Inhibition of Autotaxin by Lysophosphatidic Acid and Sphingosine 1-Phosphate

Laurens A. van Meeteren‡, Paula Ruurs‡, Evangelos Christodoulou§, James W. Goding*,
Hideo Takakusai*, Kazuya Kikuchi*, Anastassiss Perrakis‡, Tetsuo Nagano†,
and Wouter H. Moolenaar‡‡

From the‡Division of Cellular Biochemistry and Center for Biomedical Genetics and§Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, ¶Department of Pathology and Immunology, Monash Medical School, Alfred Hospital, Prahran 3181, Victoria, Australia, and
Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan

Autotaxin (ATX) or nucleotide pyrophosphatase/phosphodiesterase 2 (NPP2) is an NPP family member that promotes tumor cell motility, experimental metastasis, and angiogenesis. ATX primarily functions as a lysophospholipase D, generating the lipid mediator lysophosphatidic acid (LPA) from lysophosphatidylcholine. ATX uses a single catalytic site for the hydrolysis of both lipid and non-lipid phosphodiester, but its regulation is not well understood. Using a new fluorescence resonance energy transfer-based phosphodiesterase sensor that reports ATX activity with high sensitivity, we show here that ATX is potently and specifically inhibited by LPA and sphingosine 1-phosphate (S1P) in a mixed-type manner ($K_i \sim 10^{-7} \text{M}$). The homologous ecto-phosphodiesterase NPP1, which lacks lysophospholipase D activity, is insensitive to LPA and S1P. Our results suggest that, by repressing ATX activity, LPA can regulate its own biosynthesis in the extracellular environment, and they reveal a novel role for S1P as an inhibitor of ATX, in addition to its well established role as a receptor ligand.

Autotaxin (ATX) is a member of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family of ecto-enzymes that hydrolyze phosphodiester bonds in various nucleotides and nucleotide derivatives (1–3). ATX, also termed NPP2, was originally isolated as an autocrine motility factor for melanoma cells (4, 5) and later found to enhance the invasive and metastatic potential of Ras-transformed NIH3T3 cells in nude mice and to finally isolated as an autocrine motility factor for melanoma cells (4, 5) and later found to enhance the invasive and metastatic potential of Ras-transformed NIH3T3 cells in nude mice and to

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

Published, JBC Papers in Press, March 15, 2005, DOI 10.1074/jbc.M413183200

Autotaxin (ATX) or nucleotide pyrophosphatase/phosphodiesterase 2 (NPP2) is an NPP family member that promotes tumor cell motility, experimental metastasis, and angiogenesis. ATX primarily functions as a lysophospholipase D, generating the lipid mediator lysophosphatidic acid (LPA) from lysophosphatidylcholine. ATX uses a single catalytic site for the hydrolysis of both lipid and non-lipid phosphodiester, but its regulation is not well understood. Using a new fluorescence resonance energy transfer-based phosphodiesterase sensor that reports ATX activity with high sensitivity, we show here that ATX is potently and specifically inhibited by LPA and sphingosine 1-phosphate (S1P) in a mixed-type manner ($K_i \sim 10^{-7} \text{M}$). The homologous ecto-phosphodiesterase NPP1, which lacks lysophospholipase D activity, is insensitive to LPA and S1P. Our results suggest that, by repressing ATX activity, LPA can regulate its own biosynthesis in the extracellular environment, and they reveal a novel role for S1P as an inhibitor of ATX, in addition to its well established role as a receptor ligand.

The mode of action of ATX/NPP2 has long been elusive because the biological effects of ATX could not be explained by nucleotide hydrolysis. The surprise came when it was discovered that ATX is identical to plasma lysophospholipase D (lyso-PLD) and acts by hydrolyzing lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) (11, 12), a lipid mediator that signals cell proliferation, migration, and survival via specific G protein-coupled receptors (13). It has now become clear that de novo production of LPA can fully account for the biological effects of ATX observed in cell culture. The lysophospholipid substrate range of ATX has recently been broadened by showing that the enzyme can also hydrolyze sphingosylphosphorylcholine (SPC) to yield sphingosine 1-phosphate (S1P) (14), a bioactive lipid with signaling properties very similar to those of LPA while acting on distinct receptors (15–17). The physiological significance of the SPC-to-S1P conversion is debatable, however, because the reported $K_m$ for ATX for SPC (14) is 3 orders of magnitude higher than the normal SPC levels in plasma and serum (18). Rather than through SPC hydrolysis, S1P is thought to originate largely from the phosphorylation of sphingosine by sphingosine kinases (19).

Mutational analysis has revealed that the lyso-PLD and nucleotide phosphodiesterase activities of ATX originate from the same catalytic site (20, 21). Unexpectedly, the other two members of the NPP family (NPP1 and NPP3) lack intrinsic lyso-PLD activity despite their close homology to ATX (21). Given the differences in substrate specificity, it is not surprising that the NPPs appear to have largely unrelated physiological functions. The founding member, NPP1, hydrolyzes ATP into pyrophosphate, an inhibitor of calcification, and thereby regulates bone mineralization, whereas the third member, NPP3, promotes differentiation and invasion of glial cells by an unknown mechanism (3).

An unresolved question concerns the regulation of ATX activity. One puzzling observation is that LPA levels in plasma or freshly isolated blood are very low (22–24), yet plasma ATX is constitutively active and its substrate LPC abundantly present (> 100 µM) (25). This suggests that ATX is negatively regulated in vivo, but physiological or pharmacological inhibitors of ATX have not been identified to date. In the present study we sought to examine how ATX activity is regulated in the extracellular milieu. To this end, we used a newly invented fluorescence resonance energy transfer (FRET)-based phosphodiesterase sensor (termed CPF4; see Ref. 26) that, as we show here, reports ATX activity in conditioned media with superior sensitivity. Using this assay system, we demonstrate that ATX,
secreted by the classical export route, is potently and specifically inhibited by LPA and S1P at biologically relevant concentrations. These results have important implications for lysosphospholipid action and signaling in general and ATX targeting in particular.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials—**HEK293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. All phospholipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Brefeldin A, monensin, BSA, fatty acid-free BSA (99%), para-nitrophenyl thymidine-5’-monophosphate (pNP-TMP), and bis(p-nitrophenyl) phosphate (bis-pNPP) were from Sigma. Highly purified PLD from Streptomyces chromofuscus, sphingomyelinase D from Locacesla laeta, and sphingomyelinase D from Corynebacterium pseudotuberculosis were kindly provided by M. Roberts (Boston College), D. Tambourgi (Instituto Butantan, Sao Paulo, Brazil), and S. Billington (University of Arizona), respectively.

cDNA Cloning—RNA extracted from human diploid foreskin fibroblasts was used to generate cDNA using Invitrogen reverse transcriptase. ATX cDNA was isolated using ATX-specific primers. The stop codon was removed and KpnI and NotI restriction sites were introduced at the 5’/3’ sites, respectively. After digestion, ATX was ligated in pFastBac I vector (Invitrogen). The resulting plasmid was then used for generating recombinant baculovirus to infect S9 insect cells, which were grown in SP-900 II medium (Invitrogen); the multiplicity of infection was ~5. After 60 h of infection, medium containing secreted ATX was collected by low speed centrifugation and applied onto a Q-Sepharose column (Amersham Biosciences). The flow-through was applied onto a hydroxyapatite column (Bio-Rad), and the bound proteins were eluted with a linear potassium phosphate gradient. The ATX-containing fractions were applied onto an isopropl column (Amersham Biosciences), and bound proteins were eluted with a decreasing linear gradient of ammonium sulfate. Purity of ATX-containing fractions was >95% as shown by SDS-PAGE and Coomassie Blue staining. The approximate yield was about 3 mg of pure protein from 10 liters of culture supernatant.

Recombinant ATX—The Bac-to-Bac baculovirus expression system (Invitrogen) was used for ATX production. ATX cDNA fused to the honeybee melittin signal sequence at the 5’ end was cloned into the pFastBacI vector (Invitrogen). The resulting plasmid was then used for generating recombinant baculovirus to infect S9 insect cells, which were grown in SP-900 II medium (Invitrogen); the multiplicity of infection was ~5. After 60 h of infection, medium containing secreted ATX was collected by low speed centrifugation and applied onto a Q-Sepharose column (Amersham Biosciences). The flow-through was applied onto a hydroxyapatite column (Bio-Rad), and the bound proteins were eluted with a linear potassium phosphate gradient. The ATX-containing fractions were applied onto an isopropl column (Amersham Biosciences), and bound proteins were eluted with a decreasing linear gradient of ammonium sulfate. Purity of ATX-containing fractions was >95% as shown by SDS-PAGE and Coomassie Blue staining. The approximate yield was about 3 mg of pure protein from 10 liters of culture supernatant.

Recombinant NPP1—A soluble secreted form of human NPP1 was made by in-frame ligation of its extracellular domain to the BamHI site.
of the cleavable signal sequence from influenza hemagglutinin, and cloned into a mammalian expression vector driven by the SR-/H9251 promoter. After linearization, the DNA was transfected into Chinese hamster ovary cells, and a highly expressing stable transfectant was isolated by single cell cloning. The soluble enzyme was purified from culture supernatants by sequential chromatography consisting of large scale anion exchange chromatography, Blue-Sepharose, AMP-Sepharose, and final polishing on a Uno-Q column (Bio-Rad). Purity was confirmed by SDS-PAGE and silver staining.

**lyso-PLD Assay—** To measure lyso-PLD activity, radiolabeled LPC (1-[1-14C]palmitoyl, Amersham Biosciences) and unlabeled LPC (1 M) were dried under nitrogen, and the mixture was reconstituted in Tris-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 50 mM Tris, pH 8.0) and sonicated, and BSA (2 mg/ml) was added. The reaction was started by the addition of ATX-containing conditioned medium. Lipids were extracted with butan-1-ol. All of the solutions were made 0.02 M in acetic acid and extracted with 0.5 volume of butan-1-ol as described previously (22). In brief, after thorough mixing and centrifugation, the butan-1-ol phase was removed, and the water phase was extracted once again. Butanol fractions were washed with 1 volume of butanol-saturated water and dried under nitrogen. Phospholipids were separated by thin layer chromatography on silica gel-60 plates in chloroform/methanol/acetic acid/water (50:30:8:4). Lipids were detected by autoradiography.

**Phosphodiesterase Assays—** Phosphodiesterase activity toward pNP-TMP and bis-pNPP was measured by light absorbance. 40 μl of HEK293T-conditioned medium was added to 160 μl of Tris-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 50 mM Tris, pH 8.0) containing pNP-TMP or bis-pNPP in 96-well plates. After incubation for 3 h at 37 °C, the amount of liberated para-nitrophenolate (pNP) was determined by reading the absorbance at 405 nm in a Victor Wallac plate reader.

**CPF4 FRET Assay—** CPF4 was synthesized as described previously (26) and maintained as a 10 mM stock solution in Me2SO. Recombinant ATX in Tris-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 50 mM Tris, pH 8.0) or ATX-Myc-containing conditioned Dulbecco's modified Eagle's medium (buffered with 50 mM Tris, pH 8.0) was incubated with or without the indicated reagents, and CPF4 was added at a concentration of 2 μM unless indicated otherwise. CPF4 fluorescence was monitored (at 37 °C) in a BMG Fluorstar 96-well plate reader (excitation at 355 nm, emission at 460 and 520 nm). Curve fitting was carried out using GraphPad software.

**Fluorescence Microscopy—** Cells transfected with ATX-GFP or LPA1-GFP (28) were fixed with ice-cold methanol at 24 h after transfection. ATX and LPA were detected by anti-GFP antibody using a Leica confocal microscope.

**RESULTS AND DISCUSSION**

**ATX Processing in HEK293 Cells—** ATX is synthesized as a type II transmembrane glycoprotein of ~125 kDa, consisting of a very short N-terminal region, a single transmembrane domain, and a large catalytic ectodomain. ATX undergoes membrane-proximal cleavage to yield a soluble enzyme (29), yet
little is known about ATX biosynthesis and proteolytic processing. In particular, it remains unclear whether the transmembrane form of ATX is expressed on the cell surface.

As a starting point for the present studies, we expressed full-length ATX in HEK293T cells using three differently HA- and Myc-tagged constructs and analyzed ATX expression in cell lysates as well as in the culture medium. As shown in Fig. 1A, full-length ATX is detected only in cell lysates, whereas ATX in the medium lacks the N-terminal HA tag, consistent with secreted ATX being produced by cleavage of the N terminus. The Golgi-disturbing agents brefeldin A and monensin blocked ATX secretion, indicating involvement of the classical export route (supplemental Fig. 1A). When analyzed by confocal microscopy, ATX is detected in intracellular vesicles but not at the plasma membrane; in contrast, LPA1 receptors localize primarily to the cell surface under the same experimental conditions (Fig. 1B). From these results we conclude that ATX follows the classical secretory pathway and that proteolytic removal of its N terminus, including the transmembrane region, occurs intracellularly rather than at the plasma membrane.

ATX Activity toward Lipid and Non-lipid Substrates—Having established that ATX is not detectable as a plasma membrane-anchored ecto-enzyme, we set out to examine the catalytic activity of soluble ATX. To this end, we used conditioned medium from ATX-Myc-transfected HEK293T cells without further purification or, in some experiments, ATX purified from Sf9 cell supernatant (supplemental Fig. 1B). The catalytically inactive mutant ATX(T210A)-Myc served as a negative control (27).

The lyso-PLD activity of ATX was measured by the conversion of LPC(16:0) to LPA using thin layer chromatography (Fig. 2A). LPC hydrolysis by ATX proceeded at a constant rate for at least 90 min (Fig. 2C, left panel). The $K_m$ for LPC was estimated at $110 \pm 150$ M, in agreement with previously reported values (100–260 M) (11, 12, 14) and in the range of normal LPC levels in plasma (25). When screening multiple lipids as potential modulators of lyso-PLD activity, we observed that ATX-catalyzed LPC hydrolysis was significantly inhibited by LPA and S1P (1 μM, complexed to albumin). Other phospholipids tested, including phosphatidic acid, did not show such an effect (Fig. 2 and results not shown). This suggests that ATX is subject to product inhibition by LPA. Detailed analysis of lyso-PLD inhibition is obscured, however, by limitations with the standard LPC hydrolysis assay, which generates concentrations of LPA in excess of its inhibition constant (see below). As a result, formation of LPA during the course of the assay causes the apparent inhibition of ATX by added LPA to be less pronounced than the true inhibition.

To avoid the complications of lipid product inhibition and the limitations of end-point LPC hydrolysis assays, we took advantage of the fact that ATX uses a common reaction mechanism for the hydrolysis of lipid and non-lipid substrates (20, 21). As non-lipid substrates, we tested pNP-TMP, a standard NPP substrate, and the symmetric phosphodiester bis-pNPP, a sub-
found that its ability to inhibit ATX was not affected. As can be inferred from Fig. 3D, the IC₅₀ value for the LPA-BSA (1:1) complex to inhibit ATX was close to 0.1 μM, using a substrate concentration of 1 mM (i.e., equal to the Kₘ).

**CPF4, a FRET-based Sensor of ATX Activity**—Because the pNP-TMP colorimetric assay showed only moderate sensitivity (detection limit, ~0.15 μg of ATX/ml), we explored a newly developed FRET-based phosphodiesterase sensor termed CPF4 (26). CPF4 is a bis-pNPP-derived probe, in which both phenyl moieties are linked to coumarin and fluorescein, respectively, resulting in FRET with high efficiency (Fig. 4A). Cleavage of the phosphodiesterase group by a non-specific phosphodiesterase from snake venom causes loss of FRET, providing a convenient ratiometric readout of enzyme activity (26). Notably, the CPF4 fluorescence ratio is insensitive to pH in the physiological range (pH 7.0–8.0) (26). A major advantage of fluorescence-based sensors like CPF4 is their high sensitivity, allowing detection of very low concentrations of enzyme.

We examined whether CPF4 serves as a substrate for ATX. As shown in Fig. 4B, ATX-Myc causes a prominent loss of FRET (increased donor fluorescence and a concomitant decrease in acceptor fluorescence), indicative of substrate hydrolysis, which can be monitored in real time (Fig. 4C). No CPF4 signal change was detected with the inactive mutant ATX(T210A). A plot of the initial hydrolysis rates versus CPF4 concentration revealed Michaelis-Menten kinetics with an average Kₘ as low as ~4 μM (range, 2.5–6 μM) for ATX-Myc in HEK293 cell-conditioned medium (Fig. 4D). About the same Kₘ value was found with highly purified ATX from SF9 cell supernatant (see below and Fig. 6), suggesting that conditioned medium does not contain significant amounts of competitive inhibitors of ATX (which would increase the apparent Kₘ).

It is noteworthy that the apparent affinity of ATX for CPF4 is 2–3 orders of magnitude higher than that for lysophospholipids and nucleotides (Kₘ = 0.1–1.0 mM; one notable exception is diadenosine triphosphate with an apparent Kₘ of 8 μM (33)). Also with bis-pNPP, a “non-Michaelis-Menten” substrate, half-maximal hydrolysis rates are observed at concentrations as high as ~0.5 mM. Thus attachment of the coumarin-fluorescein tandem to bis-pNPP, which increases hydrophobicity and disturbs structural symmetry, converts bis-pNPP into a high-affinity substrate for ATX, suggesting that the coumarin-fluorescein tandem fits into a hydrophobic pocket involved in substrate binding. Although the overall catalytic efficiency (Vₘₐₓ/Kₘ) of CPF4 hydrolysis was about 3-fold lower than that observed with the other substrates, the FRET-based assay showed superior sensitivity; even at a 5000-fold dilution of HEK293T cell-conditioned medium, ATX activity was still detectable. The estimated detection limit of the CPF4 assay was 3 ng of ATX/ml, exceeding that of the standard colorimetric assay by at least 50-fold. This makes CPF4 the preferred probe for measuring ATX activity in conditioned media and body fluids, although it remains to be seen to what extent the presence of binding proteins and/or nonspecific phosphodiesterases may limit the usefulness of CPF4 in assessing ATX activity in complex biological fluids.

We also examined whether CPF4 is a substrate for nonmammalian secreted lyso-PLDs, notably the broad specificity PLD from *S. chromofuscus* and the sphingomyelin- and LPC-specific PLDs from *C. pseudotuberculosis* and *Loxosceles laeta* (28). When assayed under the conditions used for ATX, none of these exogenous PLDs (10 nM) was able to cleave CPF4 (results not shown). It thus appears that among the known secreted PLDs, only ATX can hydrolyze CPF4.

**Inhibition of ATX by LPA and S1P**—Because of its superior sensitivity and convenient readout, the CPF4 assay was used

---

**Fig. 5. ATX inhibition by LPA and S1P.** A, dose-response curves for the inhibitory effects of LPA (1-oleyl) and S1P on the activity of ATX-Myc and purified NPP1 (about 2 μg/100 μl of assay volume). CPF4 concentration, 2 μM. B, dependence of ATX inhibition on the acyl chain length of LPA (1 μM). The rightmost bar shows that the inhibitory effects of LPA and S1P (both added at 0.5 μM) are not additive.
in our further analysis of ATX inhibition. We determined the concentration dependence of LPA and S1P for inhibiting ATX activity using a substrate concentration close to the $K_m$. Under those conditions, the IC$_{50}$ value for 1-oleoyl-LPA is ~0.1 μM (Fig. 5A), very similar to the value found with pNP-TMP or LPC as substrate (Fig. 3D and results not shown). S1P inhibited ATX activity with the same dose dependence as observed for LPA (Fig. 5A). Other natural lipids, including dioleoylphosphatidic acid, 1-oleoyl-glycerol, sphingosine (10 mM), glycerol 3-phosphate (1 mM), and free fatty acids, did not affect ATX activity. Although AMP has been reported to inhibit the NPP reaction (1, 2), we did not observe any effect of either AMP or ATP (1 mM) on ATX activity (results not shown).

Importantly, the inhibitory effects of LPA and S1P were specific for ATX in that the activity of the structurally related ecto-phosphodiesterase NPP1 was insensitive to either lipid (Fig. 5A). Inhibition of ATX by LPA was dependent on the length of the acyl chain; maximal inhibition was induced by 1-oleoyl-LPA, 1-palmitoyl-LPA, and 1-myristoyl-LPA, whereas short-chain LPA(6:0) had no measurable effect (Fig. 5B). The apparent requirement of a long acyl chain suggests that LPA and S1P directly interact with a hydrophobic pocket on ATX. The inhibitory effects of LPA and S1P were nonadditive (Fig. 5B), suggesting that both lipids act on the same regulatory site. We conclude that, in marked contrast to the G protein-coupled receptors (17), ATX does not discriminate between LPA and S1P as ligands.

We next examined the mechanism of inhibition by LPA and S1P. Substrate titration studies revealed that LPA and S1P are mixed-type inhibitors, producing a reduction in $V_{max}$ and an increase in $K_m$ (Fig. 6). In other words, inhibition of ATX by LPA/S1P has both a noncompetitive and a competitive compo-
Inhibition of Autotaxin by LPA and S1P

REFERENCES

1. Bollen, M., Gijbers, B., Ceulemans, H., Stalmanis, W., and Stefan, C. (2000) *Cirtr. Rev. Biochem. Mol. Biol.* **35**, 393–432

2. Clair, T., Lee, H. Y., Liotta, L. A., and Stracke, M. L. (1997) *J. Biol. Chem.* **272**, 996–1001

3. Clauser, W. T., Goding, J. W., Grobben, B., and Slegers, H. (2003) *Biochim. Biophys. Acta* **1638**, 1–19

4. Stracke, M. L., Krutzsch, H. C., Unsworth, E. J., Arendt, A., Ciocio, V., Schifflmann, E., and Liotta, L. A. (1992) *J. Biol. Chem.* **267**, 2524–2529

5. Murata, J., Lee, H. Y., Clair, T., Krutzsch, H. C., Arendt, A. A., Sobel, M. E., Liotta, L. A., and Stracke, M. L. (1994) *J. Biol. Chem.* **269**, 30473–30484

6. Nam, S. W., Clair, T., Campo, C. K., Lee, H. Y., Liotta, L. A., and Stracke, M. L. (2000) *Oncogene* **19**, 241–247

7. Nam, S. W., Clair, T., Kim, Y. S., McManlin, A., Schifflmann, E., Liotta, L. A., and Stracke, M. L. (2001) *Cancer Res.* **61**, 6938–6944

8. Mills, G. B., and Moalenaar, W. H. (2003) *Nat. Rev. Cancer* **3**, 582–591

9. Fuss, B., Baba, H., Phan, T., Tushy, V. K., and Macklin, W. B. (1997) *J. Neurosci.* **17**, 9065–9073

10. Bachner, D., Ahrens, M., Betat, N., Schroder, D., and Gross, G. (1999) *Mech. Dev.* **84**, 121–125

11. Uemoto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G. B., Inoue, K., Aoki, J., and Aras, H. (2002) *J. Cell Biol.* **158**, 227–233

12. Tokumura, A., Majima, E., Kariya, Y., Tominga, K., Kogure, K., Yasuda, K., and Fujikawa, K. (2002) *J. Biol. Chem.* **277**, 39436–39442

13. Moalenaar, W. H., van Meeteren, L. A., and Giepmans, B. N. (2004) *BioEssays* **26**, 670–681

14. Clair, T., Aoki, J., Koh, E., Randle, B. W., Nam, S. W., Paasynyko, M. M., Mills, G. B., Schifflmann, E., Liotta, L. A., and Stracke, M. L. (2003) *Cancer Res.* **63**, 5446–5453

15. Fassina, F. R., Jalink, K., Hengeveld, T., and Moalenaar, W. H. (1996) *EMBO J.* **15**, 2388–2392

16. Ila, T., Lee, M. J., Ancellin, N., Paik, H. J., and Kluk, M. J. (2001) *Science* **294**, 1875–1878

17. Ishii, I., Fukushima, N., Ye, X., and Chun, J. (2004) *Annu. Rev. Biochem.* **73**, 321–354

18. Liliom, K., Sun, G., Bunemann, M., Virag, T., Nusser, N., Baker, D. L., Wang, D. W. J., Fabian, M. J., Brzozowski, S., Bender, K., Eickel, A., Malik, K. U., Miller, D. D., Desiderio, D. M., Tigyi, G., and Pot, L. (2001) *Biochem. J.* **355**, 189–197

19. Spiegel, S., and Milstien, S. (2003) *Nat. Rev. Mol. Cell. Biol.* **4**, 397–407

20. Koh, E., Clair, T., Woodhouse, E. C., Schifflmann, E., Liotta, L. A., and Stracke, M. L. (2003) *Cancer Res.* **63**, 2042–2045

21. Gijsbers, R., Aoki, J., Arai, H., and Bollen, M. (2003) *FEBS Lett.* **539**, 69–74

22. Eichholtz, T., Jalink, K., Fahrenheit, I., and Moalenaar, W. H. (1993) *Biochem. J.* **291**, 567–680

23. Baker, D. L., Lapidus, P., Miller, B., Relye, C. A., Tilley, B., Westermann, A. M., Bonifer, J. M., Ruis, E., Moalenaar, W. H., and Tigyi, G. (2002) *J. Am. Med. Assoc.* **287**, 3081–3082

24. Sano, T., Baker, D., Virag, T., Wada, A., Yamayoshi, Y., Igarashi, Y., and Tigyi, G. (2002) *J. Biol. Chem.* **277**, 21197–21206

25. Croset, M., Brousard, N., Polette, A., and Lagarde, M. (2000) *Biochem. J.* **345**, 61–67

26. Takakusa, H., Ikikuchi, K., Uram, Y., Sakamoto, S., Yamaguchi, K., and Hama, K. (2000) *J. Am. Chem. Soc.* **122**, 1653–1657

27. Lee, H. Y., Clair, T., Mulvaney, P. T., Woodhouse, E. C., Aznavoorian, S., Liotta, L. A., and Stracke, M. L. (1996) *J. Biol. Chem.* **271**, 24408–24412

28. van Meeteren, L. A., Frederiksen, F., Giepmans, B. N., Pedrosa, M. F., Billington, S. J., Jess, B. H., Tambourgi, D. V., and Moalenaar, W. H. (2004) *J. Biol. Chem.* **279**, 10833–10836

29. Stracke, M. L., Clair, T., and Liotta, L. A. (1997) *Adv. Enzyme Reg.* **37**, 135–144

30. Kelly, S. J., Dardinger, D. E., and Butler, L. G. (1975) *Biochemistry* **14**, 4983–4988

31. Rudolph, A. E., Stuckey, A. J., Zhao, Y., Matthews, H. R., Patton, W. A., Moss, J., and Drexhage, H. (1999) *J. Biol. Chem.* **274**, 11824–11831

32. Zambonelli, C., Casali, M., and Roberts, M. F. (2003) *J. Biol. Chem.* **278**, 52282–52289

33. Vollmayer, P., Clair, T., Goding, J. W., Sano, K., Servos, J., and Zimmermann, H. (2003) *Eur. J. Biochem.* **270**, 2971–2978

34. Yang, L., Yamotomi, Y., Miura, Y., Satoh, K., and Ozaki, Y. (1999) *Br. J. Haematol.* **107**, 292–293

35. Clair, T., Krutzsch, H. C., Liotta, L. A., and Stracke, M. L. (1997) *Biochem. Biophys. Res. Commun.* **236**, 449–454