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Myelin basic protein stimulates plasminogen activation via tissue plasminogen activator following binding to independent L-lysine-containing domains

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ABSTRACT

Myelin basic protein (MBP) is a key component of myelin, the specialized lipid membrane that encases the axons of all neurons. Both plasminogen (Pg) and tissue-type plasminogen activator (t-PA) bind to MBP with high affinity. We investigated the kinetics and mechanisms involved in this process using immobilized MBP and found that Pg activation by t-PA is significantly stimulated by MBP. This mechanism involves the binding of t-PA via a lysine-dependent mechanism to the Lys^{91} residue of the MBP NH_2-terminal region Asp^{82} -Pro^{99}, and the binding of Pg via a lysine-dependent mechanism to the Lys^{122} residue of the MBP COOH-terminal region Leu^{109}-Gly^{126}. In this context, MBP mimics fibrin and because MBP is a plasmin substrate, our results suggest direct participation of the Pg activation system on MBP physiology.

1. Introduction

Both plasminogen (Pg) and tissue-type plasminogen activator (t-PA) are serine proteases commonly associated with fibrinolysis [1]; however, they also play important roles in the central nervous system (CNS) [2,3]. t-PA is primarily involved in synaptic formation and plasticity [4] via mechanisms both dependent [5,6] and independent [7–9] of its proteinase activity. In addition to their fibrinolytic functions [10], both t-PA and Pg are also involved in the neuroinflammation observed in patients suffering from pathologies, such as multiple sclerosis (MS) and encephalitis [11], in which demyelination and axonal damage are responsible for neurological deficits [12,13].

Pg activation in the normal brain is tightly regulated, possibly because it is neurotoxic [10]; however, plasmin (Pm) may play a role in the generation of long-term potentiation (LTP) in the rat hippocampus [14]. Furthermore, a recent report suggests that Pm, along with t-PA, is involved in the blood-brain barrier (BBB) disruption that occurs during t-PA-induced thrombolysis in ischemic stroke [15]. Both t-PA and Pg are expressed in neurons, astrocytes and microglia [10]. Oligodendrocytes are responsible for the production and maintenance of myelin, the specialized lipid membrane that encases the axons of all neurons in the brain [16]. Myelin is composed of lipids and two proteins, myelin basic protein (MBP) and proteolipid protein [16]. The integrity of the myelin sheath may be disrupted by Pg conversion to Pm by t-PA because Pm may hydrolize MBP [17].

We found that both t-PA and Pg bind to MBP with high affinity, and that Pg activation by t-PA is stimulated by MBP. This mechanism involves the binding of t-PA via a lysine-dependent mechanism to the MBP NH_2-terminal region, Asp^{82}-Pro^{99}, and the binding of Pg via a lysine-dependent mechanism to the MBP COOH-terminal region, Leu^{109}-Gly^{126}.

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2. Materials and methods

2.1. Materials

Culture media were purchased from Life Technologies (Gaithersburg, MD). The chromogenic substrates V-L-K-pNA (S-2251) and I-P-R-pNA (S-2288) were purchased from Diapharma (West Chester, OH). MBP peptides D^{82}ENPVVHFFKNIVTPRTP^{99} (Asp^{82}-Pro^{99}), T^{98}PPPSQGKGRGLSLSRFS^{115} (Thr^{98}-Ser^{115}), L^{109}SLSRFSWGAEGQKPGFG^{126} (Leu^{109}-Gly^{126}) and ARGQG-PYFSWGGFSEKIG (scrambled L^{109}-G^{126}) were obtained from Bachem Americas, Inc. (Torrance, CA). Tranexamic acid (TXA) was purchased from Sigma (St. Louis, MO). The HyperPAGE dye-conjugated M_r markers (10 kDa–190 kDa) were purchased from Bioline USA, Inc. (Taunton, MA). Dithiotreitol (DTT) was purchased from Sigma (St. Louis, MO). The other reagents used were of the highest grade available.

2.2. Proteins

Human Pg was purified by affinity chromatography on L-lysine-Sepharose [18]. Human t-PA and urokinase-type Pg activator (u-PA) were purchased from Calbiochem-EMD Chemicals, Inc. (San Diego, CA). Human brain MBP was purchased from Sigma (St. Louis, MO). Porcine MBP was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Human α_2-antiplasmin was purchased from Sigma (St. Louis, MO).

2.3. Antibodies

The goat polyclonal IgG against human Pg (H-14), goat polyclonal IgG against the NH2-terminal region of human t-PA (N-14), and the goat polyclonal IgG against an internal region of human MBP (I-15) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-goat IRDye 680 LT IgG was purchased from LI-COR Biotechnology Lincoln, NE.

2.4. Analysis of Pg and t-PA binding to MBP

All assays were performed on porcine MBP coated Immulon® ultra-high binding polystyrene microtiter plates from Thermo (Milford, MA). Briefly, the plates were coated by incubating overnight at 24 °C with 200 μl MBP (10 μg/ml) in 0.1 M Na_2CO_3, pH 9.6, containing 0.01% NaN_3, followed by rinsing with phosphate-buffered saline (PBS) and incubation with 3% bovine serum albumin (BSA) in 0.1 M Na_2CO_3, pH 9.6, containing 0.01% NaN_3 to block non-specific sites. After rinsing the plates with PBS, the plates were stored at 4 °C until further use. The amount of MBP bound to the plates was calculated after reaction with the goat anti-MBP I-15 IgG followed by reaction with a rabbit anti-goat alkaline phosphatase-conjugated IgG, rinsing with PBS and a final incubation with the alkaline phosphatase substrate p-nitrophenylphosphate (1 mg/ml) in 0.1 M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 10.4. The absorbance was monitored at 405 nm using a Molecular Devices SPECTRAmax kinetic plate reader (Molecular Devices, LLC, Sunnyvale, CA). The Pg and t-PA binding assays were performed in triplicate, and the bound Pg or t-PA was calculated from calibration curves constructed from immobilized Pg or t-PA reacted with the H-14 anti-Pg or N-14 anti-t-PA antibodies. This was followed by a reaction with a rabbit anti-goat alkaline phosphatase-conjugated IgG, rinsing with PBS and a final incubation with the alkaline phosphatase substrate p-nitrophenylphosphate (1 mg/ml) in 0.1 M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 10.4. The absorbance was measured at 405 nm as described above. The bound Pg or t-PA was expressed as nmol Pg or t-PA/nmol MBP. The K_d and B_{max} were determined using the statistical program GraphPad Prism® 6 from GraphPad Software, Inc. (San Diego, CA).

2.5. Determination of Pg activation rate

Coupled assays were used to evaluate the initial rate of Glu-Pg activation by t-PA by monitoring the amidolytic activity of the generated Pm [19]. Glu-Pg (100 nM) was incubated in 96-well microtiter plates at 37 °C in 20 mM HEPES, pH 7.4, in a total volume of 200 μl with the Pm substrate S-2251 (0.3 mM). Pg activation was initiated by the addition of 0.55 nM t-PA. The resulting Pm hydrolysis of S-2251 was monitored as described above. The initial velocities (v_i) were calculated from plots of A_{405nm} *vs.* time^2 using the equation $v_i = b(1 + K_m/S_0)/\varepsilon k_e$, where K_m is the apparent Michaelis constant of S-2251 hydrolysis by Pm, k_e is the empirically determined catalytic rate constant for Pm hydrolysis of S-2251 [3.2×10^4 M min^{-1}(mol of Pm)$^{-1}$] and ε is the molar extinction coefficient of p-nitroanilide at 405 nm (10,000 M^{-1} cm^{-1}) [19].

2.6. Determination of Pm amidolytic activity

The Pm amidolytic activity was determined after incubation of Glu-Pg with u-PA (2 pM) in 20 mM HEPES, pH 7.4, in a total volume of 175 μl. The Pm substrate, VLK-pNA (0.3 mM, 25 μl), was added to the mixture, and substrate hydrolysis was monitored at 405 nm as described above.

2.7. SDS-PAGE and immunoblotting analyses of plasmin digested MBP

MBP was digested by incubation with Pg (2 nM) and t-PA (30 nM) for 1 h at 37 °C in the presence or absence of α_2-AP (2 μM), followed by electrophoretic separation of the digested proteins on 15% polyacrylamide gels (1.2 mm thick, 14 × 10 cm) containing 0.1% SDS under reducing conditions. A discontinuous Laemli buffer system was used [20]. The proteins were transferred from the gels to nitrocellulose membranes [21]. The membranes were thoroughly rinsed with PBS and then incubated with 3% BSA for 1 h at room temperature to block non-conjugated areas. Then, the membranes were incubated with a goat anti-human MBP (5 μg/ml) in 3% BSA in PBS overnight at room temperature. Then the membranes were washed three times for 5 min each with PBS containing 0.1% Triton X-100 (PBS-T), followed by incubation with a 1:10,000 dilution of an anti-goat IRDye 680 LT IgG for 1 h at room temperature in the dark. The blots were then washed twice for 5 min each with PBS-T, followed by a final 5-min wash with PBS. The probed membranes were scanned on a Li-Cor Odissey infrared imager (LI-COR Biotechnology, Lincoln, NE).

2.8. Analysis of the capacity of Pm digested MBP to bind Pg

All assays were performed on porcine MBP coated Immulon® ultra-high binding polystyrene.

Microtiter plates from Thermo (Milford, MA) as described above. MBP was digested by incubation with Pg (2 nM) and t-PA (30 nM) for 1 h at 37 °C. Then, the plates were extensively washed with 6-aminohexanoic acid (50 mM) in PBS to remove any residual Pg/Pm or t-PA bound to MBP. The Pg binding assays were performed in triplicate as described above. The amount of MBP bound to the plates was calculated after reaction with the goat anti-MBP I-15 IgG, as described above.

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