Binding of Tissue-type Plasminogen Activator to the Glucose-regulated Protein 78 (GRP78) Modulates Plasminogen Activation and Promotes Human Neuroblastoma Cell Proliferation in Vitro*

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**Background:** Glucose-regulated protein 78 (GRP78) is a plasminogen (Pg) and tissue-type plasminogen activator (t-PA) receptor on the cell surface.

**Results:** Binding of t-PA to GRP78 stimulates its amidolytic activity, activation of Pg, and cell proliferation in vitro.

**Conclusion:** Cell surface GRP78 enhances Pg activation by t-PA and mediates cell proliferation via binding to t-PA and microplasminogen.

**Significance:** This complex may play a significant role in brain physiology.

The glucose-regulated protein 78 (GRP78)2 is a plasminogen (Pg) receptor on the cell surface. In this study, we demonstrate that GRP78 also binds the tissue-type plasminogen activator (t-PA), which results in a decrease in $K_m$ and an increase in the $V_{max}$ for both its amidolytic activity and activation of its substrate, Pg. This results in accelerated Pg activation when GRP78, t-PA, and Pg are bound together. The increase in t-PA activity is the result of a mechanism involving a t-PA lysine-dependent binding site in the GRP78 amino acid sequence $^{90}$LIGRTWNDP5VQD1KFL. We found that GRP78 is expressed on the surface of neuroblastoma SK-N-SH cells where it is co-localized with the voltage-dependent anion channel (VDAC), which is also a t-PA-binding protein in these cells. We demonstrate that both Pg and t-PA serve as a bridge between GRP78 and VDAC bringing them together to facilitate Pg activation. t-PA induces SK-N-SH cell proliferation via binding to GRP78 on the cell surface. Furthermore, Pg binding to the COOH-terminal region of GRP78 stimulates cell proliferation via its microplasminogen domain. This study confirms previous findings from our laboratory showing that GRP78 acts as a growth factor-like receptor and that its association with t-PA, Pg, and VDAC on the cell surface may be part of a system controlling cell growth.

The glucose-regulated protein 78 (GRP78)2 is expressed primarily in the endoplasmic reticulum (1) but is also expressed on the cell surface (2). In the endoplasmic reticulum, GRP78 participates in protein folding, transportation, and degradation processes (1, 2), whereas on the cell surface, it recognizes a variety of extracellular ligands and acts as a transducer of proliferative signals, particularly in tumor cells (2). In our laboratory, we have demonstrated that GRP78 acts as a growth factor-like receptor on the cell surface and that signaling initiated by GRP78 is either pro-proliferative or pro-apoptotic depending on whether its NH$_2$-terminal or COOH-terminal are bound by the ligand, respectively (2). In a recent study, we demonstrated that the ability of GRP78 to differentially regulate opposite signaling pathways in tumor cells is the result of the surface topology of the protein (3).

On the cell surface, GRP78 is co-expressed with multiple binding partners, which modulate its signaling capacities (2, 4). One partner of GRP78 on the surface is the voltage-dependent anion channel (VDAC) (5). Both GRP78 and VDAC serve as receptors for plasminogen (Pg), a protein that binds via its kringle 5 domain (K5) to either GRP78 or VDAC (5, 6). In addition, Pg binds to the COOH-terminal region of GRP78 via its microplasminogen domain (5). Both Pg fragments induce Ca$^{2+}$ signaling cascades; however, K5 acts through VDAC and microplasminogen acts via GRP78 (5). Cell surface VDAC is also a receptor for tissue-type plasminogen activator (t-PA), and as a result of this interaction, t-PA activity and Pg activation are enhanced (7).

GRP78 is expressed in the hippocampus of human Alzheimer patients and also in a mouse model of Alzheimer disease (8, 9). In the mouse brain, Pg and t-PA are highly expressed in the hippocampus (10), a region where VDAC expression is also enhanced (11). GRP78 is expressed on the cell surface of human brain glioma cells (12) as well as in mouse brain astrocytes and neurons (13). In our laboratory, we have extensively studied the functions of GRP78 in the cell surface (2), particularly in human prostate cancer (14) and malignant melanoma cells (15).
Both Pg and t-PA are key components of the central nervous system (CNS) (16) where, in addition to their fibrinolytic roles, they play roles in long term potentiation, synaptic plasticity, and neuronal migration (17, 18). Because t-PA activity is enhanced by VDAC (7) and because both t-PA and Pg activation are affected by the interaction of these ligands with either VDAC or GRP78, we hypothesized a functional cell surface interaction between these four components in the brain. For these studies, we used the human neuroblastoma cell line SK-N-SH as an in vitro model because these cells express VDAC on the cell surface (19). We investigated the expression of GRP78 in these cells and found that GRP78 co-localizes with VDAC on the cell surface. Kinetic studies and binding of t-PA to GRP78 or binding of GRP78 to VDAC were assessed using GRP78 or VDAC immobilized to microtiter plates, respectively. Our studies show that GRP78 enhances t-PA activity and induces SK-N-SH cell proliferation via t-PA binding to a lysine residue in the GRP78 amino acid sequence 98LIGRTWNDPSVQQDIKFL115. We also found that Pg binding to the COOH-terminal region of GRP78 via its microplasminogen domain induces cell proliferation and this mechanism is mediated by a Pg benzamidine-binding site. Furthermore, cross-linking studies show that both t-PA and Pg function as bridges between GRP78 and VDAC on the surface of SK-N-SH cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**Culture media were purchased from Invitrogen. The chromogenic proteins V-L-K-p-nitroanilide (S-2251) and l-P-R-p-nitroanilide (S-2288) were purchased from Diapharma (West Chester, OH). The VDAC 94GKSARVDVFTKGYGFG-LIKLDL30 (Gly10-Leu30) and GRP78 98LIGRTWNDPSVQQDIKFL115 (Leu98-Leu115), 98LIGRTWNDPSVQQDIVFL115 (Leu98-Leu115 K113V) and scrambled GTNKSQDLWIPQLRDVF1 (Leu98-Leu115 K113V scram) peptides were obtained from Genemed Synthesis, Inc. (San Antonio, TX). The homobifunctional cross-linker 3,3’-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was purchased from Pierce. The wide spectrum protease inhibitor mixture Halt™ was purchased from Thermo Scientific (Rockford, IL). Tranexamic acid (TXA) was purchased from Sigma. The other reagents used were of the highest grade available.

**Proteins—**Human Pg was purified by affinity chromatography on 1-lysine-Sepharose (5). Human t-PA and urokinase-type Pg activator (u-PA) were purchased from Calbiochem-EMD Chemicals, Inc. (San Diego, CA). Human VDAC clones (Genecopeia, Germantown, MD) were expressed in Escherichia coli and purified as previously described (20). Recombinant human microplasminogen (Genecopeia) was expressed in E. coli and purified from clones as previously described (21). Recombinant murine GRP78 and the COOH-terminal domain of GRP78 containing amino acids 516–636 (Lys516-Gly636), a kind gift from Dr. Syl- vie Y. Blond, College of Pharmacy University of Illinois, Chicago, IL, were purified as previously described (22).

**Antibodies—**The goat polyclonal IgG against the NH2-terminal region of human GRP78 (N-14) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The sheep polyclonal IgG against murine GRP78 was raised and purified as previously described (14).

**Cell Culture—**Human neuroblastoma SK-N-SH cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in MEM containing 2 mM l-glutamine, 1.5 g/liter of sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS), and 100 units/ml of penicillin/streptomycin, which were all purchased from Invitrogen. Pg-free FBS was prepared by adsorption of FBS to lysine-Sepharose as described previously (6).

**Cell Proliferation Assays—**SK-N-SH cells were resuspended in MEM containing 5% Pg-free FBS at a density of 0.1 × 10^6/ml and plated in 96-well culture plates (0.1 ml/well) containing increasing concentrations of the tested ligands in a final volume of 0.2 ml/well. Cell proliferation was determined at 72 h using a BrdU labeling and colorimetric immunoassay detection method (Roche Applied Science). The results were expressed as the absorbance at 372 nm (reference wavelength: 492 nm). Control cell proliferation was determined in the absence of any ligand.

**t-PA Binding Analysis—**All assays were performed on GRP78-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 of GRP78 (10 µg/ml) in 0.1 M Na2CO3, pH 9.6, containing 0.01% NaNO3, followed by rinsing with phosphate-buffered saline (PBS) and incubation with 3% bovine serum albumin (BSA) in 0.1 M Na2CO3, pH 9.6, containing 0.01% NaNO3 to block nonspecific sites. After rinsing the plates with PBS, the plates were stored at 4°C until further use. A similar procedure was used to coat the microtiter plates with the human recombinant VDAC or t-PA. The amount of GRP78 bound to the plates was calculated after reaction with the goat anti-GRP78 N-20 IgG followed by reaction with a rabbit anti-goat alkaline phosphatase-conjugated IgG, rinsing with PBS, and final incubation with the alkaline phosphatase substrate p-nitrophenyl phosphate (1 mg/ml) in 0.1 M glycine, 1 mM MgCl2, and 1 mM ZnCl2, pH 10.4. The absorbance was monitored at 405 nm using a Molecular Devices SPECTRAmax kinetic plate reader (Molecular Devices, LLC, Sunnyvale, CA). The t-PA binding assays were performed in triplicate, and the bound t-PA was calculated from calibration curves constructed with immobilized t-PA reacted with the N-14 anti-t-PA antibody followed first 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-nitrophenyl phosphate (1 mg/ml) in 0.1 M glycine, 1 mM MgCl2, and 1 mM ZnCl2, pH 10.4. The absorbance was measured at 405 nm as described above. The bound t-PA was expressed as nanomole of t-PA/nmol of GRP78. The Kd and Bmax were determined using the statistical program GraphPad Prism® 6 from GraphPad Software, Inc. (San Diego, CA).

**t-PA Amidolytic Activity Assay—**The steady-state amidolytic activity of t-PA toward S-2288 was analyzed in the presence of a single concentration of t-PA (10 nM) in 0.1 M Tris-HCl and 100 mM NaCl, pH 8.0. The velocity of S-2288 hydrolysis was calculated as the increase of absorbance at 405 nm using a Molecular Devices SPECTRAmax kinetic plate reader. The molar extinc-
tion coefficient ($\varepsilon$) of $p$-nitroanilide at 405 nm is 10,000 M$^{-1}$ cm$^{-1}$ (21). The kinetic parameters ($K_m$, $V_{max}$, and $k_{cat}$) were calculated using the GraphPad Prism 6 program from GraphPad Software, Inc.

**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 plasmin (Pm) (21). 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 plasmin 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 $A_{405}$ nm versus time$^2$ using the equation: $v_i = b(1 + K_m/S_0)/k_{cat}$, where $K_m$ is the apparent Michaelis constant of S-2251 hydrolysis by Pm, $k_{cat}$ is the empirically determined catalytic rate constant for Pm hydrolysis of S-2251 (3.2 $\times$ 10$^4$ M min$^{-1}$ (mol of Pm)$^{-1}$), and $\varepsilon$ is the molar extinction coefficient of $p$-nitroanilide at 405 nm (10,000 M$^{-1}$ cm$^{-1}$) (23).

**Determination of Plasmin and t-PA Amidolytic Activities**—The plasmin 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 plasmin substrate, VLK-$p$-nitroanilide (0.3 mM), was added to the mixture, and substrate hydrolysis was monitored at 405 nm as described above. The t-PA amidolytic activity was measured by incubating t-PA (5.5 nM) with the chromogenic substrate, S-2288 (0.3 mM), for 20 min at 37 °C in 20 mM HEPES, pH 7.4, in a total volume of 200 µl. The reaction was monitored at 405 nm.

**Cross-linking of t-PA and Pg to SK-N-SH Cell Membrane Proteins**—Cross-linking of Pg or t-PA to SK-N-SH cell membrane proteins was performed by incubating 1-ml cell suspensions (5 $\times$ 10$^6$ cells/ml) in serum-free MEM culture medium with Pg (1 µM) or t-PA (0.5 µM) at 37 °C for 30 min. Excess ligand was removed by centrifugation. After rinsing the cells with serum-free MEM, they were resuspended in 1 ml of ice-cold PBS, and the homobifunctional cross-linker DTSSP was added to a final concentration of 10 nM. The cross-linking reaction was performed on ice for 60 min and terminated by the addition of 0.2 ml of 1 M Tris-HCl, pH 7.5, containing 1 M glycine. The cells were then lysed with 1 ml of 100 mM N-octyl glucopyranoside in 50 mM Tris-HCl, pH 7.5, containing 10 µl of Halt protease inhibitor mixture (Thermo Scientific). After mixing, the samples were incubated at room temperature for 30 min to extract the membrane proteins. Cell debris was removed by centrifugation at 10,000 $\times$ g for 30 min at 4 °C. Supernatants containing the proteins cross-linked to cell surface GRP78 were immunoaffinity purified with sheep anti-GRP78 IgG covalently attached to Sepharose-4B. In a separate experiment, proteins cross-linked to t-PA were also purified by immunoaffinity with goat anti-tPA IgG covalently attached to Sepharose-4B. After elution with 1 M guanidine HCl in 50 mM Tris-HCl, pH 8.0, and extensive dialysis against 50 mM Tris-HCl, pH 8.0, the protein solutions were concentrated to 0.5 ml with Amicon concentration filters (EMD Millipore, Billerica, MA). The cross-linked proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (24), which breaks the DTSSP bridges between Pg or t-PA and GRP78.
After separation, the proteins were electroblotted to nitrocellulose membranes (25) and singly probed with anti-human GRP78 IgG (N-20), anti-human Pg IgG (H-14), anti-human VDAC IgG (N-18), or anti-human t-PA IgG (N-14). The blots were washed 3 times for 5 min each with phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and then incubated with a rabbit anti-goat IgG antibody conjugated to an IR-800 nm label (Rockland, Gilbertsville, PA) or a donkey anti-goat IgG 680 (Invitrogen) diluted 1:10,000 in Rockland blocking buffer (Rockland, Gilbertsville, PA) 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 Odyssey instrument (Li-Cor Biosciences, Lincoln, NE). The HyperPAGE dye-conjugated molecular mass markers (10 to 190 kDa) were purchased from Bioline USA, Inc. (Taunton, MA).

**Immunofluorescence Staining of Non-permeabilized Cells—** SK-N-SH cells were grown on chamber slides in complete medium to 80% confluence.

**FIGURE 2.** Kinetics of t-PA amidolytic activity in the presence of GRP78. The amidolytic activity was determined by monitoring the generation of p-nitroanilide (pNA) as described under “Experimental Procedures.” A, t-PA (10 nM) was incubated with increasing concentrations of S-2288 (0.1–5 mM) in the absence (○) or presence of 100 nM GRP78 (□) or 200 nM GRP78 (△). B, t-PA (10 nM) was incubated with increasing concentrations of S-2288 (0.1–5 mM) in the presence of 100 nM GRP78 only (○) or 100 nM GRP78 in combination with 100 (□) or 200 (△) nM VDAC. C, effect of increasing concentrations of the GRP78 Leu<sup>98</sup>-Leu<sup>115</sup> peptide on t-PA amidolytic activity at a single concentration of S-2288 (2 mM). D, inhibition of the stimulation of t-PA amidolytic activity induced by 100 nM GRP78 (●) or 10 μM GRP78 Leu<sup>98</sup>-Leu<sup>115</sup> peptide (▲) with increasing concentrations of TXA (0–20 mM). E, effect of increasing concentrations of the GRP78 Leu<sup>98</sup>-Leu<sup>115</sup> K113V peptide on t-PA (10 nM) amidolytic activity at a single concentration of S-2288 (2 mM). F, effect of increasing concentrations of the scrambled GRP78 Leu<sup>98</sup>-Leu<sup>115</sup> scrm peptide on t-PA (10 nM) amidolytic activity at a single concentration of S-2288 (2 mM). The data are the mean ± S.D. (error bars) from experiments performed in triplicate (n = 6).
**GRP78 Is a Plasminogen and t-PA Receptor**

Temperature in 1% fresh paraformaldehyde in PBS. The slides were then incubated in 5% donkey serum in PBS for 60 min at room temperature. Excess blocking buffer was drained from the slide, and the cells were incubated with anti-GRP78 antibody (1:100), anti-VDAC antibody (1:100; Santa Cruz Biotechnology), or a combination of the two in 1% donkey serum in PBS overnight at 4 °C. The cells were then rinsed three times in PBS-T and incubated with a 1:1,000 dilution of Alexa Fluor 488-conjugated donkey anti-sheep IgG, Alexa Fluor 568-conjugated donkey anti-goat IgG, or a combination of the two for 60 min at room temperature in the dark. As controls, the cells were incubated with secondary IgG alone. The cells were washed three times with PBS and then incubated with Hoechst 33342 to stain the nuclei for 10 min at room temperature. The slides were mounted with medium consisting of 90% glycerol, 0.1 M n-propanol, and 0.01% sodium azide. Fluorescence microscopy was performed with a Leica DMI 4000B fluorescence microscope with a ×40/0.22 Fluotar lens (Leica).

**Statistics**—GraphPad Prism, version 6.0 software (GraphPad 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 was determined by a one-way analysis of variance and unpaired Student’s t tests.

**RESULTS**

**Binding of t-PA to Immobilized GRP78**—t-PA binds to immobilized GRP78 in a dose-dependent manner (Fig. 1A) with high affinity ($K_d = 4.07 \pm 0.46$ nM). The binding of t-PA is inhibited by TXA (1–20 mM), suggesting that interaction of t-PA with GRP78 is lysine-dependent (Fig. 1B). Binding of t-PA to GRP78 is not inhibited by the VDAC Gly10-Leu30 peptide (Fig. 1C), which we previously found to mediate binding of t-PA to VDAC (7); however, the GRP78 Leu98-Leu115 peptide inhibits its binding (Fig. 1D), suggesting that t-PA binds to the N-terminal site in GRP78, which also binds activated $\alpha_v$-macroglobulin ($\alpha_v$-M*) or anti-GRP78 autoantibodies found in prostate cancer or malignant melanoma patients (14, 15).

**Effect of GRP78 on the Amidolytic Activity of 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 immobilized GRP78. Increasing amounts of GRP78 (100 and 200 nM) stimulated the amidolytic activity of t-PA (Fig. 2A). Because VDAC also stimulates t-PA amidolytic activity (7), we evaluated the effect of VDAC on t-PA activity in the presence of a single concentration of GRP78 (100 nM) and two concentrations of VDAC (100 and 200 nM) (Fig. 2B). In both cases, the velocity isotherms show a dose-dependent increase after the addition of GRP78 or GRP78 mixed with VDAC, suggesting a synergistic effect on t-PA enzymatic activity when combined. 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 GRP78 or combined GRP78 and VDAC are caused primarily by decreases in $K_m$ and increases in $V_{max}$, which lead to a 3-fold increased catalytic efficiency ($k_{cat}/K_m$).

**Table 1**

| Protein | $V_{max}$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ |
|---------|-----------|-------|-----------|---------------|---------------|
| None    | $1.92 \times 10^{-4}$ | $1.176$ | $4.96$ | $4.21$ | $2.11$ |
| GRP78 (100 nM) | $2.036$ | $1.087$ | $9.69$ | $8.91$ | $2.48$ |
| GRP78 (200 nM) | $3.065$ | $1.023$ | $12.76$ | $10.46$ | $2.73$ |
| VDAC (100 nM) | $2.656$ | $1.006$ | $11.57$ | $11.50$ | $2.73$ |
| GRP78 (100 nM) and VDAC (100 nM) | $2.889$ | $0.947$ | $12.03$ | $12.70$ | $3.01$ |

*a The abbreviations used are: pNA, p-nitrophenyl phosphate.

**FIGURE 3. Kinetics of Pg activation by t-PA in the presence of GRP78 and VDAC.** 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. A, Glu-Pg (100 nM) 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 100 nM GRP78 (●) or 200 nM GRP78 (●) followed by the addition of plasmin substrate S-2251 (0.3 mM) and t-PA (0.55 nM). B, Glu-Pg (100 nM) 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 presence of 100 nM GRP78 and absence of VDAC (●), and in the presence of either 100 nM GRP78 and 100 nM VDAC (●) or 100 nM GRP78 and 200 nM VDAC (●) followed by the addition of plasmin substrate S-2251 (0.3 mM) and t-PA (0.55 nM). The data are the mean ± S.D. (error bars) from experiments performed in triplicate ($n = 6$).

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25170 JOURNAL OF BIOLOGICAL CHEMISTRY

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idue at position 113 (Lys\textsuperscript{113}), we hypothesized that such a residue was responsible for binding of t-PA to GRP78 on the cell surface. For this purpose, we measured t-PA activity using increasing concentrations of the GRP78 Leu\textsuperscript{98}-Leu\textsuperscript{115} peptide where the Lys\textsuperscript{113} was replaced by a valine residue (Fig. 2E) or a scrambled Leu\textsuperscript{98}-Leu\textsuperscript{115} peptide containing the sequence GTNKSPDLWIPQLRDVFI (Fig. 2F). Neither of these two peptides had any effect on t-PA activity, demonstrating that Lys\textsuperscript{113} in GRP78 is not only important for anchoring t-PA but also for enhancing its catalytic properties.

Effect of GRP78 on t-PA-mediated Plasminogen Activation—The initial velocities of Pg activation by t-PA were obtained under steady-state conditions. The velocity curves in the presence of GRP78 (100 and 200 nM) show a dose-dependent increase in the activation velocity (Fig. 3A). Activation of Pg by t-PA at a single concentration of GRP78 (100 nM) and two concentrations of VDAC (100 and 200 nM) also show a modest increase in the activation velocity (Fig. 3B) which, as demonstrated above, act together on t-PA activity. The data for the rates of plasmin formation versus the initial Pg concentration were extrapolated to the Michaelis-Menten equation by nonlinear regression to obtain the \(k_{cat}\) and \(K_m\) (Table 2). This analysis shows that GRP78 alone or in combination with VDAC increases the \(k_{cat}/K_m\) for the activation reaction from 0.92 to a maximum of 3.62 \(\mu M^{-1} s^{-1}\), resulting in an overall 3.93-fold increase in activation efficiency, as previously observed with VDAC (7). Similar to VDAC (7), GRP78 enhances only t-PA activity and has no significant effect on Pm activity after it is formed (data not shown).

TABLE 2
Kinetic parameters of Glu-Pg activation by t-PA in the presence of GRP78 and VDAC

| Protein   | \(V_{max}\) (mol Pg/min \(\times 10^{-13}\)) | \(K_m\) (\(\mu M\)) | \(k_{cat}\) (\(s^{-1}\)) | \(k_{cat}/K_m\) | \(k_{cat}/K_m\) fold increase |
|-----------|------------------------------------------|----------------------|-------------------------|----------------|-------------------------------|
| None      | 34.80                                    | 0.156                | 0.145                   | 0.92           |                                |
| GRP78 (100 nM) | 38.17                                    | 0.088                | 0.159                   | 1.67           | 1.85                          |
| GRP78 (200 nM) | 57.94                                    | 0.065                | 0.158                   | 2.43           | 3.21                          |
| VDAC (100 nM) | 58.05                                    | 0.045                | 0.163                   | 3.62           | 3.93                          |

Immunofluorescent Microscopy of GRP78 and VDAC1 on the Surface of Human Neuroblastoma SK-N-SH Cells—We previously found that GRP78 and VDAC1 are co-localized on the surface of human prostate 1-LN cells (5). Non-permeabilized SK-N-SH neuroblastoma cells were first stained with specific anti-GRP78 (AF-488, green) or anti-VDAC (AF-568, red) antibodies (Fig. 4, A and B) and then with Hoechst 33342 (H33342, blue) to stain the nuclei (Fig. 4A). Immunofluorescence analysis shows that GRP78 and VDAC are highly expressed on the surface of SK-N-SH cells. These proteins also appear co-localized and concentrated in areas of contact between cells (Fig. 4D). Control experiments performed with secondary antibodies alone show very little nonspecific staining (Fig. 4, E–H).

Identification of GRP78 and VDAC as Binding Proteins of t-PA and Pg on the Surface of SK-N-SH Neuroblastoma Cells—To further identify the Pg- and t-PA-binding proteins, SK-N-SH cells were treated with Pg (1 \(\mu\)g) or t-PA (0.1 \(\mu\)g) and cross-linked to cell surface proteins with DTSSP, followed by GRP78-specific immunoaffinity chromatography. Electrophoretic separation was performed under reducing conditions, which breaks the DTSSP bridge between Pg or t-PA and cell surface proteins, allowing each protein to be resolved separately in the gel before Western blot analysis with specific antibodies against GRP78, VDAC, t-PA, or Pg, as described under “Experimental Procedures.” The migration of proteins purified by immunoaffinity were compared with the migration of recombinant GRP78, t-PA, and VDAC and purified plasma Pg (100 ng/lane) (Fig. 5A, lanes 2–5, respectively). First, we analyzed the proteins (30 \(\mu\)l) co-precipitated with the GRP78 antibody from non-cross-linked cell lysates (Fig. 5, lane 6) and compared these to proteins (30 \(\mu\)l) co-precipitated with this antibody from cell lysates in which the cell surface proteins were cross-linked with DTSSP (Fig. 5A, lane 7). These results show almost no difference in the intensity of the protein bands for GRP78 or VDAC, suggesting that both are physically bound on the surface of SK-N-SH cells. Immunoaffinity purification of proteins using an anti-GRP78 antibody from cell lysates treated with t-PA or Pg cross-linked to the cell surface (Fig. 5A, lane 8) shows the presence of GRP78, VDAC, and t-PA. A similar purification of protein lysates from cells treated with Pg cross-linked to the cell surface (Fig. 5A, lane 9) shows the presence of protein.
bands corresponding to GRP78, VDAC, and Pg. We also performed immunoaffinity purification of proteins from a cell lysate containing t-PA cross-linked to the cell surface with an immobilized anti-t-PA antibody. The migration of proteins purified using this technique was compared with the migration of GRP78, t-PA, and VDAC (100 ng/lane) (Fig. 5B, lanes 1–3, respectively). The SDS-PAGE separation and analysis of the proteins immunoprecipitated with anti-t-PA IgG (Fig. 5B, lane 4) shows the presence of GRP78, t-PA, and VDAC. Both experiments clearly show the formation of ternary complexes between GRP78, VDAC, and either Pg or t-PA on the cell surface.

Effect of t-PA on Neuroblastoma SK-N-SH Cell Proliferation—t-PA is a known mitogen for human aortic smooth cells (26) and cancer cells (27). Because t-PA binds the NH2-terminal GRP78 amino acid sequence Leu98-Leu115, a region that stimulates growth when bound to ligands (14), and because, when expressed on the surface of SK-N-SH cells, GRP78 may contain sites for t-PA binding, we hypothesized that t-PA induces a pro-proliferative effect via GRP78 in these cells. Incubation of SK-N-SH cells with increasing concentrations of t-PA stimulates cell proliferation (Fig. 6A). Because in addition to GRP78, t-PA also binds to VDAC (7), we incubated the cells with a single concentration of t-PA (20 nM) and increasing concentrations of VDAC Gly10-Leu30 peptide as competitors. The results (Fig. 6B) show that only the GRP78 peptide inhibits cell proliferation, whereas the VDAC peptide, which is known to inhibit binding of t-PA to either immobilized VDAC or SK-N-SH cells (7), was not effective. Therefore, the pro-proliferative effect of t-PA is mediated by GRP78 on the cell surface.

Effect of Microplasminogen on Neuroblastoma SK-N-SH Cell Proliferation—The increased Pg activation by t-PA bound to GRP78 suggests that Pm is rapidly generated on the cell surface of these and other cell systems in which both GRP78 and VDAC are co-localized. Pm induces smooth muscle cell proliferation.

FIGURE 5. Cross-linking of t-PA and Pg to neuroblastoma SK-N-SH cell membrane proteins. Cells incubated with t-PA (0.5 µM) or Pg (1 µM) were lysed and the cross-linked proteins were purified by immunoaffinity. Aliquots (30 µl) were resolved by 10% SDS-PAGE under reducing conditions and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with goat anti-GRP78, anti-t-PA, anti-VDAC, and anti-Pg antibodies and developed using an anti-goat IgG conjugated to IR-800 nm. All procedures were performed as described under “Experimental Procedures.” A, immunoaffinity on anti-GRP78 IgG Sepharose. Lane 1, molecular mass markers (27–126 kDa). Lane 2, recombinant GRP78 (100 ng) immunoblotted with anti-GRP78 IgG. Lane 3, recombinant t-PA (100 ng) immunoblotted with anti-t-PA IgG. Lane 4, recombinant VDAC (100 ng) immunoblotted with anti-VDAC IgG. Lane 5, plasma Pg (100 ng) immunoblotted with anti-Pg IgG. Lane 6, immunoprecipitate of non-cross-linked cells immunoblotted with anti-GRP78 and anti-VDAC IgGs. Lane 7, immunoprecipitate of DTSSP-cross-linked cells immunoblotted with anti-GRP78 and anti-VDAC IgGs. Lane 8, immunoprecipitate of cells cross-linked with t-PA and immunoblotted with anti-GRP78, anti-VDAC, and anti-t-PA IgGs. Lane 9, immunoprecipitate of cells cross-linked with Pg and immunoblotted with anti-GRP78, anti-VDAC, and anti-Pg IgGs. B, immunoaffinity on anti-t-PA IgG Sepharose. Lane 1, molecular mass markers (27–126 kDa). Lane 2, recombinant GRP78 (100 ng) immunoblotted with anti-GRP78 IgG. Lane 3, recombinant t-PA (100 ng) immunoblotted with anti-t-PA IgG. Lane 4, recombinant VDAC (100 ng) immunoblotted with anti-VDAC IgG. Lane 5, immunoprecipitate of cells cross-linked with t-PA and immunoblotted with anti-GRP78, anti-VDAC, and anti-t-PA IgGs.

FIGURE 6. Effect of t-PA on human neuroblastoma SK-N-SH cell proliferation. A, cells in 96-well culture plates (1 × 10⁴ cells/well) were incubated for 72 h in MEM containing 5% FBS in the absence or presence of increasing concentrations of t-PA. B, SK-N-SH cells were incubated with a single concentration of t-PA (20 nM) and increasing concentrations of VDAC Gly10-Leu30 (●) or GRP78 Leu98-Leu115 (Δ) peptides (0–10 µM) as competitors. Cell proliferation was measured as described under “Experimental Procedures.” The data are the mean ± S.D. (error bars) from experiments performed in triplicate (n = 6).
via a lysine binding-dependent mechanism (28); however, the effect of the Pg microplasminogen domain on cell proliferation has not been thoroughly investigated. Because microplasminogen binds to the COOH-terminal domain of GRP78 to induce a Ca\textsuperscript{2+} release from intracellular stores (5) that leads to initiation of the unfolded protein response (UPR) followed by cell proliferation (4), we hypothesized a pro-proliferative response in SK-N-SH cells incubated with microplasminogen. The results (Fig. 7A) show an increase in cell proliferation with increasing concentrations of microplasminogen. We then assessed the proliferative response in cells incubated with a single concentration of microplasminogen (500 nM) and increasing concentrations of the GRP78 Leu\textsuperscript{98}-Leu\textsuperscript{115} peptide (●) or the GRP78 COOH-terminal domain Lys\textsuperscript{516}Gly\textsuperscript{636} peptide (▲) as competitors. C, SK-N-SH cells were incubated with increasing concentrations of the anti-GRP78 (C-20) IgG. D, SK-N-SH cells were incubated with a single concentration of microplasminogen (500 nM) and increasing concentrations of the anti-GRP78 (C-20) IgG as competitors. E, SK-N-SH cells were incubated with increasing concentrations of p-aminobenzamidine in the absence (□) or presence (●) of microplasminogen (500 nM). F, SK-N-SH cells were incubated with a single concentration of microplasminogen (500 nM) and increasing concentrations of aprotinin. Cell proliferation was measured as described under “Experimental Procedures.” The data are the mean ± S.D. (error bars) from experiments performed in triplicate (n = 6).
that the Lys<sup>516</sup>-Gly<sup>636</sup> region of GRP78 acts as a receptor site for Pg on the cell surface (5). The pro-proliferative effect of microplasminogen is the opposite of the effect observed with antibodies against the GRP78 COOH-terminal domain, which induce apoptosis in human prostate cancer cells (29, 30). Therefore, we tested the effect of the anti-GRP78 (C-20) antibody in SK-N-SH cells and observed that cells incubated with increased amounts of this antibody show significant inhibition of proliferation (Fig. 7C). Furthermore, SK-N-SH cells incubated with a single concentration of microplasminogen (500 nM) and increasing amounts of the anti-GRP78 (C-20) antibody show a reversal of the pro-proliferative effect of microplasminogen (Fig. 7D). These results suggest that although both microplasminogen and the anti-GRP78 (C-20) antibody may bind to sites on GRP78 COOH-terminal region, their signaling pathways are opposed. Binding of microplasminogen to GRP78 depends on a benzamidine binding site (31). Addition of increasing concentrations of p-aminobenzamidine inhibit the pro-proliferative effects of microplasminogen (Fig. 7E), demonstrating involvement of the benzamidine binding site for GRP78. Incubation of SK-N-SH cells with microplasminogen in the presence of the chromogenic substrate S-2251 demonstrates conversion of microplasminogen into microplasmin (data not shown). Cells incubated with microplasminogen and increasing concentrations of the protease inhibitor aprotinin show inhibition of proliferation (Fig. 7F), demonstrating that conversion to microplasmin is required for cell proliferation.

**DISCUSSION**

t-PA, a member of the fibrinolytic system, is a serine protease that converts the zymogen Pg into the active protease Pm, which then proteolyzes its target proteins (32). Although the Pm proteolytic cascade is primarily involved with fibrin degradation, it plays a prominent role in the mammalian brain where both t-PA and Pg are synthesized and are important modulators of neuronal survival and function (33, 34). Pg binds to a wide variety of cell surface receptors (35) that act to decrease the $K_m$ between Pg and its activators to promote Pm formation even in the absence of fibrin (36). This mechanism is facilitated by several receptors, which in addition to Pg, also bind t-PA. These receptors include annexin II (37) and VDAC (7). However, binding of both t-PA and Pg to annexin II is lysine-dependent (38), whereas binding of Pg to VDAC is K5-dependent (6) and binding of t-PA to VDAC is lysine-independent (7). It is also known that neuronal cells express a non-fibrin cofactor for t-PA-mediated plasmin generation following cellular injury (39).

The results of this study show that GRP78, in addition to serving as a receptor for Pg (5), may also function as a receptor for t-PA. Our results also show that binding of t-PA to GRP78 is mediated via a lysine-dependent mechanism with an affinity 10-fold ($k_d = 4.07 \pm 0.46$ nM) higher than that of the lysine-independent mechanism involving the interaction of t-PA with VDAC ($k_d = 43 \pm 8.01$ nM) (8). However, the net result is an increased catalytic efficiency of t-PA for its substrate Pg in both cases. The co-localization of GRP78 and VDAC on the cell surface may facilitate Pg activation, a mechanism that operates via dual binding of t-PA and Pg to these proteins. As discussed above, t-PA also functions as a mitogen for neuroblastoma SK-N-SH cells. This occurs via binding of t-PA to a Lys<sup>113</sup> residue located in the GRP78 NH<sub>2</sub>-terminal domain containing amino acids Leu<sup>98</sup>-Leu<sup>115</sup>. Previous studies from our laboratory have demonstrated that GRP78 acts as a growth factor-like receptor and that signaling initiated by ligation of agonists to the GRP78 NH<sub>2</sub>-terminal domain on cell surface GRP78 is pro-proliferative (14). Our results suggest that t-PA interacts with this domain to induce cell proliferation, supporting the growth factor-like function of GRP78 on the cell surface. Although t-PA also binds to VDAC on the cell surface, our kinetic data suggest that binding of t-PA to GRP78 is preferential to that of VDAC and binding of t-PA to VDAC is not necessary for cell proliferation. Binding of t-PA to VDAC enhances its catalytic efficiency (7); however, any additional physiologic consequences of this interaction remain to be determined.
Taken together, these studies show the versatility of this Pg activating enzyme system, which as discussed above, involves binding of Pg and t-PA to the common receptors, GRP78 and VDAC, via different regions in their primary structures. Pg binds to two separate domains in GRP78. It binds via its K5 to an NH$_2$-terminal domain and via its microplasminogen domain to a COOH-terminal domain in GRP78 (5). The Pg K5 is a suppressor of cell motility and proliferation (40, 41), whereas microplasminogen induces cell proliferation. In a previous study (6), we demonstrated that microplasminogen binds to a sequence containing the amino acid Ser$^{262}$-Phe$^{273}$ site in the GRP78 COOH-terminal region. This interaction induces a release of Ca$^{2+}$ from intracellular stores in human prostate 1-LN cells, which protects these cells from apoptosis under both normal and hypoxic conditions (5), confirming that microplasminogen bound to GRP78 on the cell surface plays a protective role. Several studies from our laboratory demonstrate that antibodies against the COOH-terminus of GRP78 are pro-apoptotic and suppress proliferative signaling in cancer cells (2). The anti-GRP78 C-20 antibody used in these studies recognizes an epitope containing amino acids Lys$^{633}$, Asp$^{647}$ in the GRP78 COOH-terminal domain. The mechanisms by which these antibodies operate are opposed to those induced by ligation of the NH$_2$-terminal GRP78 Leu$^{98}$-Leu$^{115}$ region, which stimulate cell proliferation (2). Furthermore, the release of Ca$^{2+}$ from intracellular stores resulting from microplasminogen binding to the GRP78 COOH-terminal domain may also mediate cell proliferation via undetermined pathways, which are opposed to those induced by the anti-GRP78 COOH-terminal antibodies (29, 30).

In addition to its multiple roles in fibrinolysis and extracellular matrix remodeling, the association of Pg, t-PA, VDAC, and GRP78 may play a major role in the CNS under both normal and pathological conditions. Both Pg and t-PA are central to mechanisms that control multiple forms of synaptic plasticity and memory (42, 43). In a previous study, we proposed a model in which VDAC potentiates t-PA-mediated plasmin formation (7). With the results of this study, we can expand this model to include the association of VDAC with GRP78. In this new model (Fig. 8), the four intracellular domains of GRP78 (2) are shown in purple. The co-localization of GRP78 and VDAC on the surface involves a proximity between the GRP78 extra-cellular domains, highlighted in beige, where the NH$_2$-terminal domain region Leu$^{98}$-Leu$^{115}$ is the t-PA binding site, and the COOH-terminal region Lys$^{633}$, Asp$^{647}$ is the microplasminogen binding site (2). t-PA binds to the Lys$^{113}$ in GRP78 in a lysine-dependent manner, which induces a conformational change that facilitates its lysine-independent binding to VDAC. In addition to stimulating cell proliferation via GRP78, binding of t-PA to VDAC may induce further enhancement of Pg activation on the cell surface. Similarly, Pg binds to VDAC via its K5, which facilitates its conversion to Pm. As previously observed, Pg K5 may inhibit cell growth; however, its role is short-lived because it may be inactivated after reduction by the NADH-dependent activity of VDAC (7), although binding of the microplasminogen/microplasmin domain to the Lys$^{633}$, Asp$^{647}$ GRP78 COOH-terminal domain may induce a further increase of cell proliferation. Because t-PA in the brain is the most abundant serine protease (44) and because the brain is an organ usually devoid of fibrinogen, GRP78 and VDAC mimic fibrin to enhance the activation of locally synthesized Pg, which can then act on non-fibrin substrates (45), such as VDAC itself, which not only mimics fibrin, but also serves as a Pm substrate (7). Therefore, this fibrin-independent mechanism may shift the conformational equilibrium of the protease domain of t-PA to the fully active form, and may also be necessary to promote brain cell proliferation and survival.

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GRP78 Is a Plasminogen and t-PA Receptor

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