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Research Article

Neuroendocrine Targeting of Tissue Plasminogen Activator (t-PA)

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Abstract

t-PA has a widespread neuroendocrine distribution including prominent expression in chromaffin cells of the sympathoadrenal system. Chromaffin cell t-PA is sorted into catecholamine storage vesicles and co-released with catecholamines in response to sympathoadrenal activation, suggesting that catecholamine storage vesicles may serve as a reservoir for the rapid release of t-PA. Chromogranin A (CgA), a major core protein in secretory vesicles throughout the neuroendocrine system, may play a crucial role in targeting proteins into the regulated secretory pathway, by forming aggregated "granin" complexes to which other proteins destined for the regulated secretory vesicle bind and become separated from constitutively secreted proteins in the trans-Golgi network (TGN). Formation of such complexes is facilitated by conditions of the TGN (low pH, high Ca^{+2}). We tested the hypothesis that t-PA interacts specifically with CgA and that this interaction is enhanced under conditions of the TGN. Immobilized t-PA was incubated with ^{125}I-CgA. t-PA interacted specifically and saturably with CgA and the interaction was domain-specific, mediated by the EGF/finger and kringle 1 domains of t-PA and by a specific internal hydrophilic domain within CgA (KERTHQQKKHSSYEDELSEVL) as assessed by antibody and peptide competition studies. The interaction of t-PA with aggregated CgA complexes may play a role in the targeting of t-PA and its release from neurosecretory cells. These results may have broad implications for the regulation of local neurosecretory cell plasminogen activation under both normal physiological conditions and pathological conditions including cerebral ischemia.

ABBREVIATIONS

BSA: Bovine Serum Albumin; CgA: Chromogranin A; DFP: Diisopropylfluorophosphate; mAB's: monoclonal Antibodies; PBS: Phosphate Buffered Saline; SPR: Surface Plasmon Resonance; t-PA: tissue Plasminogen Activator; TGN: Trans-Golgi Network

INTRODUCTION

Tissue plasminogen activator (t-PA) [1] is a serine protease that plays the dominant role in intravascular removal of fibrin by activating plasminogen to the primary fibrinolytic enzyme, plasmin [1]. In addition to its expression by endothelial cells, t-PA has a widespread neuroendocrine distribution [2-10]. t-PA has a signal peptide allowing passage across the endoplasmic reticulum and subsequent transport through the Golgi stacks. In neurons and endocrine cells, secretory proteins are sorted at the trans-Golgi network into either the constitutive or the regulated pathway [11,12]. Proteins entering the constitutive pathway are not stored but are transported directly to the cell surface and secreted in the absence of any extracellular signal. By contrast, proteins entering the regulated pathway are concentrated and stored in vesicles and subsequently released upon stimulation by a secretagogue or other specific extracellular stimuli. We previously demonstrated that t-PA is expressed in and targeted to the regulated pathway of secretion in a neuroendocrine cell type, the chromaffin cell of the adrenal medulla [13,14]. Chromaffin cells contain abundant catecholamine storage vesicles that are prototypic examples of regulated secretory vesicles [15]. Thus, catecholamine storage vesicles may serve as a reservoir for the rapid regulated release of t-PA. In addition, other sympathoadrenal and sympathoneural tissues, including hippocampal neurons and sympathetic axons of the vessel wall also represent substantial sources for the rapid release of t-PA [16-19]. The regulated release of t-PA may play a major role in a wide variety of other neuroendocrine functions including neurite outgrowth [20,21]; synaptic transmission, NMDA receptor-mediated signaling and excitotoxin-induced neuronal degeneration [8,22]; long term potentiation, learning and memory [1,23-28]; cleavage and activation of other neuroendocrine substrates such as the neurotrophin proBDNF (brain derived neurotrophic factor) [28], β-endorphin, and α-melanocyte stimulating hormone [29]; as well as systemic metabolic and cardiovascular physiologic responses under the control of sympathoadrenal and sympathoneuronal activities [18,30-34]. However, the mechanism by which t-PA is targeted to the regulated pathway of secretion is unknown.

Chromogranin A (CgA), a major core protein in secretory vesicles throughout the neuroendocrine system [35], is a member of the granin family [reviewed in 36] whose members are released by exocytosis from neuroendocrine sites including the adrenal medulla and sympathetic axons [35,37]. Members of the granin family may play a crucial role in the targeting of proteins into the regulated secretory pathway. Granins form aggregated complexes [38,39] and it has been hypothesized that other proteins destined for the regulated secretory vesicle may bind to these complexes and thereby become separated from constitutively secreted

proteins in the TGN, before binding to specific membrane sorting receptors or lipids [40-44] and movement into the dense core regulated secretory vesicle. Formation of such complexes is facilitated under conditions found in the TGN (low pH and high Ca^{+2}) [38,45]. In the present study we provide evidence that t-PA interacts specifically with CgA and that this interaction is enhanced under the pH and calcium conditions of the TGN. Furthermore, the interaction is domain-specific and consistent with the interaction of t-PA with aggregated CgA complexes. The interaction of t-PA with aggregated CgA compexes may provide a mechanism for targeting t-PA to the regulated pathway of secretion in chromaffin cells as well as in other neuroendocrine tissues.

EXPERIMENTAL PROCEDURES

Proteins and peptides

Human recombinant single chain t-PA (rt-PA, Activase) was from Genentech (South San Francisco, CA) and was inactivated with diisopropyfluorophosphate (DFP). Recombinant CgA was expressed and purified as described [31]. CgA was labeled using the chloramine T method [46] to specific activities of 91-136 nCi/ng and was >90% precipitable in 13% trichloroacetic acid.

Anti-t-PA monoclonal antibodies (mAB's) (#3700 against the EGF/Finger domain, #3704 against kringle 2 and #372 against kringle 1) were purchased from American Diagnostica (Greenwich, CT).

Synthetic peptides (average length, 22 amino acids, range, 19-25 residues) spanning 336 amino acids, or 78% of the length of CgA were synthesized at 10-100 µmol scale by the solid-phase method [47] using t-boc or f-moc protection chemistry, and were then purified to >95% homogeneity by reversed phase high pressure liquid chromatography on C-18 silica columns. Authenticity and purity of peptides were verified by rechromatography, as well as electrospray-ionization or MALDI mass spectrometry, or amino acid composition.

Binding of CgA to Immobilized t-PA

Wells of 96 well flat bottom microtiter plates (No. 3912, Falcon, Becton Dickinson, Oxnard, CA) were coated with DFP-inactivated t-PA at 25 µg/ml (unless otherwise indicated) in 0.1 M $NaHCO_3$ pH 8.5 for 18 hr at 4°C and then postcoated with 3% ovalbumin (Sigma, St. Louis, MO) in phosphate buffered saline (PBS). The wells were washed 3 times with 200 µl PBS containing 0.1 % Tween 80. ^{125}I-CgA (1 nM) was incubated with duplicate wells in a final volume of 100 µl in 10 mM HEPES, pH 7.4, 2 mM Ca^{+2}, 190 mM NaCl containing 0.1 % Tween 80 for 4 hours at 22°C (unless otherwise indicated). The wells were washed six times with PBS containing 0.1 % Tween 80, cut out and counted in an Iso-Data gamma counter (Iso-Data, Inc., Palatine, IL). fmoles of ^{125}I-CgA bound were calculated based on the specific activities of the radiolabeled ligand.

Statistics and Analyses

Data are reported as mean ± standard error of the mean. Kd and Bmax were derived from Scatchard plots of binding studies in which varying concentrations of unlabeled CgA were added with a constant concentration of ^{125}I-CgA. The data from the Scatchard analyses were analyzed using the LIGAND program [48].

RESULTS

Concentration and Time Dependence of the Interaction of t-PA with CgA- We examined whether t-PA could interact with CgA using solid phase binding studies. t-PA was inactivated with DFP and immobilized onto microtiter wells. The influence of the t-PA coating concentration on the interaction with ^{125}I-CgA is shown in Figure 1A. The extent of the interaction increased with increasing t-PA coating concentrations and a plateau was reached at a t-PA coating concentration of 25 µg/ml. 1 nM ^{125}I CgA bound to t-PA in a saturable manner. The interaction was inhibited by 85% by 1 µM unlabeled CgA. Binding in the presence of unlabeled CgA was considered nonspecific binding (Figure 1A, circles) and was subtracted from total binding (Figure 1A, squares) to obtain specific binding (Figure 1B, triangles). The time course of the interaction of CgA with t-PA is shown in Figure 1B. An apparent steady state binding of CgA was attained between 120 and 300 minutes.

Specificity of the Interaction of t-PA with CgA- The specificity of the interaction of ^{125}I-CgA with t-PA was examined further. Under conditions in which the binding of ^{125}I-CgA was 85% inhibited by 1 µM unlabeled CgA, unrelated proteins including ovalbumin, transferrin or RNase gave ≤ 20% inhibition (Figure 2).

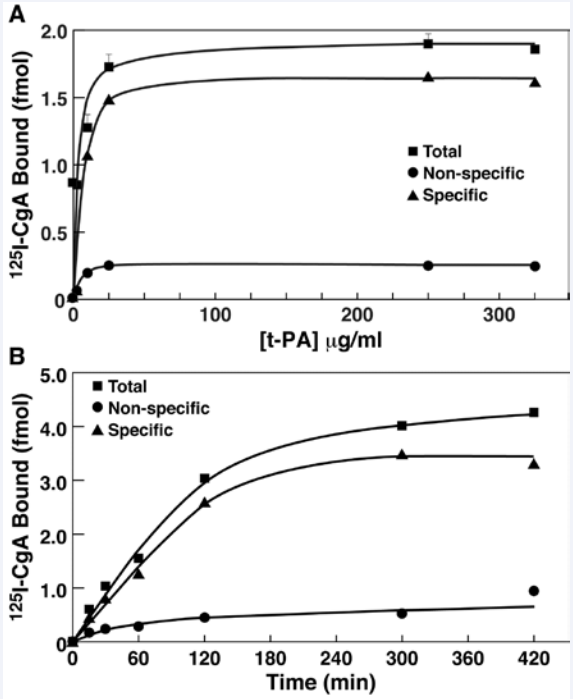

Figure 1 Concentration and time dependence of the interaction of t-PA with CgA. *A,* Microtiter wells were coated with increasing concentrations of DFP-treated t-PA and binding of 1 nM ^{125}I-CgA was determined at 4 hr as described in Experimental Procedures. *B,* Microtiter wells were coated with DFP-treated t-PA (25 µg/ml) and binding of 1 nM ^{125}I-CgA was determined at the indicated time points. The wells indicated as containing 0 µg/ml t-PA were postcoated with ovalbumin only. Nonspecific binding (circles) was determined in the presence of 1 µM unlabeled CgA and subtracted from total binding (squares) to obtain specific binding (triangles).

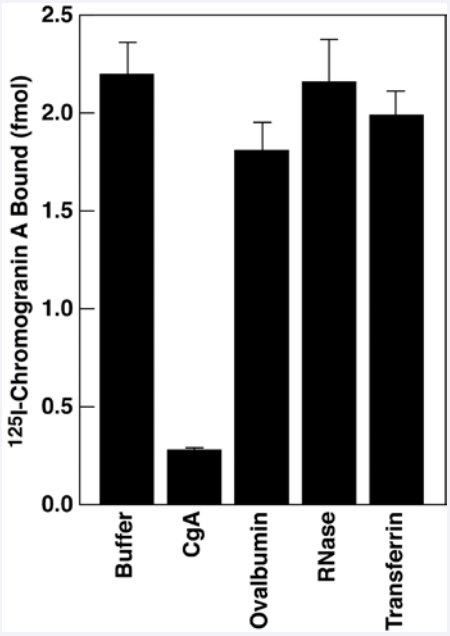

Figure 2 Specificity of the interaction of t-PA with CgA. Microtiter wells were coated with t-PA and the interaction with ^{125}I-CgA (1 nM) was determined as described in Experimental Procedures in either the absence or presence of either unlabeled CgA, ovalbumin, RNase, or transferrin (each at 1 μM) for 120 min at 22°C. Total binding is shown.

Localization of the Interactive Site in t-PA for CgA- We used anti-t-PA mAb's to approximate the interactive domain within the t-PA molecule. Both a mAb directed against the EGF-finger region and a mAb directed against kringle 1 inhibited the interaction of t-PA with CgA in a dose-dependent manner (Figure 3). The mAb directed against the EGF/finger domain produced 61% inhibition and the mAb against kringle 1 produced 58% inhibition (at a concentration of 40 μg/ml). At this concentration, control normal mouse IgG had no effect. A mAb reacting with t-PA kringle 2 did not inhibit the interaction of t-PA with CgA at the concentrations tested. The lack of effect of the antibody against t-PA kringle 2 could not be ascribed to a lower affinity of this antibody compared to the mAb against the EGF/finger domain. [The kd for the interaction of the mAbs with the target t-PA domains is 30 nM and 56 nM for the kringle 2 and EGF/finger domains, respectively (according to the manufacturer).] These data suggest that sequences within both the t-PA EGF/finger and kringle 1 domains play a role in the interaction with CgA.

Effect of pH and Ca^{+2} on the Interaction of t-PA with CgA- We examined the interaction of t-PA and CgA under conditions designed to mimic those of the trans golgi network (pH 6.4 and 10 mM Ca^{+2}) [38,45] as well as the secretory vesicle (pH 5.5, 20 mM Ca^{+2}) [49-51]. At a 1 nM input concentration of ^{125}I-CgA, its interaction with t-PA was enhanced 4.2-fold at pH 6.4 and 4.6-fold at pH 5.5, compared to the binding observed at pH 7.4 in the absence of Ca^{+2} (Figure 4). The interaction under these conditions also was specific, that is, inhibited by unlabeled CgA, but not by unrelated molecules, transferrin or RNAse.

In order to determine whether the increased CgA binding was due to changes in affinity or capacity of t-PA for CgA, binding isotherms were compared at pH 7.4, at pH 6.4 with 10 mM Ca^{+2}, and at pH 5.5, 20 mM Ca^{+2} (Figure 5). A saturable interaction of t-PA with CgA was observed under each condition. When analyzed in Scatchard plots, the data could be fit to a straight line under each condition, suggesting a single class of binding sites with respect to affinity. The apparent Kd's determined at the three pH conditions were similar: 57 nM at pH 7.4, 32 nM at pH 6.4 and 55 nM at pH 5.5. In contrast, the amount of CgA bound increased from 125 fmol at pH 7.4, to 255 fmol at pH 6.4 and 385 fmol at pH 5.5. Thus, lower pH and higher calcium ion concentrations substantially affected the maximal number of CgA molecules bound but resulted in minimal changes in affinity. These results suggest, or are at least consistent with, a model in which aggregated CgA binds t-PA.

Effect of CgA Peptides on the Interaction of t-PA with CgA- To examine possible linear sequences within the CgA molecule that might mediate the interaction with t-PA, we tested 14 synthetic peptides (average length, 22 amino acids, range, 19-25 residues) spanning 336 amino acids, or 78% of the length

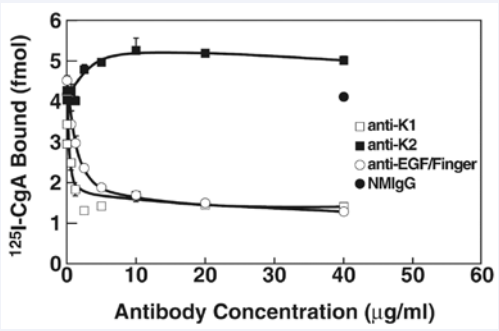

Figure 3 Effect of anti-t-PA mAb's on the interaction of t-PA with CgA. Microtiter wells were coated with t-PA and the interaction with ^{125}I-CgA was determined as described in Experimental Procedures in the presence of the indicated concentrations of anti-t-PA mAb's directed against either the EGF-finger domain (open circles), kringle 2 (closed squares), kringle 1 (open squares) or normal mouse IgG (closed circles). Specific binding is shown.

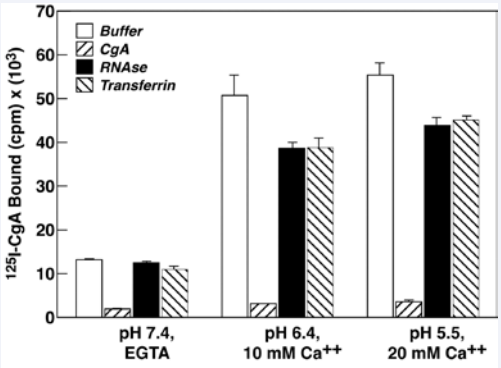

Figure 4 Effect of pH and Ca^{+2} on the interaction of t-PA with CgA. Microtiter wells were coated with t-PA and incubated with ^{125}I-CgA in either: 10 mM HEPES, pH 7.4, 1 mM EGTA; 10 mM MES pH 6.4 containing 10 mM Ca^{+2}; or 10 mM MES, pH 5.5 containing 20 mM Ca^{+2}. All buffers contained 100 mM NaCl and 0.1%Tween 80. Incubations were performed in the presence of buffer alone or in the presence of either 1 :M CgA, RNAse, or transferrin as indicated.

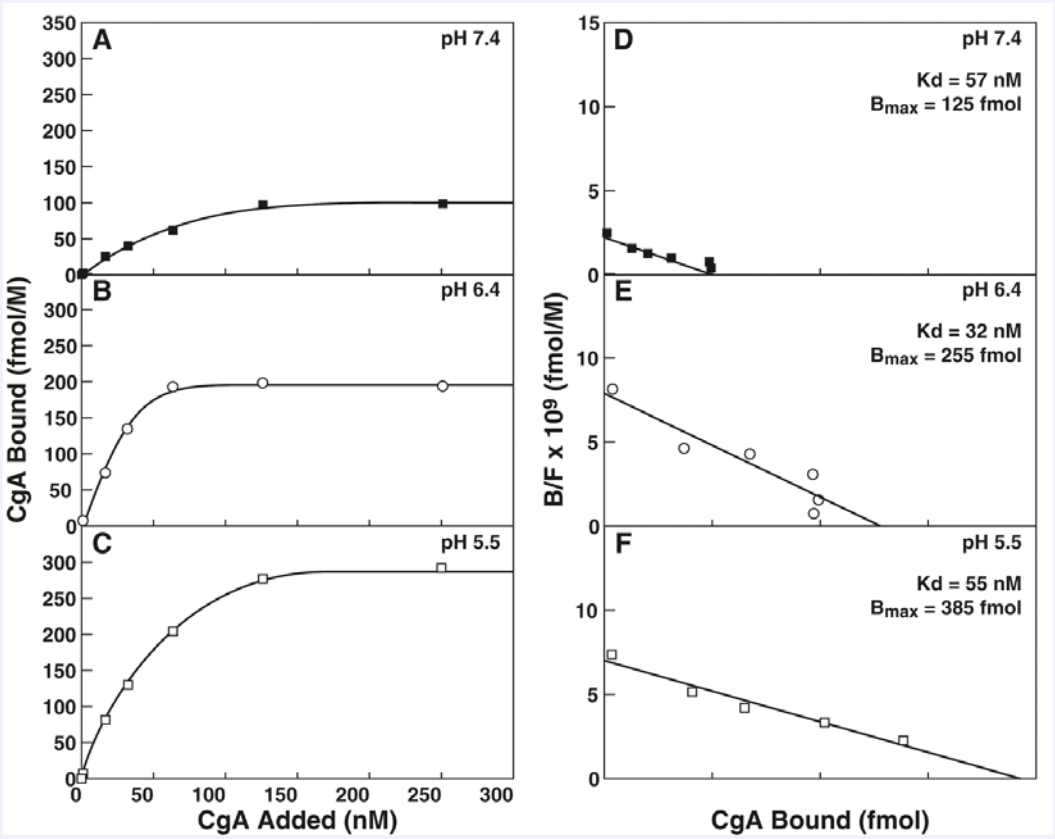

Figure 5 Effect of pH and Ca^{+2} on the interaction of t-PA with CgA as a function of CgA concentration. t-PA was immobilized onto wells of microtiter plates. ^{125}I-CgA (1 nM) was incubated with the plates for 4 hours at 22°C in the presence of increasing concentrations of unlabeled CgA. The inhibition curves obtained were transformed to binding isotherms using the LIGAND program. Isotherms were constructed and replotted as Scatchard plots in 10 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EGTA (panels A and D), 10 mM MES pH 6.4, 100 mM NaCl 10 mM Ca^{+2} (panels B and E) or 10 mM MES pH 5.5, 100 mM NaCl, 20 mM Ca^{+2} (panels C and F).

of bovine chromogranin A (mature protein =431 amino acids) for the ability to inhibit the interaction of t-PA with CgA. Only one peptide, corresponding to bovine CgA residues 70-90 and having the sequence, KERTHQQKKHSSYEDELSEVL**,** inhibited the interaction of t-PA with CgA (Figure 6A). The extent of inhibition was concentration-dependent, achieving 82% inhibition at 100 µM (Figure 6B). None of the other peptides tested was inhibitory.

DISCUSSION

The trafficking of t-PA to secretory vesicles in neuroendocrine cells is a key mechanism for the acute release of t-PA in response to stress [13,14,17-19]. CgA, a major protein in secretory vesicles throughout the neuroendocrine system, is a member of the granin family [reviewed in 36] that may play a crucial role in the targeting of proteins into the regulated secretory pathway. Here we show that t-PA interacted specifically with CgA in a high affinity interaction. The extent of the interaction of t-PA with CgA increased under pH and divalent ion conditions of the TGN and secretory vesicle, consistent with formation of a complex of t-PA and aggregated CgA. Furthermore, we have identified interactive domains that mediate the interaction of t-PA with CgA.

At physiological pH, CgA bound directly to t-PA and the interaction was competed by unlabeled CgA, demonstrating that the interaction was saturable. Unrelated proteins, ovalbumin, transferrin and RNAse, had no effect on the interaction, demonstrating that the interaction was specific for CgA. Interestingly, these proteins that did not compete for the interaction of CgA with t-PA are constitutively secreted and excluded from the aggregation process [52].

The interaction of t-PA with CgA was markedly enhanced under conditions of the TGN (pH 6.4 and 10 mM Ca^{+2}) as well as under conditions within the secretory vesicle (pH 5.5, 20 mM Ca^{+2}), compared to the interaction at pH 7.4. Scatchard analysis of binding isotherms under these conditions, as well as binding isotherms constructed at pH 7.4, in the absence of Ca^{+2}, showed that the interaction had a similar apparent Kd under the different conditions, but that the maximal number of CgA molecules bound was enhanced under conditions of the TGN and the secretory vesicle, B_{max} = 255 fmol and 385 fmol, respectively, compared with a B_{max} of 125 fmol at pH 7.4. Thus, lower pH and higher calcium ion concentrations substantially affected the maximal number of CgA molecules bound but resulted in minimal changes in affinity. It is noteworthy that the concentration of CgA within the catecholamine storage vesicle is 1.8 mM [35,53], suggesting that the majority of t-PA molecules within the TGN and catecholamine storage vesicles are bound to CgA. Thus, these results suggest, or are at least consistent with, a model in which CgA aggregates and binds t-PA.

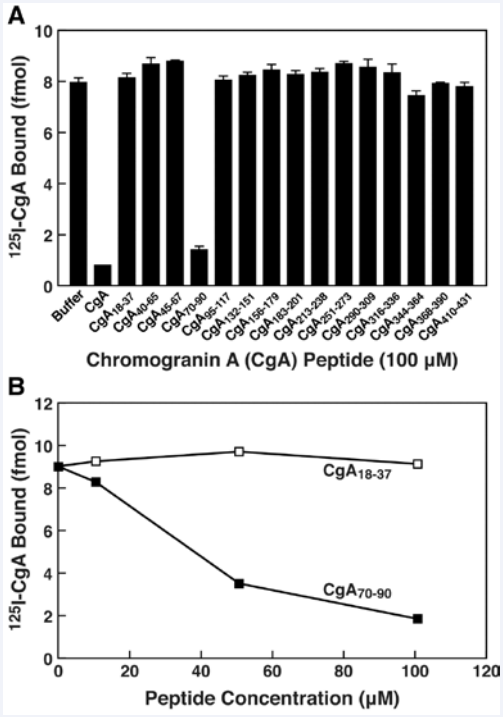

Figure 6 Effect of CgA peptides on the interaction of t-PA with CgA. *A,* Microtiter wells were coated with t-PA and incubated with ^{125}I-CgA in the presence of either buffer, unlabeled CgA or each of fourteen synthetic peptides corresponding to bovine CgA (100 μM). *B,* ^{125}I-CgA was incubated with the wells in the presence of a peptide corresponding to residues 70-90 of CgA (closed squares), or a control peptide corresponding to residues 18-37 of CgA (open squares). Specific binding is shown.

The interaction between CgA and t-PA was competed with mAbs against t-PA kringle 1 and also mAbs against the EGF-finger domain of t-PA, but not by mAbs reacting with kringle 2. The orientation of these domains of t-PA is in the following order: finger, EGF, kringle 1, kringle 2. These data suggest that sequences within both the t-PA EGF/finger and kringle 1 domains play a role in the interaction with CgA. Interestingly, the finger region of t-PA also mediates its interaction with cells [54,55] and in the initial phases of fibrinolysis, t-PA binds specifically to fibrin via the finger domain [while the kringle 2 domain mediates the binding of t-PA during later phases of fibrinolysis following generation of C-terminal lysines in fibrin] [56].

Using a synthetic peptide library screen, we identified one peptide, corresponding to bovine CgA_{70-90} having the sequence, KERTHQQKKHSSYEDELSEVL, which uniquely blocked the interaction of t-PA with CgA. This domain is highly conserved across species [57-60]. Based on hydropathy plots, the inhibitory peptide is in one of the more highly hydrophilic regions of CgA [60], consistent with exposure of this domain on the surface of CgA, and its accessibility as a site for the interaction with t-PA.

We have previously demonstrated the presence of a local catecholaminergic cell plasminogen activation system [13,14,30,32,61-65] and have shown that proteolytic processing of CgA by locally generated plasmin (formed by cell surface plasminogen activation by t-PA) liberates bioactive peptides including those that regulate the secretory response [30-32,64]. Thus, the interaction of t-PA and CgA may serve dual roles to facilitate both t-PA trafficking and subsequently, plasmin-dependent CgA processing. These results thus may also suggest a novel paradigm in which a neuroendocrine prohormone substrate (CgA) interacts with the activator of the protease system required for the proteolytic processing of that substrate, with the interaction thus serving to facilitate both co-trafficking and processing.

In summary, we have demonstrated that t-PA interacts specifically with CgA in a high affinity domain-dependent fashion. In addition, this interaction is pH- and calcium-dependent, consistent with substantial enhancement under conditions of the TGN and secretory granule to facilitate t-PA trafficking to the regulated secretory pathway. It is noteworthy that t-PA and CgA are expressed together in a wide variety of neuronal and neuroendocrine tissues [2-4,6-8,37,66-71]. The interaction of t-PA with aggregated CgA complexes may play a role in the targeting of t-PA and its release from neurosecretory cells. Studies suggest a critical role for the plasminogen/tPA system in normal neuronal function, and in the neurotoxicity and neurodegeneration following cerebral ischemia and excitotoxicity [72,73]. These results may have broad implications for the regulation of local neurosecretory cell plasminogen activation and suggest that alterations in this system may critically influence local function of key components of the neurovascular unit, under both normal physiologic conditions and pathologic conditions including cerebral ischemia.

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