Stimulation of Tumor Cell Motility Linked to Phosphodiesterase Catalytic Site of Autotaxin*

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Hoi Young Lee, Timothy Clair, Peter T. Mulvaney, Elisa C. Woodhouse, Sadie Aznavoorian, Lance A. Liotta‡, and Mary L. Stracke

From the Laboratory of Pathology, NCI, National Institutes of Health, Bethesda, Maryland 20892

A family of extracellular type I phosphodiesterases has recently been isolated by cDNA cloning, but a physiological function linked to the phosphodiesterase active site has remained unknown. We now present evidence that the phosphodiesterase catalytic site, Thr210, is found to be essential for the motility stimulating activity of autotaxin (ATX), one member of the exophosphodiesterase family. Native ATX possesses phosphodiesterase activity at neutral and alkaline pH, binds ATP noncovalently, and undergoes threonine phosphorylation. Homogeneously purified recombinant ATX, based on the teratocarcinoma sequence, retains these same activities. A single amino acid in the phosphodiesterase catalytic site, Thr210, is found to be necessary for motility stimulation, phosphodiesterase activity, and phosphorylation. Two mutant recombinant proteins, Ala210- and Asp210-ATX, lack motility stimulation and lack both enzymatic activities; Ser210-ATX possesses intermediate activities. Another mutation, with the adjacent lysine (Lys209) changed to Leu209, ATX possesses normal motility stimulation with sustained phosphodiesterase activity but exhibits no detectable phosphorylation. This mutation eliminates the phosphorylation reaction and indicates that the dephosphorylated state is an active motility-stimulating form of the ATX molecule. By demonstrating that the phosphodiesterase enzymatic site is linked to motility stimulation, these data reveal a novel role for this family of exo/ecto-enzymes and open up the possibility of extracellular enzymatic cascades as a regulatory mechanism for cellular motility.

Autotaxin (ATX), a 125-kDa glycoprotein, was initially isolated from the culture supernatants of a human melanoma cell line (A2058). It has been shown to stimulate random and directed motility of human tumor cells at high picomolar to low nanomolar concentrations (ED50 = ~300–500 pm) (1). Production of this autocrine motility factor by a variety of cancer cell types is thought to provide a mechanism for tumor cells to initiate, sustain, and regulate their own motility, a critical feature of the metastatic cascade (2). The locomotory response to ATX was demonstrated to be sensitive to pretreatment of the cells with pertussis toxin, indicating that a G protein is involved in the signal transduction pathway (1, 3). However, little else is known about how this complex protein interacts with tumor cells to stimulate locomotion.

When ATX was cloned and sequenced (4), its cDNA sequence revealed significant homology to a family of secreted and cell surface proteins that includes a marker of B cell activation (PC-1) (5, 6), a rat brain nucleotide pyrophosphatase (PD-1α) (7), and a rat neural differentiation antigen (gp130(EB13–6)) (8). The deduced amino acid sequences of each of these proteins include two adjacent somatomedin B domains, a type I phosphodiesterase (PDE) active site, and the loop region of an EF hand. PC-1 and gp130(EB13–6) are predominantly cell surface glycoproteins with short amino-terminal intracellular regions and single transmembrane domains. ATX is cleaved near its putative transmembrane domain and secreted (4).

One member of this family, PC-1, has been previously reported to possess both type I PDE/5’-nucleotide pyrophosphatase and threonine-specific kinase activities (9). However, the physiological function of PC-1 has remained unknown. To date, only ATX has been tested for cell motility. In the present study we describe several enzymatic activities that ATX possesses. We produce mutant recombinant ATXs with changes in the PDE catalytic site and utilize these mutant proteins to explore the relationship between the enzymatic properties and motility stimulation of ATX.

EXPERIMENTAL PROCEDURES

Reagents—The GeneAmp polymerase chain reaction reagent kit with AmpliTaq was purchased from Perkin-Elmer. Restriction endonucleases and SuperScript® reverse transcriptase were obtained from Life Technologies, Inc. The 48-well microchemotaxis chambers and the polyvinylpyrrolidone-free polycarbonate membranes were purchased from NeuroProbe.

Cell Culture—The human melanoma cell line A2058, originally isolated by Todaro et al. (10), was maintained as described previously (2). COS-1 cells were maintained as described previously (11).

Production of Native ATX—The production of ATX from 200-liter batches of serum-free conditioned medium of A2058 cells has been described in detail (1). In brief, after ammonium sulfate precipitation, the conditioned medium was sequentially fractionated through phenyl-Sepharose CL 4B (Pharmacia-LKB Biotechnology), agarose-bound concanavalin A (Vector Laboratories), and ZORBAX BioSeries-WAX (MacMod) columns. The final pooled active fraction, purified approximately 1000-fold over conditioned medium, was dialyzed into 50 mM Tris-HCl (pH 7.5) with 20% (v/v) ethylene glycol and stored at −5 °C.

Production of Recombinant ATXs (rATXs)—A 2.8-kilobase pair DNA fragment (T9S2A), encoding the entire 863-amino acid teratocarcinoma ATX, was constructed in pCR II® (Invitrogen Co.) as described previously (12). The ATX cDNA fragment was excised from pCR II® by restriction enzyme digestion with HindIII and XbaI. Overhanging ends were filled in with T4 DNA polymerase. Likewise, the expression vector, pBC12BI (11), was digested with SmaI and HindIII, and the HindIII overhanging end was filled in. The T9S2A fragment was then blunt-end ligated into the prepared pBC12BI plasmid to produce the vector, pBCT92H. Point mutations were introduced into the PDE active site by utilizing the restriction enzyme MseI, which cuts out a 276-base pair fragment that encodes a portion of ATX extending from the center of the
second somatodendrin B domain to just distal to the PDE catalytic site. Complementary oligonucleotide primers containing the desired mutations were used as the MscI restriction sites were utilized to amplify this fragment by polymerase chain reaction. The mutant ATX cDNA fragment and pBCT92H were each digested with MscI and gel purified. Mutant plasmids were constructed by blunt-end ligating the mutated fragment into the digested pBCT92H plasmid (13). Each mutant plasmid was sequenced to confirm the presence of the mutation and the fidelity of the polymerase chain reaction amplification.

COS-1 cells were transfected with pBCT92H or with mutant plasmids using the DEAE-dextran method (11). After overnight recovery in complete medium, Dulbecco’s modified Eagle’s medium containing 0.1 mg/ml bovine serum albumin was added to the cells, harvested after 48 h, and concentrated using a centrifprep-30 ultrafiltration device (Amicon). The control for all experiments was COS-1 cells, transfected with the pBC12ß vector that had been digested with the same restriction enzymes and autoligated. The concentrated supernatants were partially purified by lectin affinity chromatography using A-agarose (Vector Laboratories), as described previously in detail (1). Concentrations of each recombinant protein were normalized based on the relative optic density of the ATX band on a silver-stained SDS-polyacrylamide gel. The gels were scanned with an ARCUS II scanner (Agfa Corporation), and band density was analyzed utilizing NIH Image (v1.60).

**Assay for Type I Phosphodiesterase Activity**—The 5'-nucleotide phosphodiesterase activity was measured using the colorimetric method of Razzell (14). The 20-μl ATX sample was added to 80 μl of either 50 mM Tris-Cl (pH 8.9) or 50 mM Hepes (pH 7.3) containing 5 mM p-nitrophenol-TMP (Sigma). After incubation at 37 °C for 90 min, reactions were terminated by the addition of 0.1 N NaOH (900 μl). The reaction product was quantified by reading the absorbance at 410 nm.

**Assay for ATP Binding and ATX Phosphorylation**—The noncovalent binding of ATP to ATX was detected by photoaffinity labeling (15). ATX samples were incubated in microtubes containing 100 μM MgCl₂ 50 mM Hepes (pH 7.3), and 10 μM of [γ-32P]—8-azido-ATP (10 Ci/mmol; ICN). After 90 min at 0 °C, samples were photoactivated by irradiation at 254 nm for 30 s. Immediately following photolysis, reactions were terminated by the addition of 10 μl of sample buffer (16). Reaction products were resolved by electrophoresis in SDS-polyacrylamide miniges (8–16%), and radioactive bands were detected by autoradiography.

Phosphorylation of ATX was detected by incubating samples under identical conditions as above but with 10 μM of either [γ-32P]—8-azido-ATP (10 Ci/mmol; ICN) or [γ-32P]—ATP (10 Ci/mmol; ICN).

**Western Blot Analysis**—Protein samples were separated by SDS-polyacrylamide gel electrophoresis in a Tris/glycine buffer system, as described by Laemmli (16), using prepared 8–16% gradient gels (Novex). Prestained molecular weight standards (Novex) were run concurrently in order to estimate molecular weights of positive bands. After electrophoretic separation, gels were transferred to Immobilon membranes for Western blot analysis (17, 18). Immunoblots utilized affinity-purified anti-ATX 103 peptide (1:200) as primary antibody (4) and HRP-conjugated goat anti-rabbit immunoglobulin (1:50,000) as secondary antibody (Pierce). The blot was treated with ECL reagents using the manufacturer’s protocol (Amersham Life Sciences), exposed to Hyperfilm-ECL for 0.5–5 min and then developed in an X-Omat film developer.

**Identification of Phosphoamino Acids in ATX**—In order to detect phosphoamino acids, phosphorylated ATX was fractionated by SDS-polyacrylamide (8–16%) gel electrophoresis and transferred to an Immobilon membrane as described for immunoblots. The phospho-ATX sample was added to 80 μl of either 50 mM Tris-HCl (pH 8.9) or 50 mM Hepes (pH 7.3) containing 5 mM p-nitrophenol-TMP, as described by Razzell (14). The 20-μl ATX sample was added to 80 μl of either 50 mM Tris-Cl (pH 8.9) or 50 mM Hepes (pH 7.3) containing 5 mM p-nitrophenol-TMP (Sigma). After incubation at 37 °C for 90 min, reactions were terminated by the addition of 0.1 N NaOH (900 μl). The reaction product was quantified by reading the absorbance at 410 nm.

**Enzymatic Properties of Native and Purified Recombinant ATX**—ATX had been previously found to have type I PDE activity (4). Under conditions of excess type I PDE substrate (p-nitrophenyl-TMP), we compared this activity at alkaline pH (8.9) versus neutral pH (7.3). Although ATX hydrolyzed substrate at either pH, the activity was 3–4-fold higher at alkaline pH (Fig. 1A).

Based on the homology with PC-1, we next characterized the interaction of ATX with ATP, utilizing the photoaffinity label, 8-azido-ATP. Autoradiographs of ATX after incubation with [γ-32P]—8-azido-ATP revealed radiolabeled ATX only after photocaetivation (Fig. 1B, h+), indicating both that ATX bound ATP and that this binding was noncovalent. In contrast, ATX incubated with [γ-32P]—8-azido-ATP was radiolabeled with (h+ + h−) or without (h−) photocaetivation (Fig. 1B), indicating that the γ phosphate group is covalently transferred to the protein.

Separation of the phosphoamino acids of gel-purified ATX by two-dimensional thin layer chromatography demonstrated the phosphorylation of threonine but not that of serine or tyrosine (Fig. 1C).

A full-length ATX cDNA clone from the human teratocarci-
noma cell line Ntera2D1 was isolated, and the recombinant protein was purified to homogeneity as determined by silver stain (Fig. 2B, lane 1). This clone is >99% identical to the A2058 clone at both the cDNA and the protein level except for a single 52-amino acid insert found only in the A2058 ATX (12). When rATX was compared with A2058 ATX by Western blot, both reacted with ATX-103 (4) anti-peptide antibody (Fig. 2A). The empty vector-transfected COS-1 cells did not secrete a cross-reactive protein. The rATX protein possessed PDE activity (data not shown) and was phosphorylated in the presence of [γ-32P]ATP (Fig. 2B, lane 2). In addition, rATX stimulated tumor cell motility at concentrations equivalent (4) to those of native ATX (Fig. 2C). These data demonstrate that the recombinant protein has activities identical to native ATX.

**Mutations of rATX in the PDE Active Site Alter Its Enzymatic Properties—**The amino acid sequence of the rATX PDE active site (201YMRPYPTKTPP213) is identical to that of both the A2058 and the Ntera2D1 proteins (presumably wild type). Using site-directed mutagenesis, we introduced mutations into the cDNA sequence that altered this active site amino acid sequence. Based on the homology to the active site of bovine intestinal alkaline PDE, Thr210 was presumed to be necessary for the formation of an active intermediate (22). This equivalent threonine has also been shown to be phosphorylated in PC-1 and to be required for both 5'-nucleotide PDE and phosphorylation activities (23, 24). We therefore mutated this locus into an alanine (Ala210-rATX), a phosphorylatable serine (Ser210-rATX), or a negatively charged aspartic acid (Asp210-rATX), which has been demonstrated to mimic the phosphorylated state (25). The positively charged Lys209 was mutated into a neutral leucine (Leu209-rATX). Another point mutation resulted in the replacement of Tyr201 with a histidine (His201-rATX). Finally, knowing that native ATX is phosphorylated on threonine, we also mutated Thr208 into a valine (Val208-rATX).

The seven cDNA constructs were transfected into COS-1 cells; cells transfected by autoligated vector alone (V) served as control. Immunoblots revealed that the mutant autotaxins were expressed and that all reacted with anti-peptide antibody (Fig. 3A).

Assessment of type I PDE activities demonstrated that the Ala210-rATX and Asp210-rATX mutants had greatly reduced enzymatic activity compared with wild type rATX or Val208-rATX (Fig. 3B and Table I). The Ser210-rATX, Leu209-rATX, and His201-rATX mutants were intermediate in activity, having ~17, ~61, and ~77% of the activity of rATX, respectively. Utilizing radiolabeled 8-azido-ATP to determine the effect of these mutations on ATP binding, we found that [α-32P]8-azido-ATP bound the seven rATX constructs only after photoactivation, indicating that none of these point mutations significantly altered the noncovalent ATP binding to ATX (Fig. 3C and Table I). Phosphorylation of the ATX mutants (Fig. 3D) was tested with [γ-32P]ATP. The Ala210-rATX, Asp210-rATX, and Leu209-rATX mutants failed to be phosphorylated. Phosphorylation of Ser210-rATX was marginal, varying from undetectable to <10% of wild type rATX. His201-rATX and Val208-rATX (Table I) demonstrated phosphorylation identical to that of rATX. These data indicate that a phosphorylatable amino acid in position 210 (Thr210 > Ser210) is required for the PDE activity of ATX. The cationic Lys209 is important for phosphorylation but not for significant PDE activity. None of the introduced mutations affected ATP binding.

**PDE Catalytic Site Mutation Alters Motility-stimulating Capacity—**In order to determine what role the PDE active site might play in the capacity of autotaxin to stimulate motility, we utilized each construct as attractant in concurrent chemotaxis assays. Motility stimulation by His210-rATX, Val208-rATX, and Leu209-rATX is equivalent to that of wild type rATX, i.e., at least 3–5-fold increased over background (Fig. 4 and...
Table I. Comparison of different ATX constructs with changes in the phosphodiesterase active site

| Mutant        | Sequence     | PDE activity\(^a\) | Phosphorylation\(^a\) | ATP binding\(^b\) | Motility\(^c\)  |
|---------------|--------------|---------------------|-----------------------|-------------------|------------------|
| rATX (wild)   | YMRPVYPTKFNP| +                   | +                     | +                 | +                |
| H201-rATX     | YMRPVYPTKDFNP| Low\(^d\)           | +                     | +                 | +                |
| V208-rATX     | YMRPVYPTKDFNP| +                   | +                     | +                 | +                |
| L209-rATX     | YMRPVYPTKDFNP| Low\(^d\)           | +                     | +                 | +                |
| A210-rATX     | YMRPVYPTKDFNP| 1                   | +                     | +                 | +                |
| D210-rATX     | YMRPVYPTKDFNP| 2                   | +                     | +                 | +                |
| S210-rATX     | YMRPVYPTKDFNP| Low\(^d\)           | Low\(^d\)             | +                 | Low\(^d\)       |

\(^a\) Indicates >90% of wild type activity. \(^b\) Means not significantly different from vector-transfected control.

\(^c\) Cleavage of p-nitrophenol-TMP was quantitated by measuring absorption at wavelength 410 nm on a Beckman DU-600 spectrophotometer. Compared with wild type PDE activity, these average activities (± SD) were as follows: for Ser\(^{210}\)-rATX, 16.6 ± 4.9% (n = 5); for Leu\(^{209}\)-rATX, 61.1 ± 17.2% (n = 3); and for His\(^{201}\)-rATX, 77.1 ± 9.3% (n = 3).

\(^d\) The relative optic density of autoradiographic bands, quantitated utilizing NIH image 1.60 software, was <10% of those for rATX.

\(^e\) Motility stimulating capacity, quantitated by counting the number of cells(400×) high power field on an American Optics microscope, was 24–47% as great as wild type rATX (n = 5).

Fig. 3. Effect of point mutations in the PDE catalytic site on nucleotide-processing activities of the resulting mutant rATXs. A, immunoblot of the mutant recombinant proteins secreted by COS-1 cells after transfection with vectors containing wild type or mutant ATX cDNA. COS-1 supernatants were concentrated and partially purified by concanavalin A lectin affinity chromatography. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon\(\text{\textregistered}\), and analyzed by immunoblot. Anti-ATX 103 peptide was utilized as primary antibody. B, type I phosphodiesterase activity was quantitated by measuring hydrolysis of the substrate, p-nitrophenol-TMP, for 20 min as detailed in Fig. 1. The results are shown as the averages ± S.D. C, ATP binding was detected by photoaffinity labeling with [\(\gamma\)-\(^{32}\)P]8-azido-ATP as detailed in Fig. 1. D, ATX was phosphorylated by incubation with [\(\gamma\)-\(^{32}\)P]ATP. Reaction products were resolved by electrophoresis and radioactive bands were detected by autoradiography. For A, C, and D, the ATX band is indicated with an arrow. The control (Ctl) for all experiments was supernatant from COS-1 cells that had been transfected by empty plasmid vector. Wild type rATX (Wld) possessed a PDE active site identical to that deduced from the cDNA sequence of melanoma (A2058) ATX.

Fig. 4. Effect of point mutations in the PDE catalytic site on motility-stimulating capacity of the resulting mutant rATXs. The mutant rATXs were used as chemoattractants in simultaneous motility assays. These assays were performed in 48-well microchemotaxis chambers with gelatin-coated polycarbonate membranes. Supernatants from COS-1 cells that had been transfected by empty plasmid vector is control (Ctl). Wild type rATX (Wld) possessed a PDE active site identical to that deduced from the cDNA sequence of melanoma (A2058) ATX. The results are shown as the average number of migrated cells per high power (400×) field over 15 fields; standard deviations were generally <10% of the average.
PDE activity is complex. Mutants that lacked PDE activity also lacked motility stimulation, and the Ser^{210}-rATX mutant was clearly intermediate with respect to both activities. However, the association between the activities is nonlinear because both the His^{201}_2 and Leu^{209}_2-rATX mutants had full motility-stimulating capacity with less PDE activity than wild type ATX. The data are consistent with PDE activity above a certain threshold being necessary for full motility stimulation.

**DISCUSSION**

ATX is a tumor cell motility-stimulating factor that was originally purified from melanoma cell supernatants. We have now demonstrated that ATX also has multiple nucleotide-processing activities, including ATP binding, phosphorylation, and type I PDE activity. Recombinant ATX, with sequence based on the teratocarcinoma protein (12), retains all of these activities, including motility-stimulating capacity. We have utilized site-directed mutagenesis to genetically alter the PDE catalytic site and tested the resulting mutant rATXs for each of the known activities. Changing a single amino acid within this site, Thr^{210}, into either alanine or aspartate resulted in loss of phosphorylation, loss of PDE activity, and abolishment of motility stimulation; or 3) the products of an unknown phosphorylated nucleotide PDEs (12). PC-1 was first identified as a cellsurface protein capable of binding ATP and hydrolyzing phosphoester bonds of nucleotides (32, 33). Extracellular or cell surface proteins capable of binding ATP and hydrolyzing phosphoester bonds of nucleotides are known to exist, but their function has remained obscure. Data from studies with PC-1 and gp130^R^115-16 have suggested that cell surface PDEs may play roles in cellular differentiation. Our data have revealed a structure-function correlation between the PDE catalytic site and motility stimulation by ATX, indicating a biologically important functional role for the ceto/exophosphodiesterases in the stimulation of cellular motility. As an enzyme, ATX may stimulate motility directly through a cell surface receptor or indirectly through one of its enzymatic products. Based on our data, possible mechanisms of activity include the following: 1) the PDE catalytic site and its influence on the conformation of ATX may be required for binding or processing a cell surface receptor; 2) the nucleotide products of the enzymatic reaction may stimulate motility; or 3) the products of an unknown phosphorylated substrate may stimulate motility. We are now in the process of distinguishing these possibilities.

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