Neurotransmitter release by catecholaminergic cells is negatively regulated by prohormone cleavage products formed from plasmin-mediated proteolysis. Here, we investigated the expression and subcellular localization of Plg-RKT, a novel plasminogen receptor, and its role in catecholaminergic cell plasminogen activation and regulation of catecholamine release. Prominent staining with anti-Plg-RKT mAb was observed in adrenal medullary chromaffin cells in murine and human tissue. In western blotting, Plg-RKT was highly expressed in bovine adrenomedullary chromaffin cells, human pheochromocytoma tissue, PC12 pheochromocytoma cells, and murine hippocampus. Expression of Plg-RKT fused in-frame to GFP resulted in targeting of the GFP signal to the cell membrane. Phase partitioning, co-immunoprecipitation with uPAR, and FACS analysis with antibody directed against the C-terminus of Plg-RKT were consistent with Plg-RKT being an integral plasma membrane protein on the surface of catecholaminergic cells. Cells stably overexpressing Plg-RKT exhibited substantial enhancement of plasminogen activation and antibody blockade of nontransfected PC12 cells suppressed plasminogen activation. In functional secretion assays, nicotine-evoked [3H]-norepinephrine release from cells overexpressing Plg-RKT was markedly decreased (by 51±2%, p<0.001), compared with control transfected cells, and antibody blockade increased [3H]-norepinephrine release from nontransfected PC12 cells. In summary, Plg-RKT is present on the surface of catecholaminergic cells and functions to stimulate plasminogen activation and modulate catecholamine release. Plg-RKT thus represents a new mechanism and novel control point for regulating the interface between plasminogen activation and neurosecretory cell function.

Plasmin, a broad spectrum serine protease, is the major enzyme responsible for dissolving fibrin clots. Plasmin is generated by proteolytic cleavage of the circulating zymogen, plasminogen, by either of the plasminogen activators, tissue type plasminogen activator (t-PA) or urokinase (u-PA). Studies conducted in the past decade have revealed key non-fibrinolytic functions of these proteins that include major interactions between catecholaminergic and plasminogen activation pathways that may substantially influence catecholamine release. Specifically, prohormones, secreted by cells within the sympathoadrenal system are processed by plasmin into bioactive peptides that locally modulate (inhibit) sympathoadrenal catecholamine release, to provide an autocrine, homeostatic (negative feedback) mechanism to modify the function of the local neurosecretory cells and regulate catecholamine release during stress (1-3). In addition, t-PA is co-stored and co-released with catecholamines and prohormones from catecholamine storage vesicles within catecholaminergic cells of the sympathoadrenal system, including chromaffin cells of the adrenal medulla and sympathetic neurons (4-6). Furthermore, catecholaminergic cells have a high binding capacity for plasminogen and, therefore, plasminogen activation and local prohormone
processing are markedly enhanced when plasminogen is bound to the surface of these cells (1,7,8). Carboxypeptidase B (CpB) treatment decreases cell-dependent plasminogen activation by ~90%, suggesting that the binding of plasminogen to proteins exposing C-terminal basic residues on the cell surface is required to promote plasminogen activation (8). Beta/gamma actin (processed to expose a C-terminal lysine) represents a component of the CpB-sensitive cell surface plasminogen binding sites on catecholaminergic cells (8). However, while cell surface actin is an important plasminogen binding site, accounting for a substantial fraction of plasminogen binding and activation, these observations also suggest a crucial role for other cell surface plasminogen binding proteins on these cells.

Recently, we used multi-dimensional protein identification technology (MudPIT) to isolate a structurally unique plasminogen receptor, the novel protein, Plg-RKT, (9). The Plg-RKT protein includes 147 amino acids with a molecular mass of 17,261 Da. Plg-RKT is synthesized with and exposes a C-terminal basic residue (lysine) on the cell surface, in an orientation to promote cell-dependent plasminogen activation. Furthermore, Plg-RKT is highly conserved with high interspecies homology (e.g. human vs mouse = 94% similarity), high identity and no gaps in the sequence among the 20 mammalian orthologs for which sequence information is available (9). In the present study, we have examined catecholaminergic cells and tissues for expression of Plg-RKT and investigated the subcellular localization and function of Plg-RKT in catecholaminergic cells. The results of our study suggest that Plg-RKT is a key regulator of catecholaminergic cell plasminogen activation and of neurotransmitter release.

EXPERIMENTAL PROCEDURES

Proteins
Glu-plasminogen was purified from fresh human blood as described (1,10). Single chain recombinant human t-PA was from EMD (San Diego, CA). Anti-Plg-RKT polyclonal antibodies were raised in rabbits and monoclonal anti-Plg-RKT antibodies were raised in mice against the synthetic peptide, CEQSKFFSDK (corresponding to the nine C-terminal amino acids of rat Plg-RKT with an amino terminal cysteine added for coupling), coupled to keyhole limpet hemocyanin. Antibodies were selected for direct binding to immobilized CEQSKFFSDK coupled to bovine serum albumin and for the ability to inhibit specific plasminogen binding to CEQSKFFSDK. Anti-Plg-RKT mAb was panspecific, reacting with the C-terminal nonapeptides of mouse, rat and human Plg-RKT with equivalent affinity. Monoclonal anti-uPAR #3936 was from American Diagnostica (Stamford, CT). Polyclonal anti-GFP was from Invitrogen (Carlsbad, CA).

Cells
PC12 cells derived from rat pheochromocytoma (11) were obtained from Dr. David Schubert (Salk Institute, La Jolla, California, USA) and were grown as described in DMEM supplemented with 5% fetal calf serum, 10% horse serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin at 37°C, 6% CO2 (1,2,5,12).

Hoxa9-ER4 cells (13) were a kind gift from Dr. Mark P. Kamps, University of California, San Diego, and were cultured as described (14) and differentiated with murine macrophage colony stimulating factor (M-CSF) (EMD) as described (9).

Bovine chromaffin cells were isolated from bovine adrenal glands as described (1,5,8) and were cultured in minimal essential media containing 1% non-essential amino acids, 1% L-glutamine, 10% fetal calf serum, 1% amphotericin B, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Constructs and Transfections
We subcloned the full-length 443 bp Plg-RKT cDNA into the mammalian expression vector pAcGFP1-C1 (Clontech, Franklin Lakes, NJ,) using Bgl II and Sal I cloning sites to produce the pAcGFP-Plg-RKT construct (encoding the GFP-Plg-RKT fusion protein with Plg-RKT fused in frame at the C-terminus). We also subcloned the full-length 443 bp Plg-RKT cDNA into the mammalian expression vector, pCIneo (Promega, Madison, WI) driven by the CMV promoter, to produce the construct, pCIneo-Plg-RKT. Constructs were transfected into cells using Lipofectamine 2000 (Invitrogen) and stable
transfectants were selected with 1 mg/ml G418 (Promega).

**Immunohistochemistry**

Normal human and mouse adrenal samples were on tissue microarrays (TMA) (Imgenex Array-IMH-372) (Imgenex, San Diego, CA) or histological slides. After dewaxing, TMAs or histological slides were incubated with anti-Plg-RKT mAb followed by secondary anti-mouse IgG antibody and developed using the Envision Plus HRP system [DakoCytomation and diaminobenzidine (DAB) -based detection method] in an automated Dako Universal Staining System immunostainer (15). The slides were scanned on a Scanscope CM-1 scanner (Aperio Technology, Vista, CA).

**Western blotting**

Tissues were lysed in 50 mM TrisHCl, pH 7.2, containing 150 mM NaCl, 0.1% sodium dodecylsulfate (SDS), 1% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche, Palo Alto, CA). Subcellular fractionation was carried out by dounce homogenization, followed by centrifugation steps as used in our laboratory (9). Proteins were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 4-20% gradient gels under reducing conditions, transferred to nitrocellulose (Amersham Pharmacia Biotech, Arlington, Heights, IL) and incubated with anti-Plg-RKT antibodies. The membranes were incubated with an anti-mouse antibody-HRP conjugate, developed using an ECL substrate (Pierce, Rockford, IL) and exposed to Kodak Biomax MR Film (Fisher, Santa Clara, CA). Harvesting of mouse tissue was performed under an experimental protocol approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

**Laser scanning confocal microscopy**

Confocal images were captured using a Zeiss Laser Confocal Scanning Microscope (LCSM) running the latest Zen 2009 Zeiss software suite (Carl Zeiss Inc., Thornwood, NY). All images were then imported and further analyzed for quantitative colocalization using two independent software packages: LSM examiner (Zeiss) and Image J (NIH imaging; [http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)). Colocalization between fluorescently labeled Plg-RKT with wheat germ agglutinin (WGA)-alexa-555 (Invitrogen) was quantified by obtaining the threshold range of real over background signal and then using the average real threshold range to calculate the correlation coefficients (M values) of at least 40 cells in three separate experiments. To define the number and size of each fluorescently labeled aggregate, images were imported into Image Pro Plus (Media Cybernetics Inc., Bethesda MD) where each cell was outlined and a similar threshold range (as described above) was used to define a real signal within each cell. Once this range was defined the software then automatically extracts parameters, such as area, perimeter total number, and average fluorescence intensity of the fluorescently labeled proteins per cell.

**Plasminogen activation assays**

Cells were preincubated with 2.7 μM glued-plasminogen at 37°C for 30 minutes. Then 20 nM t-PA was added. Plasmin activity (expressed as O.D. 405 nM) was measured after 3 minutes by diluting the reaction mixture 1:10 into D-VLK-pNA (DiaPharma Group Inc., Franklin, OH) to a final concentration of 1 mM and monitoring absorbance at 405 nM as described (16).

**Fluorescence activated cell sorting (FACS) analysis**

Subconfluent, adherent PC12 cells that had been cultured for 48 hours without a change of media, were harvested by rinsing flasks twice with PBS at 4°C and then detached with 5 mM EDTA/PBS at 37°C for 5 minutes. All FACS analyses were performed as described (17). Briefly, for the detection of cell-surface Plg-RKT on viable PC12 cells, indirect immunofluorescence staining and dual-color FACS analyses were performed. Cells (2 X 10^5 cells) were incubated with 40 μg/ml of anti-Plg-RKT mAb IgG or isotype control IgG for 30 minutes in binding buffer (HBSS containing 0.1% BSA) at 4°C. The cells were washed once with 200 μl binding buffer and incubated with FITC-labeled secondary IgG for 30 minutes at 4°C in the dark. The cells were washed again, resuspended in 500 μl of binding buffer containing the non-vital dye, propidium iodide (PI) at 5 μg/ml and the cells immediately analyzed by dual-color FACS as described (8). Populations of cells were gated according to the fluorescence intensity of PI.
staining. The population of cells with low cell-associated PI fluorescence intensity (cells that excluded PI) were defined as viable cells while the population of cells with high PI fluorescence intensity (inclusion of PI) were defined as non-viable.

Secretagogue-stimulated catecholamine release

Chromaffin cell catecholamine secretion was determined as described (1,2,12). Briefly, PC12 cells were labeled for 2 hours with $[^{3}H]$-norepinephrine (PerkinElmer Life Sciences, Boston, MA) at 1 $\mu$Ci/ml in cell culture medium, washed twice with release buffer (150 mM NaCl, 5 mM KCl, 2 mM CaCl$_{2}$, 10 mM HEPES, pH 7.0) and incubated at 37°C for 15 minutes in release buffer in either the presence or absence of 60 $\mu$M nicotine. After aspirating the release buffer, the cells were harvested and lysed in release buffer containing 0.1% Triton X-100. $[^{3}H]$-norepinephrine content of release buffer and cell lysates was determined by liquid-scintillation counting. Per cent release was calculated as percentage of secretion [amount released/(amount released + amount in cell lysate)] and results expressed as net release (% secretagogue-stimulated release minus % basal release).

In control studies, the density of secretory vesicles was assessed and compared in transfected PC12 cells. Electron micrographs were obtained using methods similar to those we have used previously to evaluate transfected PC12 cells (12). Briefly, PC12 cells were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate with 3 mM calcium chloride, washed twice with 0.2 M sodium cacodylate and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate. The cells were enrobed with 2% agarose and cut into 1 mm cubes, stained en bloc with 4% uranyl acetate in 50% ethanol. A graded ethanol series was used for dehydration and propylene oxide was used as a transitional solvent. Cell cubes were embedded in Embed 812 and polymerized in a 60°C oven. Semi-thin sections of 1 $\mu$m were cut on a Reichert Ultracut S ultramicrotome and stained with toluidine blue. Thin sections were cut at 70 nm, collected on 200 mesh copper grids, and stained with 2% uranyl acetate followed by lead citrate. Grids were imaged at 80 kV using a Zeiss EM 10C transmission electron microscope equipped with a Gatan ES1000W digital camera. Secretory vesicle density was determined in random electron micrographs, evaluating cytoplasmic area within 1 $\mu$m of the plasma membrane surface, using Image-Pro Plus software v. 6.3, Media Cybernetics, Inc. (Bethesda, MD).

Statistics

Data are presented as means ± standard error of the mean. Results were analyzed by ANOVA followed by Student-Newman-Keuls post hoc tests for multiple comparisons.

RESULTS

Plg-R$_{KT}$ is prominently expressed in catecholaminergic tissues and cells

In our previous analysis of catecholaminergic cell plasminogen receptors, a major unknown CpB-sensitive protein migrating with an $M_{r}$ of 17.2 X 10$^{3}$ was detected in plasminogen-ligand blotting of 2-D gels of PC12 membrane preparations, in addition to actin (8). Therefore, we investigated expression of Plg-R$_{KT}$, a novel, structurally unique plasminogen receptor ($M_{r}$ = 17.2 X 10$^{3}$) (9), in human and murine adrenal tissues. Prominent staining with anti-Plg-R$_{KT}$ mAb was observed in adrenal medullary chromaffin cells in both human (Fig. 1A) and murine (Fig. 1C) adrenal tissue samples. In specificity controls, no immunostaining was detected in cells preincubated with the peptide used for immunization, CEQSKFFSDK (corresponding to the nine C-terminal amino acids of rat Plg-R$_{KT}$ with an amino terminal cysteine added for coupling) (Fig. 1B,D).

Expression of Plg-R$_{KT}$ was also detected in western blotting of catecholaminergic cells and tissues (Fig. 2). Plg-R$_{KT}$ was detected as a specific immunoreactive band migrating with an $M_{r}$ of 17.2 X 10$^{3}$ in bovine adrenal medullary chromaffin cells. The Plg-R$_{KT}$ protein was also prominently expressed in human pheochromocytoma, a catecholamine-producing tumor of the adrenal medulla, and hence, a source of human chromaffin cells, and PC12 cells [a well-established chromaffin cell line with abundant catecholamine storage vesicles, derived from rat pheochromocytoma (11)]. Plg-R$_{KT}$ was also highly expressed in murine hippocampus, representing a non-adrenal source of catecholaminergic cells. The bands migrated with the same $M_{r}$ as the protein expressed by M-
CSF-differentiated Hox9-ER4 cells, from which Plg-RKT was initially isolated (Fig. 2). In additional specificity controls, minimal expression of Plg-RKT was detected in membranes of undifferentiated progenitor Hox9-ER4 cells as described (9) and no reactivity was detected with isotype control (not shown). These results demonstrate prominent expression of the Plg-RKT protein in catecholaminergic cells and tissues.

**Plg-RKT is localized in the plasma membrane of catecholaminergic cells**

To assess subcellular localization of Plg-RKT and to determine whether the Plg-RKT protein contains a dominant plasma membrane trafficking signal, PC12 cells were transiently transfected with pAcGFP-Plg-RKT (in which the Plg-RKT cDNA was inserted in frame for expression of a GFP-Plg-RKT fusion protein, with Plg-RKT at the C-terminus), or with the pAcGFP empty vector. Cell lysates were western blotted with anti-Plg-RKT polyclonal IgG (Fig. 3A, lanes 1 and 2) or anti-GFP antibody (Fig. 3A, lanes 3 and 4). In cells transfected with pAcGFP-Plg-RKT an immunoreactive band migrating with a Mr app of ~40 X 10^3 was detected with both anti-Plg-RKT and with anti-GFP antibodies (Fig. 3A, lanes 2 and 4), consistent with expression of the GFP-Plg-RKT fusion protein. In cells transfected with the pAcGFP empty vector a band with a Mr app of 23 X 10^3 was detected using the anti-GFP antibody, corresponding to GFP expressed by the vector without insert (Fig. 3A, lane 3). A band migrating with a Mr app of 17.2 X 10^3, corresponding to the endogenous Plg-RKT protein, was also detected with the anti-Plg-RKT antibody in cells transfected with pAcGFP-Plg-RKT or transfected with the pAcGFP empty vector control (Fig. 3A, lanes 1 and 2). In controls, no bands were detected with preimmune IgG (data not shown).

Confocal microscopy was performed to examine the subcellular localization and expression of the GFP-Plg-RKT fusion protein. Cells expressing GFP-Plg-RKT showed membrane localization that was highly colocalized (79 ± 5%) with WGA, a well-established cell surface marker on non-permeabilized fixed cells (Fig. 3B). In contrast the GFP fluorescent signal in cells transfected with vector alone was diffuse and cytoplasmic and did not significantly colocalize (9 ± 5%) with WGA. Thus, fusion of GFP to Plg-RKT resulted in trafficking of GFP to the plasma membrane.

We also tested whether endogenously expressed (native) Plg-RKT behaved as an integral membrane protein in catecholaminergic cells. Nontransfected PC12 cells were subjected to phase separation in Triton X-114 as described (18,19). In this technique, integral membrane proteins are recovered in the detergent phase, whereas hydrophilic proteins remain in the aqueous phase. An immunoreactive band corresponding to Plg-RKT was detected in the detergent phase, but was not detected in the aqueous phase (Fig. 4A). In controls for the method, when the cell lysates were spiked with BSA and subjected to phase partitioning, BSA was detected in the aqueous, but not the detergent phase.

To further assess the plasma membrane localization of endogenous Plg-RKT we assessed the physical association of Plg-RKT with the urokinase-type plasminogen activator receptor (uPAR), a glycosylphosphatidylinositol-anchored cell surface receptor (20). In co-immunoprecipitation studies anti-Plg-RKT antibody immunoprecipitated both Plg-RKT and uPAR from membrane fractions of nontransfected PC12 cells (Fig. 4B).

To evaluate the cell surface expression of the C-terminus of endogenous Plg-RKT, PC12 cells were subjected to FACS analysis with anti-Plg-RKT mAb, raised against the C-terminal peptide of Plg-RKT. Prominent binding of anti-Plg-RKT mAb to the cell surface was observed (Fig. 4C). These data are consistent with Plg-RKT behaving as an integral plasma membrane protein on the surface of catecholaminergic cells.

**Plg-RKT regulates plasminogen activation on catecholaminergic cells**

Plasminogen activation is enhanced when plasminogen is bound to catecholaminergic cells (1,7,8). Therefore, we investigated the role of Plg-RKT in plasminogen activation. We subcloned the full-length 443 bp Plg-RKT cDNA into the mammalian expression vector, pCIneo, for constitutive overexpression of full-length Plg-RKT driven by the CMV promoter. Transfection of PC12 cells with pCIneo-Plg-RKT resulted in prominent enhanced expression of Plg-RKT on the cell surface (Fig. 5A) that was substantially greater than the surface expression of endogenous Plg-RKT.
by cells stably expressing the control pCIneo vector without insert (Fig. 5B).

We compared plasminogen activation on cells stably overexpressing Plg-RKT with plasminogen activation on control cells stably expressing the pCIneo empty vector. Cells stably transfected with the pCIneo empty vector stimulated plasminogen activation in a cell-concentration-dependent fashion, consistent with our previously published results (1,7,8). With cells stably overexpressing Plg-RKT, stimulation of plasminogen activation was markedly enhanced compared to stimulation with cells transfected with empty vector (p < 0.001 at all cell concentrations tested) (Fig. 5C). These results support a prominent role of Plg-RKT in cell surface plasminogen activation.

Role of Plg-RKT in catecholamine secretion
Localization of plasminogen on catecholaminergic cell surfaces results in processing of prohormones (released from catecholamine storage vesicles after secretagogue stimulation) to bioactive peptides that inhibit secretagogue-stimulated catecholamine release (1-3). Therefore, we tested the effect of overexpression of Plg-RKT on secretagogue-stimulated catecholamine release from catecholaminergic cells. We compared secretagogue-stimulated catecholamine release from PC12 cells stably overexpressing Plg-RKT with catecholamine release from control cells transfected with empty vector. The cells were labeled with [3H]-norepinephrine, and stimulated with the chromaffin cell secretagogue, nicotine, acting through nicotinic cholinergic receptors, at 37°C for 15 minutes and catecholamine release was measured by liquid scintillation counting. Norepinephrine release in response to nicotine was markedly suppressed in cells overexpressing Plg-RKT (by 52.2 ± 7.6%, n = 9, p < 0.001) compared with release by cells expressing vector alone (Fig. 6).

In controls, catecholamine levels in cells stably overexpressing Plg-RKT were comparable to those of cells transfected with empty vector (2321 ± 97 cpm [3H]-norepinephrine per 10⁶ cells, n = 9, for Plg-RKT overexpressing cells, versus 2281 ± 81 cpm [3H]-norepinephrine per 10⁶ cells, n = 9, for cells transfected with empty vector, p = 0.756). In additional controls, secretory vesicle density obtained from scanning of electron micrographs did not differ between PC12 cells overexpressing Plg-RKT and cells transfected with empty vector (4.65 ± 0.44 vesicles/µm², for Plg-RKT overexpressing cells, versus 4.16 ± 0.50 vesicles/µm², for control transfected cells, n = 12 random electron micrographs evaluated for each cell type, p = 0.470).

In other controls, we measured catecholamine release stimulated by another secretagogue, 55 mM KCl (high-K⁺), acting through membrane depolarization. Release stimulated by high K⁺ from Plg-RKT overexpressing cells did not differ from that from control transfected cells (21.1 ± 1.6%, n = 6, versus 23.0 ± 1.5%, n = 6, p = 0.407). These results suggest that the observed differences in catecholamine release are specific for nicotine-evoked release.

We also evaluated the functional role of endogenous Plg-RKT on nontransfected PC12 cells. In the presence of function blocking anti-Plg-RKT mAb, cell-dependent plasminogen activation was markedly suppressed (Fig. 7A). Correspondingly, when nontransfected PC12 cells were preincubated with function blocking anti-Plg-RKT mAb, norepinephrine release was significantly increased compared with secretion from cells pre-treated with isotype control (Fig. 7B).

DISCUSSION
Plasminogen binding sites on catecholaminergic cells markedly stimulate plasminogen activation and, consequently, prohormone processing by plasmin (1,3,8). For example, plasmin processes the prototypical prohormone chromogranin A (CgA) to liberate a specific peptide [human CgA-(360-373)] that inhibits nicotine-stimulated catecholamine release (1,2). These interactions represent a local proteolytic system on catecholaminergic cells that markedly influences catecholamine secretion.

Here, we have examined the expression and subcellular localization of the novel plasminogen receptor, Plg-RKT, in catecholaminergic cells and tissues and investigated the role of Plg-RKT, in cell surface plasminogen activation and regulation of catecholamine release. We found that 1) Plg-RKT was highly expressed in catecholaminergic cells
and tissues of different species; 2) cloning of GFP upstream of the Plg-RKT cDNA directed GFP to the plasma membrane of catecholaminergic cells; 3) Plg-RKT behaved as an integral plasma membrane protein on the surface of catecholaminergic cells; 4) Plg-RKT exposed its C-terminus (with a C-terminal lysine) on the catecholaminergic cell surface in an orientation to promote plasminogen activation; 5) overexpression of Plg-RKT resulted in substantial enhancement of plasminogen activation on the catecholaminergic cell surface, and antibody blockade of endogenous Plg-RKT resulted in inhibition of plasminogen activation; and 6) overexpression of Plg-RKT resulted in inhibition of nicotine-stimulated catecholamine release, and antibody blockade of endogenous Plg-RKT resulted in augmentation of nicotine-stimulated catecholamine release. Taken together, these results suggest a key role for Plg-RKT in the regulation of catecholamine release through promoting local, cell surface plasminogen activation.

Our data suggest that Plg-RKT is highly expressed in catecholaminergic tissues. Prominent, specific immunochemical staining of chromaffin cells within the medulla of both human and murine adrenal tissue was detected. These results were corroborated in western blotting in which a specific immunoreactive band migrating with an Mr app of 17.2 X 10^3 (consistent with the Mr app of Plg-RKT) was detected also in human pheochromocytoma tissue, bovine adrenal medullary chromaffin cells and PC12 cells, demonstrating the presence of this novel protein in these catecholaminergic tissues. Plg-RKT was also highly expressed in murine hippocampus, representing a non-adrenal source of catecholaminergic cells. In addition, results obtained by searching the Gene Expression Atlas at http://www.ebi.ac.uk/gxa/ (E-GEOD-3594) indicated prominent expression of the Plg-RKT transcript in murine adrenal tissue and in murine hippocampal tissue in expression profiling of tissues from 5 inbred mouse strains, consistent with our results demonstrating expression of Plg-RKT protein in these tissues.

Our results show that Plg-RKT is located in the plasma membrane of catecholaminergic cells. In subcellular localization studies, PC12 cells were transfected with pAcGFP-Plg-RKT (in which the Plg-RKT cDNA was inserted in frame for expression of a GFP-Plg-RKT fusion protein, with Plg-RKT at the C-terminus) or with the pAcGFP empty vector. Confocal microscopy showed marked colocalization (79 ± 5%) of the GFP-Plg-RKT fusion protein with WGA, a cell surface marker. In contrast, transfection with GFP vector without insert led to expression of fluorescence in a diffuse cytoplasmic pattern that did not significantly colocalize with WGA, demonstrating that Plg-RKT provided a dominant trafficking signal to direct GFP to the cell membrane. In additional studies addressing subcellular localization, endogenous Plg-RKT was immunochemically detected in the detergent phase in phase partitioning experiments. Furthermore, Plg-RKT co-immunoprecipitated with uPAR, a glycosylphosphatidylinositol-anchored cell surface receptor (20). In addition, the C-terminus of endogenous Plg-RKT was detected on the surface of PC12 cells in FACS analysis. Thus, Plg-RKT behaves as an integral plasma membrane protein on the surface of catecholaminergic cells.

Our results indicate that Plg-RKT functions to promote plasminogen activation on the catecholaminergic cell surface. In FACS analysis of intact PC12 cells Plg-RKT was detected with a mAb directed against the C-terminal nonapeptide of Plg-RKT, demonstrating that the C-terminus of Plg-RKT (with its C-terminal lysine) was expressed on the surface of catecholaminergic cells, thus providing an orientation for interaction with the kringle domains of plasminogen. Cell-dependent plasminogen activation was markedly augmented on PC12 cells stably overexpressing Plg-RKT compared to that on cells transfected with empty vector (Fig. 5C). Conversely, specific antibody blockade of Plg-RKT markedly decreased cell-dependent plasminogen activation. These results support a prominent role of Plg-RKT in cell surface plasminogen activation.

Our results suggest that Plg-RKT serves as a key control point for modulation of catecholamine release. We compared secretagogue-stimulated catecholamine release from cells stably overexpressing Plg-RKT with cells transfected with empty vector. Norepinephrine release in response to nicotinic cholinoergic stimulation was markedly suppressed in cells overexpressing Plg-RKT (by 52.2 ± 7.6%, n = 9, p < 0.001) compared with release by cells
expressing vector alone. This result is consistent with plasmic processing of prohormones to produce peptides that feed back to inhibit catecholamine release as we have demonstrated previously (1-3). Consistent with these results, antibody blockade of Plg-RKT markedly enhanced catecholamine release.

The current study, thus, identifies a key component, Plg-RKT, as a crucial molecular focal point in the regulation of the cell-surface-dependent mechanism underlying the ability of catecholaminergic cells to promote local plasminogen activation. Plg-RKT is unique among plasminogen binding proteins in that it is an integral membrane protein synthesized with a C-terminal basic residue. It is noteworthy that Plg-RKT was highly expressed in human, mouse and bovine adrenal medullary tissues as well as hippocampal tissue. Expression of Plg-RKT and additional binding sites for plasminogen (1,7) and t-PA (1,21), along with trafficking of t-PA to catecholamine storage vesicles (5,22), constitute a local catecholaminergic cell plasminogen activation system that regulates cell-surface-dependent neuroendocrine prohormone processing that plays a key role in the regulation of neurotransmitter release. Molecules of the plasminogen activation system are expressed broadly in neuroendocrine sites, including the cerebral cortex (23), cerebellum (23-25), hippocampus (23,25-29), sympathetic neurons (30,31) as well as the adrenal medulla (5,25). Notably, the transcript for Plg-RKT is expressed in all of these tissues (http://www.ebi.ac.uk/gxa/). Hence, Plg-RKT may play a key role in the regulation of local neurosecretory cell plasminogen activation in both central and peripheral nervous systems, with important implications for a variety of noteworthy neuronal/neuroendocrine plasminogen-dependent processes, including: neurite outgrowth (32,33); synaptic transmission, NMDA receptor-mediated signaling and excitotoxin-induced neuronal degeneration (34,35); long term potentiation, learning and memory (26,28,36-40); cleavage and activation of other neuroendocrine substrates such as the neurotrophin proBDNF (brain derived neurotrophic factor) (40), β-endorphin, and α-melanocyte stimulating hormone (41); as well as systemic metabolic and cardiovascular physiologic responses under the control of sympathoadrenal and sympatheuneral activities (1-3,30). Our current results identifying Plg-RKT as a novel cell membrane plasminogen receptor on catecholaminergic cells implicate Plg-RKT as a focal point for regulating the interface between plasminogen activation and catecholaminergic neurosecretary cell function. These interactions between fibrinolytic and neurosecretory pathways thus may have major implications for regulating catecholamine secretion during sympathoadrenal activation/stress responses. Taken together with results demonstrating widespread expression of Plg-RKT in neuronal and neuroendocrine tissues, these results may also suggest a broader paradigm for regulating cell surface proteolysis and neurotransmitter release in other neuronal and neuroendocrine sites.

**ACKNOWLEDGMENTS**

Supported by National Institutes of Health Grants (HL050398 to R.J.P and HL081046 to L.A.M.) and Department of Veterans Affairs (to R.J.P.).

Reference List


FOOTNOTES

1 Abbreviations:
- CgA, chromogranin A
- CpB, carboxypeptidase B
- DAB, diaminobenzidine
- FACS, fluorescence activated cell sorting
- LCSM, laser confocal scanning microscope
- MCSF, macrophage colony stimulating factor
- MudPIT, multidimensional protein identification technology
- PI, propidium iodide
- SDS, sodium dodecylsulfate
- SDS-PAGE, SDS polyacrylamide gel electrophoresis
- TMA, tissue microarrays
- t-PA, tissue plasminogen activator
- u-PA, urokinase
- uPAR, urokinase type plasminogen activator receptor
- WGA, wheat germ agglutinin

FIGURE LEGENDS

Fig. 1. Expression of Plg-RKT in adrenal medulla. Human (A,B) and mouse (C,D) adrenal medulla tissue sections were stained with anti-Plg-RKT mAb, as described in Experimental Procedures, in the absence (A,C) or presence (B,D) of the decapeptide used for immunization, CEQSKFFSDK (corresponding to the nine C-terminal amino acids of rat Plg-RKT with an amino terminal cysteine added for coupling). Positive tissues are indicated by brown staining with DAB. Scale bar = 100 µm.

Fig. 2. Expression of Plg-RKT in catecholaminergic cells. Membrane fractions were prepared from PC12 cells, Hoxa9-ER4 monocyte progenitor cells and Hoxa9-ER4 cells differentiated with M-CSF as described (9). Lysates were prepared from human pheochromocytoma tissue, bovine adrenomedullary chromaffin cells and mouse hippocampus. A quantity of 40 µg protein was loaded in each lane and western blotted with anti-Plg-RKT mAb. No bands were detected with isotype control (not shown). In additional specificity controls with anti-Plg-RKT mAb, minimal expression of Plg-RKT was detected in membranes of progenitor Hox9-ER4 cells, but the 17.2 kDa Plg-RKT band was highly expressed in M-CSF-differentiated Hoxa9-ER4 cells as described (9).

Fig. 3. Plg-RKT re-directs GFP to the cell membrane. PC12 cells were transiently transfected with pAcGFP-Plg-RKT (in which the Plg-RKT cDNA was inserted in frame for expression of a GFP-Plg-RKT fusion protein, with Plg-RKT at the C-terminus) or with control pAcGFP vector. (A) Cell lysates were prepared and western blotted with polyclonal anti-Plg-RKT IgG (lanes 1 and 2) or anti-GFP IgG (lanes 3 and 4). No reactivity was observed with preimmune IgG (not shown). (B) The transfected cells were grown on coverslips and fixed in 2% formaldehyde and then washed and stained with a combination of DAPI and WGA-rhodamine at 20°C in PBS. Cells were washed and mounted in Immunofloure Mounting Medium. Images were captured using a Zeiss Confocal Laser Scanning Microscope, then imported into LSM Examiner and Image J for further processing as described in Experimental Procedures. The first and third panels of images represent maximum projections of a series of optical slices through the cells. The GFP-Plg-RKT signal was localized primarily to the plasma membrane. The second and fourth panels of images represent sagittal (apical-basal) slices through the same cells along the white dotted line indicated. Here the peripheral plasma membrane localization of GFP-Plg-RKT is also evident throughout the vertical stacks of images that were acquired. Scale bar in bottom right corner of image represents 10 microns.

Fig. 4. Plg-RKT is an integral plasma membrane cell surface protein. (A) Phase partitioning of Plg-RKT. Nontransfected PC12 cells were solubilized in 3% Triton X-114. After heating at 37°C and separation of the phases by centrifugation, an aliquot of both phases was electrophoresed and western blotted with polyclonal anti-Plg-RKT. An immunoreactive band corresponding to the Mr app of Plg-RKT was detected in
the detergent phase, but not in the aqueous phase. In controls for the method, when the cell lysates were spiked with BSA and subjected to phase partitioning, BSA was detected in the aqueous, but not the detergent phase (data not shown). No bands were detected using control preimmune IgG (data not shown). (B) Co-immunoprecipitation of Plg-RKT with uPAR. Membrane fractions from nontransfected PC12 cells were prepared as described in Experimental Procedures and immunoprecipitated with polyclonal anti-Plg-RKT IgG or preimmune IgG. Membrane fractions and immunoprecipitates were electrophoresed on SDS-PAGE and immunoblotted for uPAR and for Plg-RKT. No bands were detected using control preimmune IgG isotype control for immunoblotting (data not shown). (C) FACS analysis of Plg-RKT expression on intact PC12 cells. Nontransfected PC12 cells were analyzed by dual color FACS analysis as described in Experimental Procedures. Viable cells were gated from non-viable cells and histogram plots of viable cells are shown. Dotted tracings = anti-Plg-RKT mAb IgG. Black tracings = isotype control IgG. Dashed tracings = autofluorescence.

Fig. 5. Overexpression of Plg-RKT increases cell surface plasminogen activation. PC12 cells stably overexpressing Plg-RKT (pCIneo-Plg-RKT) (A) or vector alone (pCIneo) (B) were analyzed by dual color FACS analysis as described in Experimental Procedures. Viable cells were gated from non-viable cells and histogram plots of viable cells are shown. Dotted tracings = anti-Plg-RKT mAb IgG. Black tracings = isotype control IgG. Dashed tracings = autofluorescence. (C) PC12 cells stably overexpressing Plg-RKT (pCIneo-Plg-RKT) or vector alone (pCIneo) were incubated with plasminogen (2.7 μM) for 30 minutes and then t-PA (20 nM) was added and plasminogen activation was measured as cleavage of the tripeptide substrate D-VLK-pNA (1 mM) after 3 minutes. Cell-mediated plasminogen activation was substantially greater in Plg-RKT overexpressing cells than in control cells (p<0.001 at each cell concentration tested).

Fig. 6. Effect of overexpression of Plg-RKT on catecholamine release. PC12 cells stably overexpressing either Plg-RKT (filled bars) or vector alone (open bars) were treated with 60 μM nicotine (Nicotine) or buffer (Basal) at 37°C for 15 minutes and catecholamine release was measured by liquid scintillation counting as described in Experimental Procedures. Per cent release was calculated as percentage of secretion [amount released/(amount released + amount in cell lysate)] and results expressed as net release (% secretagogue-stimulated release minus % basal release). Results are mean ± SEM, n= 9 for each experimental group. **p < 0.001 for the Plg-RKT transfectants stimulated with nicotine compared with corresponding values for the vector control tested.

Fig. 7. Effect of antibody blockade of endogenous Plg-RKT. (A) Nontransfected PC12 cells (3 x 10^5) were preincubated with 20 μg/ml of either anti-Plg-RKT mAb IgG (closed bars) or isotype control IgG (open bars) for 30 min at 37°C and then incubated with plasminogen (2.7 μM) for 30 minutes and then t-PA (20 nM) was added and plasminogen activation was measured as cleavage of the tripeptide substrate D-VLK-pNA (1 mM) after 3 minutes. (B) Nontransfected PC12 cells were preincubated with either anti-Plg-RKT mAb IgG (closed bars) or isotype control (open bars) for 30 min at 37°C and then were treated with 60 μM nicotine (Nicotine) or buffer (Basal) at 37°C for 15 minutes and catecholamine release was measured by liquid scintillation counting as described in Experimental Procedures. Per cent release was calculated as percentage of secretion [amount released/(amount released + amount in cell lysate)] and results expressed as net release (% secretagogue-stimulated release minus % basal release). Results are mean ± SEM, n= 6 for each experimental group. **p<0.001 for cells incubated with anti-Plg-RKT mAb compared with isotype control.
Figure 2

PC12 cells
Bovine chromaffin cells
Human pheochromocytoma
Hoxa9-ER4
Hoxa9-ER4 - M-CSF
Mouse hippocampus

28 kDa —
18 kDa —
15 kDa —
6 kDa —
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>Anti-Plg-RKT</th>
<th>Anti-GFP</th>
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<tbody>
<tr>
<td>pAcGFP</td>
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<td>pAcGFP-Pig-RKT</td>
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17.2 kDa  23 kDa  40.2 kDa

1  2  3  4

B

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<tr>
<th>GFP-Construct</th>
<th>WGA-Rhodamine</th>
<th>DAPI-Nuclear</th>
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<tr>
<td>GFP-Pig-RKT</td>
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</tr>
<tr>
<td>basal</td>
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</table>

| GFP Control   |               |             |        |
| apical        |               |             |        |
| basal         |               |             |        |

Scale bar: 10 μm
Figure 4

A

Membrane Fraction

Aqueous Phase

Detergent Phase

18 kDa

15 kDa

B

IgG IP

Plg-RKT IP

Membrane Fraction

64 kDa

49 kDa

37 kDa

18 kDa

15 kDa

uPAR

Plg-RKT

C

Counts

10^0 10^1 10^2 10^3 10^4

FITC

Auto

Isotype

anti-Plg-RKT
Figure 5

A. pClneo-Plg-RKT

B. pClneo

C. Plasminogen Activation

Counts

Counts

Plasminogen Activation

FitC

FitC

OD405

Cell Concentration (cells x 10^5/ml)
Figure 6

[Graph showing the comparison of Net $^3$H-Norepinephrine Release (%) between Vector Control and Plg-R$_{KT}$ Transfectants under Basal and Nicotine conditions. The graph indicates a significant increase in release upon Nicotine treatment compared to Basal conditions for Plg-R$_{KT}$ Transfectants.]
Figure 7
The novel plasminogen receptor, plasminogen receptor_{KT} (Plg-R_{KT}), regulates catecholamine release

Hongdong Bai, Nagyung Baik, William B. Kiosses, Stan Krajewski, Lindsey A. Miles and Robert J. Parmer

J. Biol. Chem. published online July 27, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.218693

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