Site-directed Mutations in the Tumor-associated Cytokine, Autotaxin, Eliminate Nucleotide Phosphodiesterase, Lyso phospholipase D, and Motogenic Activities

Eunjin Koh, Timothy Clair, Elisa C. Woodhouse, Elliott Schiffmann, Lance Liotta, and Mary Stracke

ABSTRACT

The exoenzyme autotaxin/NPP2 (ATX/NPP2) is a potent stimulator of cell migration, invasion, metastasis, and angiogenesis. Recently, ATX/NPP2 was found to possess lyosphospholipase D (lyso-LPD) activity, generating the bioactive mediator lysophosphatic acid from precursors. In the present study, we used site-directed mutagenesis to delineate the active domain of lysospholipid catalytic activity and to examine potential overlap with the nucleotide phosphodiesterase domain. We found four amino acid residues obligatory for the phosphodiesterase, lyso-LPD, and migration-stimulating activities of ATX/NPP2, suggesting that 5′-nucleotide phosphodiesterase (PDE) and lyso-LPD share a common reaction mechanism and inviting design of enzymatic inhibitors as therapeutic agents for neoplastic disease.

INTRODUCTION

ATX/NPP2, a Mr 125,000 glycoprotein, is a member of the exo/exo-NPP family of enzymes. Expression of this cytokine increases tumorigenicity and metastatic potential in transformed cells (1) and elicits an angiogenic response in Matrigel plug assays (2). Recently, two separate groups (3, 4) have demonstrated that, in addition to its PDE activity, ATX/NPP2 possesses lyso-LPD activity that can generate LPA from LPC. In contrast to both PDE and PLD, the active site for PLD activity, ATX/NPP2 possesses lyso-LPD activity that can generate LPA from LPC. LPA, in turn, might mediate many of the activities attributed to ATX/NPP2 itself, including motility stimulation, invasiveness, and late-stage angiogenesis. A functionally similar enzyme, PLD, which hydrolyzes phosphatidylcholine to produce phosphatic acid, has been cloned from many species and studied extensively (reviewed in Refs. 5 and 6). Its active site consists of duplicated HxKxxxxD sequences, commonly referred to as HKD motifs (7, 8). In contrast to both PDE and PLD, the active site for lyso-LPD, which might provide insight into the enzymatic mechanism of its action, has not yet been described. In this report, we have used published reports detailing the amino acid requirements of the NPP-associated PDE reactive center (9) as well as the HKD motif for PDE activity (7) as our models for constructing eight separate mutant ATX/NPP2s. We present evidence that all three of the PDE, lyso-LPD, and motogenic actions of ATX/NPP2 require at least four identical amino acids for activity.

MATERIALS AND METHODS

Reagents. LPC (18:1), LPA (18:1), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS), 4-aminoantipyrine (4-AAP), horseradish peroxidase, choline oxidase, p-nitrophenyl-TMP, 5′-AMP, and CPT were from Sigma (St. Louis, MO).

Cell Culture. A2058 (human melanoma) and COS-1 (green monkey kidney) cell lines were maintained as described previously (10). Construction of ATX/NPP2 Mutants and Preparation of Mutant Proteins. Site-directed point mutations of ATX/NPP2 were made by overlap extension PCR methodology as described previously (11). ATX/NPP2 cDNA, derived from MDA 435 cells, was cloned into pcDNA3.1 vector (pcDNA3.atx/npp2) and used as a template. PCR was performed using the Platinum Pfx DNA polymerase (Invitrogen Life Technologies, Inc., Carlsbad, CA) and oligonucleotides designed for each mutant (Table 1). Each mutant plasmid was sequenced to confirm the presence of the mutation and the fidelity of the PCR amplification. COS-1 cells were transiently transfected with pcDNA3.atx/npp2 or with each mutant plasmid using LipofectAMINE 2000 reagent (Invitrogen Life Technologies, Inc.) per manufacturer’s protocol. A nonvector control was produced by adding appropriate medium and identical treatments but no vector to COS-1 cells. Each medium was partially purified with concanavalin A-agarose as described previously (12).

Immunoblot Analysis. After Tris-Glycine SDS-PAGE (8–16%), proteins were transferred onto Immobilon membranes (Invitrogen Life Technologies, Inc.). Affinity-purified anti-ATX/NPP2 (13) polyclonal antibodies were used as primary antibodies, and horseradish peroxidase-conjugated goat antirabbit IgG (Pierce, Rockford, IL) was used as secondary antibodies, followed by signal development using enhanced chemiluminescence (Amersham Life Sciences). Quantification of each recombinant protein was assessed by densitometric scanning and image analysis (Personal Densitometer SI and ImageQuant software; Molecular Dynamics).

PDE and Lyso-LPD Assays. Partially purified conditioned medium from transfected COS cells was adjusted to equal ATX/NPP2 protein concentrations based on the intensity of immunoblot signals. Twenty-μl samples (100-μl reaction volume) were incubated in DMEM-BSA at 37°C in the presence of 0.5 mM of either p-nitrophenyl-TMP (for PDE assays) or LPC (for lyso-LPD assays). DMEM-BSA was used to mimic the conditions of the motility assays. For determination of PDE activity, reactions were stopped after 30 min by the addition of 900 μl of 0.1 N NaOH, and the nitrophenol product was detected by reading the absorbance at 410 nm (A410 nm × 6 = nmol). To determine lyso-LPD activity, released choline was detected by a modification of the enzymatic photometric assay described previously (14, 15). A 900-μl cocktail containing 50 mM Tris-HCl (pH 8), 5 mM CaCl2, 0.3 mM N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS), 0.5 mM 4-aminoantipyrine (4-AAP), 5.3 units/ml horseradish peroxidase, and 2 units/ml choline oxidase were added to the 100-μl reaction mixture and were incubated for 15 min at 37°C. Absorbance was read at 555 nm and converted to nmol of choline by comparison with a choline standard curve.

Cell Motility Assays. A2058 cells were prepared and used for motility assays as described previously in detail (16). Partially purified conditioned medium from transfected COS cells was adjusted to equal ATX/NPP2 protein concentrations based on the intensity of immunoblot signals. Migrated cells were counted under light microscopy at ×200 (medium power field).

RESULTS AND DISCUSSION

Expression of Wild-Type and Mutant ATX/NPP2 Protein in COS-1 Cells. ATX/NPP2 possesses both PDE (12) and lyso-LPD enzyme activities (3, 4). We have found that the amino acid residue T210 is obligatory for both PDE and motogenic activities (10), suggesting that an intact PDE-reactive center is necessary for motility stimulation. On the basis of sequence similarity to the known structure of alkaline phosphatase, Gijsbers et al. (9) found three histidines and three aspartates in PC1/NPP1 that were required for PDE activity and were proposed to coordinate the binding of two metal ions within the reaction center. A functionally similar enzyme, PLD, has been shown...
to require an intact HKD motif (HxKxxxxD) for its catalytic activity (7, 8). Using these two known reactions as our model for selecting appropriate amino acids, we introduced point mutations into ATX/NPP2 cDNA using overlap extension PCR methodology as described in “Materials and Methods.”

Because histidines are crucial to catalysis in both of our enzyme models and many other phosphoreactive catalytic sites, most of the introduced mutations involved changes in histidine residues, with the exception being T210A. Three mutations were placed into the ATX/NPP2 homologues of histidines proposed to coordinate the binding of metal ions: H316Q, H360Q, and H475Q. Although ATX/NPP2 has no canonical HKD, mutations were created in those histidines from the two most similar sites: H587 (HxKxxxxE), mutated into H587Q, and H453 (HxxRKxxD), mutated into H453Q. The final two mutations were chosen because of their proximity to the PDE catalytic site to create H243Q and H298Q. The locations of these eight mutations relative to known domains in ATX/NPP2 are shown schematically in Fig. 1A. The residues associated with PDE activity are shown in red, others are shown in blue.

Plasmids containing wild-type or mutant ATX/NPP2 cDNA were transiently transfected into COS-1 cells. After 48 h, each medium was collected and partially purified, then verified and quantified via immunoblot as shown in Fig. 1B. A non-vector-transfected medium served as a negative control and failed to reveal a protein band on immunoblot, suggesting that COS1 cells secrete undetectable levels of ATX/NPP2.

Effect of Point Mutations on PDE and lyso-PLD Enzymatic Activities. Each mutant ATX/NPP2 was tested for PDE and lyso-PLD activities, using well-established colorimetric assays: p-nitrophenyl-TMP

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<tr>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>T210A-s</td>
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<tr>
<td>T210A-as</td>
<td>5'-aaagccccacctttctactaagctttctac-3'</td>
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<sup>a</sup>-s and -as refer to sense and antisense primers.
<sup>b</sup>Mutated sequences are underlined.

Fig. 1. Effect of point mutations introduced into ATX/NPP2 on enzyme activities. A, schematic representation of the known domains of ATX/NPP2 with our introduced mutations shown. Red, point mutations that have been associated with PDE activity; blue, other point mutations. B, immunoblot analysis of the expression of wild-type (wt) ATX/NPP2 and each mutant ATX/NPP2 in transiently transfected COS-1 cells. Proteins were quantified by densitometric scanning and image analysis. C, PDE (Ⅲ) and lyso-PLD (Ⅲ) activities were measured as described in “Materials and Methods” by using the partially purified protein preparations shown in B. Non-vector-transfected COS-1 medium (w/o vector) served as negative control. The results shown are the mean of duplicate experiments. Samples with results significantly different from the measured activity of wild-type ATX/NPP2 are marked as follows: *, P < 0.001; #, P < 0.01.
hydrolysis for PDE (12) and choline release from LPC for lyso-PLD (3). Wild-type ATX/NPP2 served as positive control, and partially purified conditioned medium from non-vector-transfected COS-1 cells as negative control in these experiments. For both types of assays, results were expressed as a percentage of wild-type activity.

As shown in Fig. 1C, mutant ATX/NPP2s that were altered in the PDE-reactive center (T210A, H316Q, H360Q, and H475Q) possessed significantly reduced PDE activity compared with wild-type ATX/NPP2 (P < 0.01 for H316Q and H475Q; P < 0.001 for T210A and H360Q). This was an expected finding because Gijsberg et al. (9) had found that mutations in the homologous amino acids of PC-1/NPP1 resulted in similarly reduced activity. In novel findings, these same four mutant ATX/NPP2s (T210A, H316Q, H360Q, and H475Q) also had greatly diminished lyso-PLD activity compared with wild-type ATX/NPP2 (P < 0.001 for all). In fact, these activity-negative forms of ATX/NPP2 were not significantly different from the nonvector control for the activity of either enzyme, even at substrate concentrations that were saturating for the activity-positive forms. This fact precludes comparison of substrate concentration dependence. In contrast, those ATX/NPP2 mutant proteins that most closely corresponded to the HKD motif (H587Q and H453Q) had PDE and lyso-PLD activities that were not significantly different from those of native autotaxin. Similarly, the ATX/NPP2 mutants chosen for their proximity to the PDE-reactive site (H243Q and H298Q) also retained the activities of both enzymes, which were not significantly different from the activity of native ATX/NPP2.

We have shown that the hydrolysis of LPC by ATX/NPP2 has structural requirements for threonine and histidine identical to those for nucleotide hydrolysis. Despite the similarity of substrates, phospholipids versus lysophospholipids, the PLD and lyso-PLD enzymes seem to use different catalytic strategies for the hydrolysis of phosphodiester bonds. The precise correspondence between the effects of four separate point mutations on both PDE and lyso-PLD activities strongly indicates the presence of a common enzymatic site in ATX/NPP2, suggesting that ATX/NPP2/lyso-LPD uses a common reaction mechanism for the hydrolysis of nucleotides and lysophospholipids.

**Effect of Point Mutations on the Migration Responses of A2058 Cells.** To determine the effects of these same eight mutations on the capacity of the resulting ATX/NPP2s to stimulate motility, each was tested via a modified Boyden chamber assay for its capacity to induce a migration response in A2058 responder cells. Wild-type ATX/NPP2 served as the positive control in these experiments. Conditioned medium from non-vector-transfected COS-1 cells, as well as DMEM-BSA alone, served as negative controls. The result of a representative motility assay is shown in Fig. 2A. The same four mutant ATX/NPP2s that lacked both PDE and lyso-PLD activity (T210A, H316Q, H360Q, and H475Q) also failed to stimulate motility (P < 0.001 compared with wild-type ATX/NPP2). These enzyme-negative mutants were essentially no different from the nonvector control. In contrast, the mutations that had no significant effect on any enzymatic activity (H243Q, H298Q, H453Q, and H587Q) induced a migration response that was equivalent to that of native autotaxin.

Similar results were obtained when 500 nM LPC was added to each ATX/NPP2 sample, incubated at 37°C for 5 h, and then heated to inactivate ATX/NPP2 before the migration assay. Heating in this manner eliminates ATX/NPP2-stimulated motility but has no effect on LPA stimulation (data not shown). In these substrate experiments, wild-type ATX/NPP2 served as a positive control for the enzyme action of ATX/NPP2, and 500 nM LPA served as the product control. Negative controls included non-vector-transfected COS-1 medium and 500 nM LPC alone. As shown in Fig. 2B, incubation of LPC with T210A, H316Q, H360Q, or H475Q failed to produce a heat-stable product and did not result in motility stimulation (P < 0.001 compared with wild-type ATX/NPP2; no significant difference from nonvector control). However, incubation of LPC with H243Q, H298Q, H453Q, or H587Q resulted in a heat-stable product that stimulated migration in a manner equivalent to incubation with native ATX/NPP2.

For the first time, we have shown that the capacity of mutant ATX/NPP2s to induce migration correlates directly with their capacity to hydrolyze both nucleotides and lysophospholipids. These data present a cogent argument that the enzyme reactive center of ATX/NPP2 must be intact for the protein to produce a migration response, strongly implying that this response results from its enzymatic products.

**Adenosine Receptor Does Not Mediate ATX/NPP2 Migration Response.** The correlation of both PDE and lyso-LPD activities with the stimulation of a motile response raises the possibility that nucleotides might also be important to ATX/NPP2-stimulated motility. Platelets have been shown to release both adenosine nucleotides and LPA (17), providing a physiological mixture of alternative substrates. Tokumura et al. (4) found that high concentrations (5–10 mM) of ATP or p-nitrophenyl-TMP inhibited choline release from LPC, suggesting the possibility of complex substrate interactions in the microenvironment of the cell. In addition, extracellular nucleosides and nucleotides are known to elicit numerous physiological responses by binding to specific membrane receptors. In A2058 cells, both adenosine and 5′-AMP elicit similar pertussis-toxin-sensitive migration responses, mediated by the A1 subclass of the PI purinoceptor family (18).

We, therefore, tested the effect of the A1-specific adenosine analogue CPT on the migration response of A2058 cells to both native ATX/NPP2 and 5′-AMP. The results are shown in Fig. 3. CPT completely blocked high levels of ATP in platelet suspensions. The possibility of complex substrate interactions in the microenvironment of the cell is supported by the finding of Tokumura et al. (4) that high concentrations (5–10 mM) of ATP or p-nitrophenyl-TMP inhibited choline release from LPC, suggesting the possibility of complex substrate interactions in the microenvironment of the cell. In addition, extracellular nucleosides and nucleotides are known to elicit numerous physiological responses by binding to specific membrane receptors. In A2058 cells, both adenosine and 5′-AMP elicit similar pertussis-toxin-sensitive migration responses, mediated by the A1 subclass of the PI purinoceptor family (18).

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the 5′-AMP response \((P < 0.001\) for CPT treated versus untreated) but had no significant effect on ATX/NPP2-stimulated migration, indicating that the ATX/NPP2 response is independent of the adenosine A1 receptor. Although ATX/NPP2 possesses 5′-nucleotide PDE activity, the migration elicited by the product of this reaction, AMP, is independent of the motogenic effect of ATX/NPP2.

ATX/NPP2 Acts as a Member of the NPP Family. Our data indicate that ATX/NPP2/lyso-PLD behaves like a member of the NPP family of ecto/exo-enzymes (19, 20), using a common reactive site to extend its capacity to hydrolyze nucleotide phosphoester bonds to those of lysophospholipids. Aoki et al. (17) have recently shown that ATX/NPP2 also hydrolyzes other glycerophospholipids, including lysophosphatidylserine, lysophosphatidylethanolamine, and lysophosphatidylcholine, into LPA. Furthermore, this intact PDE/lyso-PLD reactive site is obligatory for the motogenic activity of ATX/NPP2. Because the product of the PDE enzymatic activity, AMP, is not necessary for autotaxin-stimulated motility, our data strongly imply that the motogenic activity of ATX/NPP2 is mediated entirely by its production of bioactive phospholipids. This is a striking example of a highly potent cytokine exerting its effects by directly controlling the synthesis of an equally potent biological agent, LPA. The emerging importance of such a phenomenon has widespread implications for the regulation of the cellular microenvironment and for novel therapeutic approaches to a variety of diseases that might affect any facet of this regulation.

REFERENCES


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