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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.

2.9. Statistics

Graph Pad Prism, version 6.0 software (Graph Pad Software, Inc., San Diego, CA) was used to determine the standard deviation of the experimental data. The significance of differences between the controls and different treatments ($P < 0.001$) was determined using unpaired Student's t-tests.

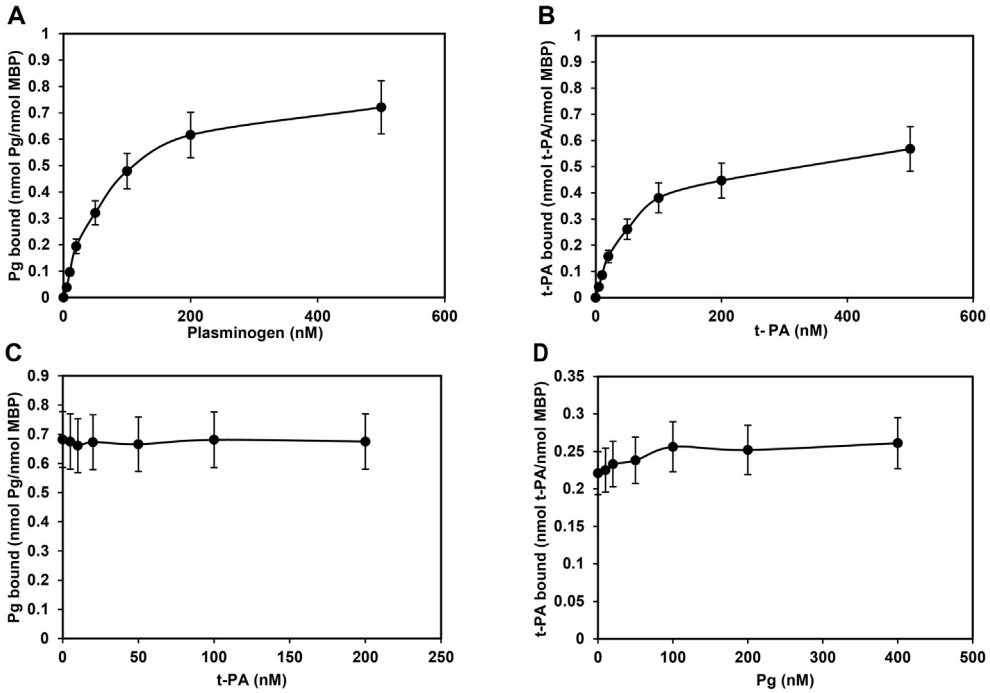

Fig. 1. Binding of Pg and t-PA to immobilized MBP. Experiments were performed in plates containing MBP immobilized on 96-well tissue culture plates. (A) Increasing concentrations of Pg were added to the plates. Bound Pg was calculated as described under *Experimental Procedures*. (B) Increasing concentrations of t-PA were added to the plates. Bound t-PA was calculated as described under *Experimental Procedures*. (C) Binding of Pg (400 nM) to immobilized MBP in the presence of increasing concentrations of t-PA. (D) Binding of t-PA (400 nM) to immobilized MBP in the presence of increasing concentrations of Pg. Data are the means ± S.D (*error bars*) from experiments performed in triplicate (n = 6).

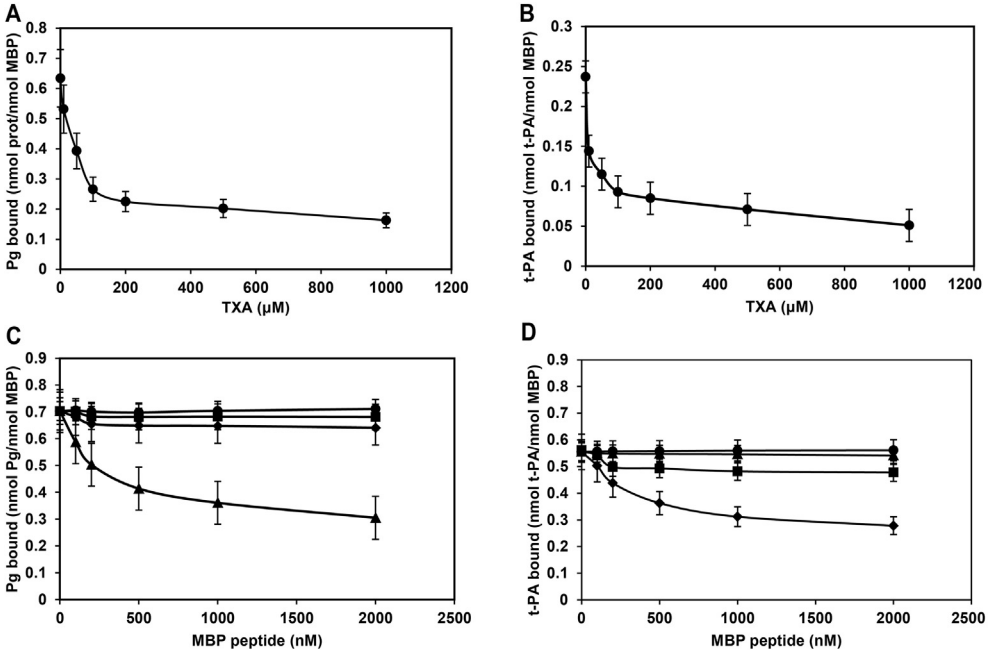

Fig. 2. Inhibition of binding of Pg and t-PA to MBP by tranexamic acid and peptides containing specific sequences of MBP. (A) Effect of increasing concentrations of TXA on Pg (400 nM) binding to MBP. (B) Effect of TXA on t-PA (400 nM) binding to MBP. (C) Inhibition of Pg binding to MBP in the presence of increasing concentrations of peptides containing the MBP regions Asp^{82}-Pro^{99} (◆), Thr^{98}-Ser^{115} (■), Leu^{109}-Gly^{126} (▲), and the scrambled Leu^{109}-Gly^{126} (●) region. (D) Inhibition of t-PA binding to MBP in the presence of increasing concentrations of peptides containing the MBP regions Asp^{82}-Pro^{99} (◆),Thr^{98}-Ser^{115} (■), Leu^{109}-Gly^{126} (▲), and the scrambled Leu^{109}-Gly^{126} (●) region. Data are the means ± S.D (*error bars*) from experiments performed in triplicate (n = 6).

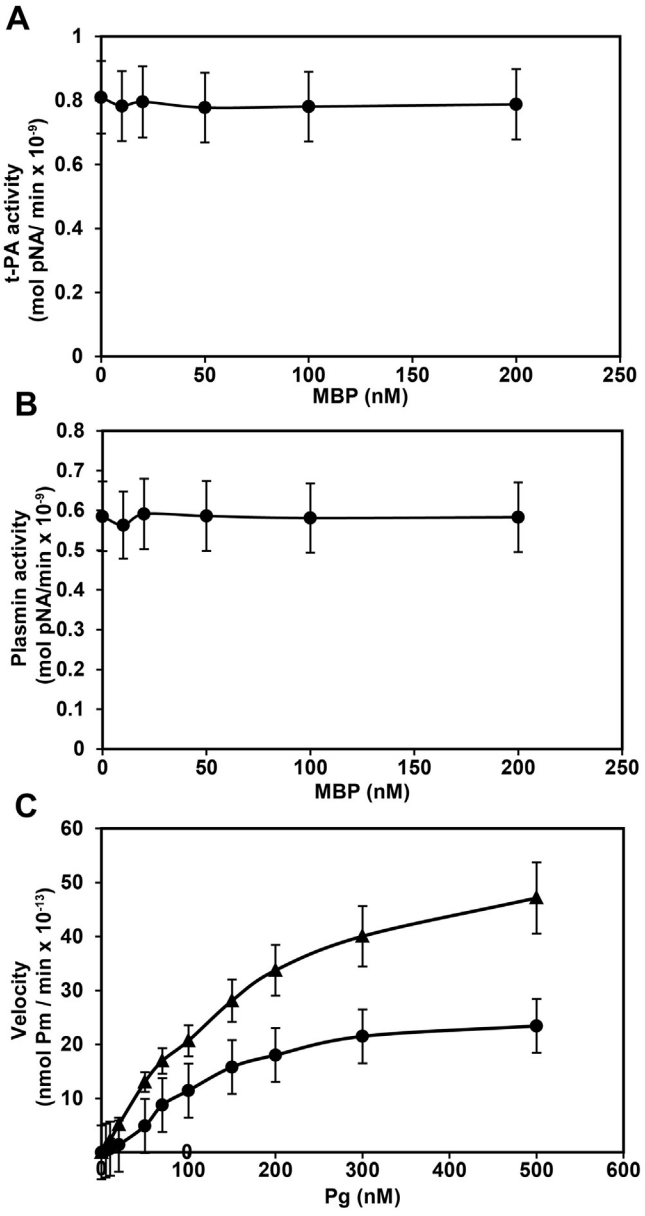

Fig. 3. Effect of MBP on catalytic properties of t-PA and Pm and the rate of activation of Pg by t-PA. (A) Effect of increasing concentrations of MBP on t-PA(10 nM) amidolytic activity. (B) Effect of MBP on Pm amidolytic activity. (C) Glu-Pg was preincubated in 96-well microtiter plates for 15 min at 37 °C in 20 mM HEPES, pH 7.4 in a total volume of 200 µl in the absence (●) or presence (▲) of MBP (100 nM) followed by the addition of the Pm substrate VLK-pNA (0.3 mM) and t-PA (0.55 nM). Data are the means ± S.D (*error bars*) from experiments performed in triplicate (n = 6).

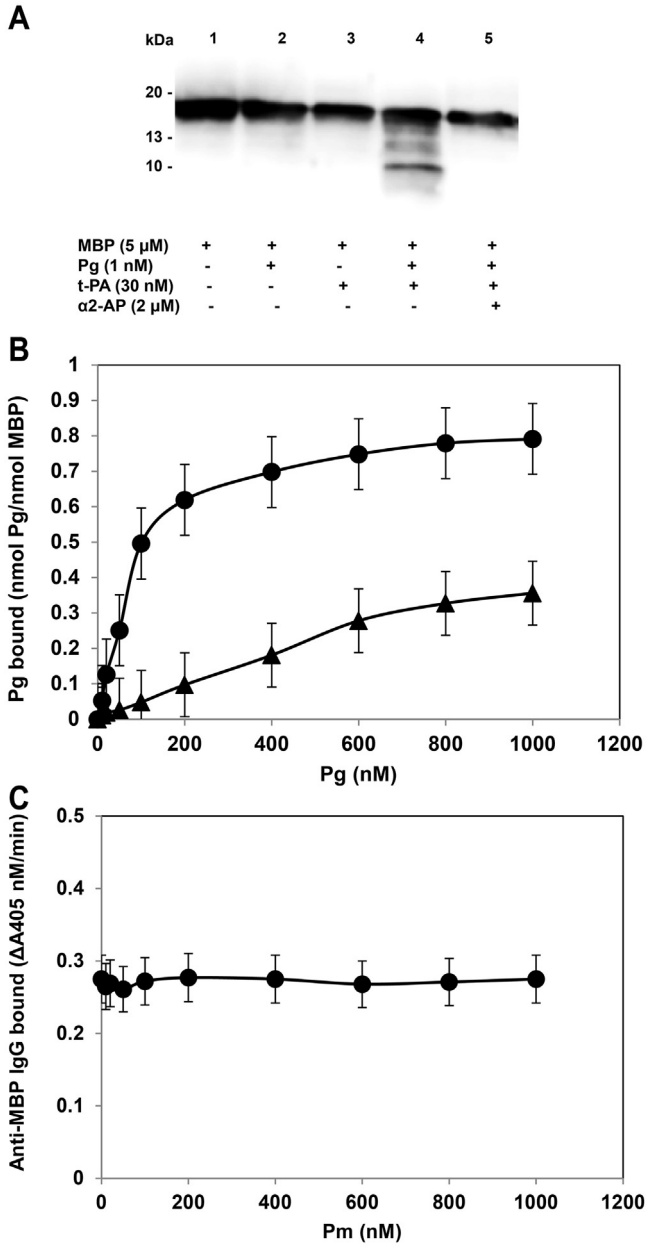

Fig. 4. Effect of Pm on the capacity of MBP to bind to Pg. (A) Incubation of human MBP with Pg and t-PA alone or in combination in the absence or presence of α_2-AP. (B) Pg binding to native immobilized MBP (●) or after proteolysis with Pm (0.1 µM) (▲) for 3 h at room temperature. (C) Reactivity of MBP with an anti-MBP (IgG) (100 ng/well) after proteolysis with increasing concentrations of Pm for 3 h at room temperature. Data are the means ± SD (*error bars*) from experiments performed in triplicate (n = 6).

Table 1
Kinetic parameters of Glu-Pg activation by t-PA in the presence of MBP.

Protein	V_{max} mol Pm/min $\times 10^{-13}$	K_m µM	k_{cat} s^{-1}	K_{cat}/K_m μM^{-1} s^{-1}	K_{cat}/K_m -fold increase
None	41.35	0.21	0.23	1.09	
MBP	81.03	0.13	0.71	5.46	5.00

3. Results

3.1. Binding of Pg and t-PA to immobilized MBP

Pg binds to immobilized MBP in a dose-dependent manner (Fig. 1A) with high affinity (K_d = 50.26 ± 8.38 nM). Similarly, t-PA binds to MBP in a dose-dependent manner (Fig. 1B) with high affinity (K_d = 49.45 ± 8.32 nM). The binding of Pg to MBP is not inhibited by t-PA (Fig. 1C) and the binding of t-PA to MBP is not inhibited by Pg (Fig. 1D), suggesting that both proteins bind to independent sites in MBP. Binding of both Pg and t-PA to immobilized MBP is inhibited by TXA (Fig. 2A and B, respectively), an inhibitor of kringle-dependent binding of Pg or t-PA to fibrin or cell receptors [22,23]. We also assessed the antagonistic effect on Pg and t-PA binding to immobilized MBP with peptides including the amino acid residues Asp^{82}-Pro^{99}, Thr^{98}-Ser^{115}, Leu^{109}-Gly^{126} and scrambled Leu^{109}-Gly^{126} of MBP. The results show that Pg binding to MBP

is significantly inhibited by the peptide Leu^{109}-Gly ^{126} (Fig. 2C), whereas t-PA binding to MBP is inhibited by the peptide Asp^{82}-Pro^{99} (Fig. 2D), confirming the observation that Pg and t-PA bind to MBP via independent lysine sites.

3.2. Effect of MBP on the kinetic parameters of Pg activation by t-PA

The amidolytic activity of the Pg activator t-PA (10 nM) was measured with the chromogenic substrate S-2288 in the presence of increasing concentrations of MBP. The results (Fig. 3A) show no significant effect of MBP on the activity of t-PA. A non-significant effect was also observed with plasmin (Pm) (200 nM), obtained by incubation of Pg with u-PA, in the presence of increasing concentrations of MBP (Fig. 3B). However, when Pg was incubated with t-PA (10 nM) in the presence of MBP (100 nM), there was an increase in the velocity of Pg activation (Fig. 3C). The data were extrapolated to the Michaelis-Menten equation by non-linear regression to determine the k_{cat} and K_m (Table 1). The stimulatory effect of MBP is caused primarily by a decrease in K_m and an increase in V_{max}, which leads to a 5-fold increase in catalytic efficiency (k_{cat}/K_m). This increased catalytic efficiency of t-PA as a Pg activator by MBP is possibly caused by a Pg conformational change, which occurs after Pg binding via its kringle domains to a lysine residue localized in the Leu^{109}-Gly ^{126} domain of MBP.

3.3. Proteolysis of human MBP by plasmin

We investigated the *in vitro* proteolysis of human MBP and found that degradation of MBP is inhibited by α_2-AP (Fig. 4A). Next, we assessed the binding of Pg to immobilized MBP not-treated and treated with Pm (0.1 μM) for 3 h at room temperature (Fig. 4B). After incubation with Pm, the plate was extensively washed with 6-aminohexanoic acid (50 mM) in PBS to remove any bound Pm before adding Pg. The binding of Pg is significantly affected after cleavage of MBP by Pm. Addition of anti-MBP IgG (100 ng/well) demonstrates that reactivity of MBP incubated with increasing Pm concentrations is similar to that of native MBP (Fig. 4C), thereby excluding removal of the peptides from the plate after Pm treatment as the cause for low Pg binding. Therefore, undegraded MBP is necessary for efficient Pg binding.

4. Discussion

The Pg activator system plays an important role in the CNS, including processes of neuronal migration, neurite outgrowth, and neuronal plasticity [24,25]. The Pg activator, t-PA, may also participate in several neuropathological conditions, such as cerebral ischemia, Alzheimer's disease and multiple sclerosis [25]. t-PA and its substrate Pg are expressed in neurons, astrocytes and microglia [10].

Pm activity in the normal brain is tightly regulated [10]. We hypothesize that MBP, a prominent component of the myelin sheath of neurons secreted by oligodendrocytes serves as a receptor for Pg and t-PA. We investigated the mechanism by which MBP regulates Pg activation by t-PA, thereby controlling Pm activity. In this report, we show that MBP binds both Pg and t-PA. Using immobilized MBP, we found that both proteins bind to independent L-lysine sites, and that Pg activation by t-PA is stimulated in the presence of MBP. This mechanism involves the binding of t-PA to Lys^{91} in the MBP NH_2-terminal Asp^{82} - Pro^{99} amino acid sequence, and the binding of Pg to a Lys^{122} in the MBP COOH-terminal Leu^{109}-Gly^{126} amino acid sequence. MBP does not influence the catalytic activity of either t-PA or Pm. Therefore, its effect on Pg activation by t-PA is likely caused by the close proximity of these proteins resulting from the conformational change of Pg after binding to MBP. In this mechanism, MBP enhances t-PA mediated Pm generation in a manner similar to fibrin [26]. Because the brain is an organ usually devoid of fibrinogen, MBP mimics fibrin to stimulate t-PA mediated Pg activation, which then acts on non-fibrin substrates [27], such as MBP itself. Our experiments show that Pm cleaves MBP at several sites and the peptides generated [17], including peptides between residues Lys^{91} and Lys^{142} [17], may facilitate release of Pm from MBP, permitting its rapid inhibition by α2-AP. Furthermore, it is evident from our results that Pg binding to MBP requires an intact MBP molecule.

Interestingly, this mechanism is similar to that of the endothelial receptor, Annexin II, which stimulates the rate of Pg activation by t-PA because it induces conformational changes in Pg and proximity between the two proteins, and not via a direct effect of Annexin II on t-PA catalytic activity [28]. MBP functions differently from other t-PA receptors, such as the glucose-regulated protein 78 (GRP78), which regulates Pg activation via a direct effect on t-PA catalytic activity [29]. Although both mechanisms involve a lysine–dependent mechanism, the affinity of t-PA for GRP78 is 10-fold (Kd = 4.07 ± 0.46 nM) higher than the interaction of t-PA with MBP (Kd = 49.45 ± 8.32 nM). This phenomenon is commonly observed with Pg, in which binding with different affinities to several receptors may be utilized by the same cell when responding to different stimuli by a single ligand [30]. Therefore, the differential affinity of t-PA for different receptors may help the cell prioritize a specific cell function. A strict regulation of the Pg activation system controlling MBP cleavage by Pm may be necessary to facilitate the integrity of the myelin sheath around the axons to facilitate rapid nerve conduction [31]. Physiologically, this process may be controlled by α2-AP, expressed by neurons [32]. Furthermore, t-PA participates directly in the migration of oligodendrocyte progenitor cells that originate myelin forming cells during developmental myelination, normal adult physiology and post-lesion remyelination in white matter [33]. This effect of t-PA is mediated via its epidermal growth factor-like domain [33].

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