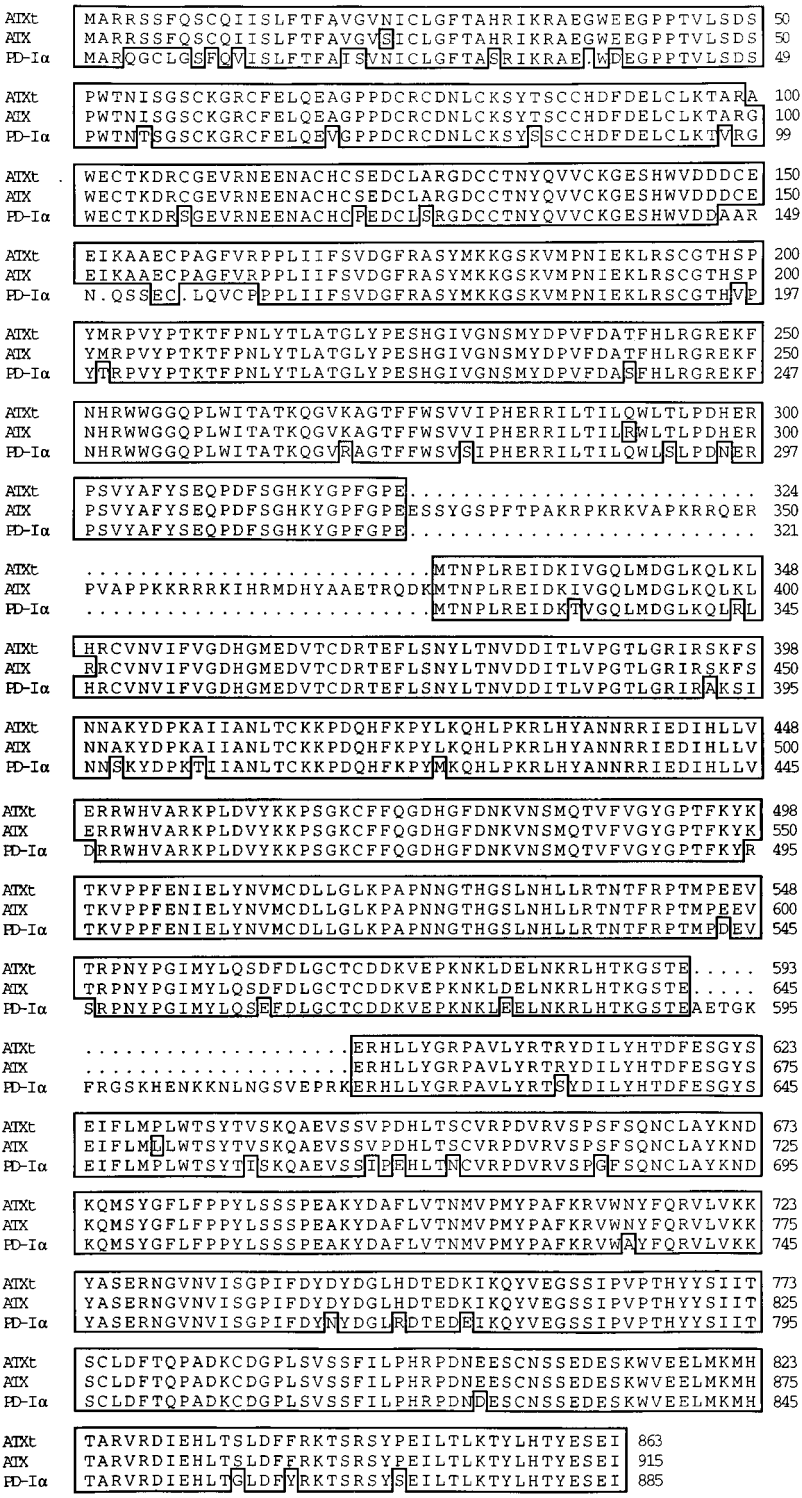

FIG. 2. Comparison of the deduced amino acid sequences of teratocarcinoma ATX (ATX_t), melanoma ATX, and rat PD-Iα. Identical sequences are blocked in together, revealing ≥84% identity between the three proteins.

intracellular tail, four potential N-linked glycosylation sites (in teratocarcinoma ATX: N^{54}, N^{411}, N^{525}, and N^{807}), two adjacent cysteine-rich somatomedin B domains near the amino end of their extracellular portions, and the loop region of a calcium-binding EF-hand (3, 10). In addition, they also have identical regions which are homologous to the bovine intestinal phosphodiesterase enzymatic domain, with conservation of the threonine that is thought to act as an enzyme intermediate binding site (11). The major difference between melanoma and teratocarcinoma ATX is a 52 amino acid insertion which only the melanoma protein contains. This highly basic insertion is located between amino acids, E^{324} and M^{325} of the teratocarcinoma ATX. Excluding this insertion, the remainder of the deduced peptide sequence is 99% identical. In fact, the peptide sequences differ by only five additional amino acids (melanoma $\rightarrow$ teratocarcinoma ATX): $S^{23} \rightarrow N^{23}$, $G^{100} \rightarrow A^{100}$, $R^{291} \rightarrow Q^{291}$, $R^{401} \rightarrow H^{348}$, and $L^{681} \rightarrow P^{628}$.

Teratocarcinoma ATX, like the melanoma-derived protein, is homologous to the activated B cell membrane marker, PC-1 (3, 4), with a 44% identity at the protein level. In addition, a recently cloned rat brain phosphodiesterase I/nucleotide pyrophosphatase (PD-Iα) is even more homologous to the two ATX molecules (Figure 2). The reported PD-Iα cDNA clone contains a 2,655-nucleotide open reading frame encoding a polypeptide of 885 amino acids. The PD-Iα cDNA sequence is 84% identical to the teratocarcinoma and 79% identical to the melanoma ATX cDNA. The deduced amino acid sequence of PD-Iα is 90% and 84% identical to those for the teratocarcinoma and melanoma proteins, respectively. As seen in Figure 2, PD-Iα lacks the same 52 amino acid insertion found in melanoma ATX, but contains a second insertion which both ATX's lack. This insertion is 25 amino acids long and is located between E^{593} and E^{594} of teratocarcinoma ATX. These data suggest that these phosphodiesterases are well conserved in mammalian systems and that ATX might be found in normal brain.

Localization of the ATX gene to human chromosome 8. In order to localize the human autotaxin gene, southern blot analysis was performed on restriction enzyme digests of DNA from a human-hamster somatic cell hybrid panel. The probe for this study was a 776 bp fragment from the 5′ end of the teratocarcinoma ATX cDNA (773 bp out of the 776 are identical in the melanoma cDNA). Analysis of the positive and negative discordance values for each chromosome localized the gene to chromosome 8, which was the only chromosome with zero discordance (data not shown).

Further localization of the ATX gene was achieved by screening the CEPH YAC library. Primers from the 3′ untranslated region of the cDNA clones were used for PCR amplification. These oligonucleotide primers yielded an expected 86 bp fragment, which is identical in both melanoma and teratocarcinoma ATX cDNA's. Five addresses were obtained and the data were used to localize the gene relative to microsatellite markers already typed in the YAC library. Two of the YACs, which were positive for the autotaxin gene, were also positive for the presence of the genetic marker D8S269. The three additional YACs overlapped YACs that were positive for genetic markers D8S522, D8S527, D8S503, D8S516, D8S504 and D8S273, already known to map in the same region of human chromosome 8. These data suggest that human autotaxin is encoded by a single gene localized within 1 Mb from the genetic marker D8S269, in the 8q23-24 cytogenetic region.

Human tissue ATX mRNA abundance. Northern blot analysis of poly-(A) mRNA from multiple human tissues was carried out in order to determine the tissue distribution of *atx* expression in normal adult tissue (Figure 3). The results indicated that expression is highest in brain, placenta, ovary, and small intestine. Expression is intermediate in kidney, prostate, testis, pancreas, colon, and lung. There is very low expression in liver, heart, skeletal muscle, spleen, thymus, and peripheral blood leukocytes.

DISCUSSION

In this manuscript, we report the sequencing of the cDNA of autotaxin from a second human tumor cell line, the teratocarcinoma-derived Ntera2D1. The deduced amino acid sequence of the

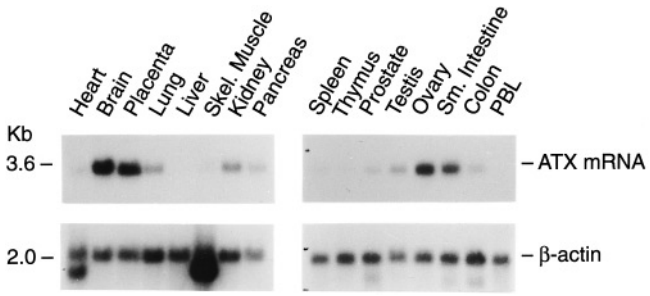

FIG. 3. Differential tissue expression of ATX. Northern blots were prepared using 2 μg/well of poly-(A) mRNA from multiple human tissues. A [^{32}P] radiolabeled 776 bp cDNA fragment from teratocarcinoma ATX was used as probe. ATX expression is highest in brain, placenta, ovary and small intestines.

teratocarcinoma protein is 94% identical to that previously isolated from a human melanoma cell line. The *atx* gene was localized to chromosome 8 by hybridizing a probe from the 5′ end of the cDNA clone to human-hamster somatic cell hybrids. Further localization near the microsatellite genetic marker D8S269 was achieved by PCR amplification of the CEPH YAC library with oligonucleotide probes from the 3′ untranslated region of the ATX cDNA. This localization corresponds to cytogenetic region 8q23-24. Finally, Northern blot analysis of mRNA from multiple human tissues revealed that ATX expression is highest in brain, placenta, ovary, and small intestine. The expression was low or undetectable in liver, heart, and skeletal muscle, as well as spleen, thymus, and peripheral blood leukocytes.

The major difference between the teratocarcinoma and melanoma ATX cDNA sequences the two molecules is a 156 bp insert, coding for 52 amino acids, found in melanoma but not teratocarcinoma ATX cDNA. With this insertion excluded from the comparison, the two molecules are 99% identical at both the protein and the DNA levels. As might be expected, both ATX's have identical domain structures. However, the teratocarcinoma ATX has a calculated molecular mass of 99 KDa, compared to 105 Kda for the melanoma cytokine. In addition, the teratocarcinoma ATX has a deduced pI of 7.3, more neutral than the pI of 9.0 predicted for melanoma ATX. These differences in both calculated molecular mass and pI can be explained by the highly basic 52 amino acid insertion found only in the melanoma ATX. Only five other widely-scattered amino acids differ between the two ATX's, all of them accounted for by single base substitutions. This high level of sequence identity suggests that these two sequences could be coded by a single chromosomal gene with the insertion explained by alternative splicing patterns.

The two ATX's have significant homology to the plasma membrane-bound phosphodiesterases, PC-1 and rat PD-Iα. PC-1, a marker of B cell activation with undefined function, is homologous to the ATX's throughout its extracellular portion but differs significantly in its intracellular and transmembrane domains (2). In this manuscript, we have now shown that the tissue distribution of the autotaxins and PC-1 are also significantly different. ATX is most strongly expressed in brain, placenta, ovary, and small intestine. It is virtually undetectable in lymphoid tissues, such as spleen, thymus, and peripheral blood leukocytes. In contrast, PC-1 was first demonstrated on the surface of plasma cells (12). It was later also localized to the distal convoluted tubule of kidney, salivary gland ducts, liver, testis, epididymis, chondrocytes, placenta, fibroblasts, and the capillary endothelium of brain (13–17). PD-Iα, recently cloned from rat brain, is even more homologous to the two autotaxin molecules than PC-1. The deduced amino acid sequence of PD-Iα is 84% and 90% identical to melanoma and teratocarcinoma ATX, respectively and this homology extends throughout their length. PD-Iα, isolated from rat brain, appears to represent a rat homologue of ATX.

In order to determine chromosomal localization of the ATX gene, we selected probes that were nearly identical for melanoma and teratocarcinoma cDNA's. The gene appears to localize to a

single site within 1 Mb from the genetic marker D8S269, equivalent to the cytogenetic locus, 8q23-24. These data provide further evidence that the teratocarcinoma and melanoma-derived ATX sequences are likely to be splice variants from a single gene.

In summary, ATX is a newly discovered motility-stimulating protein which has now been sequenced from human melanoma and teratocarcinoma cell lines. It has been proposed to play a significant role in initiating and sustaining tumor motility as a component of the metastatic cascade. It could also be important for motility-related events in such processes as development, angiogenesis, and neurite outgrowth. The cDNA and deduced amino acid sequences for the two ATX's are >99% identical except for a single insertion found only in the melanoma-derived proteins. Hybridization experiments with human-hamster somatic cell hybrid panels and PCR amplification of the CEPH YAC library indicated that ATX is encoded by a single gene which localizes to 8q23-24, within one megabase of the microsatellite marker D8S269. The normal tissue distribution of ATX is different from PC-1, another type I alkaline phosphodiesterase, suggesting that they may represent two members of a family of these proteins. The function of these ecto/exoenzymes in their respective tissues remains to be fully defined. Nevertheless, their type I alkaline phosphodiesterase and nucleotide pyrophosphatase activities would confer a capacity to alter the cellular microenvironment.

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