Overexpression of the 78-kDa Glucose-regulated Protein/Immunoglobulin-binding Protein (GRP78/BiP) Inhibits Tissue Factor Procoagulant Activity*

Received for publication, January 29, 2003
Published, JBC Papers in Press, March 5, 2003, DOI 10.1074/jbc.M301006200

Lindsay M. Watson‡, Anthony K. C. Chan¶, Leslie R. Berry†, Jun Li‡, Sudesh K. Sood‡, Jeffrey G. Dickhout‡, Ling Xu¶, Geoff H. Werstuck¶, Laszlo Bajzar‡, Henry J. Klamut**, and Richard C. Austin‡ ‡‡

From the Departments of ‡Pathology and Molecular Medicine, ¶Pediatrics, and ¶¶Medicine, McMaster University and the Henderson Research Centre, Hamilton, Ontario L8V 1C3 and the **Division of Experimental Therapeutics, Ontario Cancer Institute, Toronto, Ontario M5G 2M9, Canada

Previous studies have demonstrated that overexpression of GRP78/BiP, an endoplasmic reticulum (ER)-resident molecular chaperone, in mammalian cells inhibits the secretion of specific coagulation factors. However, the effects of GRP78/BiP on activation of the coagulation cascade leading to thrombin generation are not known. In this study, we examined whether GRP78/BiP overexpression mediates cell surface thrombin generation in a human bladder cancer cell line T24/83 having prothrombotic characteristics. We report here that cells overexpressing GRP78/BiP exhibited significant decreases in cell surface-mediated thrombin generation, prothrombin consumption, and the formation of thrombin-inhibitor complexes, compared with wild-type or vector-transfected cells. This effect was attributable to the ability of GRP78/BiP to inhibit cell surface tissue factor (TF) procoagulant activity (PCA) because conversion of factor X to Xa and factor VII to VIIa were significantly lower on the surface of GRP78/BiP-overexpressing cells. The additional findings that (i) cell surface factor Xa generation was inhibited in the absence of factor VIIa and (ii) TF PCA was inhibited by a neutralizing antibody to human TF suggests that thrombin generation is mediated exclusively by TF. GRP78/BiP overexpression did not decrease cell surface levels of TF, suggesting that the inhibition in TF PCA does not result from retentive loss of TF in the ER by GRP78/BiP. The additional observations that both adenovirus-mediated and stable GRP78/BiP overexpression attenuated TF activation by ionomicyn or hydrogen peroxide suggest that GRP78/BiP indirectly alters TF activation through a mechanism involving cellular Ca2+ and/or oxidative stress. Similar results were also observed in human aortic smooth muscle cells transfected with the GRP78/BiP adenovirus. Taken together, these findings demonstrate that overexpression of GRP78/BiP decreases thrombin generation by inhibiting cell surface TF PCA, thereby suppressing the prothrombotic potential of cells.

In eukaryotic cells, the endoplasmic reticulum (ER) is the cellular organelle where secretory proteins or proteins destined for the plasma membrane undergo a variety of modifications, including disulfide bond formation, glycosylation, folding, and oligomeric assembly. The inability of nascent polypeptide chains to fold into their native conformation generally leads to retention in the ER and degradation (1). To assist in the correct folding of newly synthesized proteins and to prevent aggregation of folding intermediates, the ER contains a wide range of molecular chaperones such as the glucose-regulated proteins, calnexin, calreticulin, protein-disulfide isomerase, and Erp72 (2, 3). These chaperones are postulated to act as a quality control system by ensuring that only correctly folded proteins are processed prior to entering the Golgi apparatus for further processing and secretion (2–4). In addition to their function as molecular chaperones, recent studies have demonstrated that some of these ER proteins protect cells against oxidative stress (5–7).

Prominent among the ER-resident chaperones is the 78-kDa glucose-regulated protein/immunoglobulin-binding protein (GRP78/BiP), a highly conserved member of the 70-kDa heat shock protein family (8, 9) and a major ER luminal Ca2+ storage protein (10, 11). GRP78/BiP transiently associates with correctly folded proteins but forms more stable complexes with misfolded or incompletely assembled proteins (1, 4, 12). This association involves the binding of GRP78/BiP to hydrophobic motifs exposed on unfolded or unassembled polypeptides (13–15). Proteins stably bound to GRP78/BiP are subsequently translocated from the ER into the cytosol for proteasome-dependent degradation (16, 17). The observation that GRP78/BiP is induced by conditions of stress that result in the accumulation of misfolded or underglycosylated proteins in the ER (8, 9) provides further evidence that GRP78/BiP plays a critical role in quality control processes during protein synthesis and folding.

Recent studies have demonstrated that alterations in GRP78/BiP protein levels mediate selective changes in the abundance of membrane or secretory proteins that transit the ER. In mammalian cells, reduced levels of GRP78/BiP increase the secretion of factor VIII and a mutant form of tissue plasminogen activator (3, 18).

1 The abbreviations used are: ER, endoplasmic reticulum; GRP78/BiP, 78-kDa glucose-regulated protein/immunoglobulin-binding protein; vWF, von Willebrand factor; TF, tissue factor; PCA, procoagulant activity; APTT, activated partial thromboplastin time; TAT, thrombin-antithrombin; MCSF, macrophage colony-stimulating factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RIPA, radioimmune precipitation assay buffer; pfu, plaque-forming unit; HRP, horseradish peroxidase; ROS, reactive oxygen species; CHO, Chinese hamster ovary; HASMC, human aortic smooth muscle cells.

2 This research was supported in part by Research Grants NA-4842 (to R. C. A.) and NA0420 (to A. K. C. C.) from the Heart and Stroke Foundation of Ontario. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 A recipient of a Premier’s Research Excellence Award Studentship and an Ontario Graduate Scholarship.

4‡‡ A Career Investigator of the Heart and Stroke Foundation of Ontario (HSFO). To whom correspondence should be addressed: Henderson Research Centre, 711 Concession St., Hamilton, Ontario L8V 1C3, Canada. Tel.: 905-527-2299 (ext. 42628); Fax: 905-575-2646; E-mail: raustin@thrombosis.hhscrc.org.
Prevention of TF PCA by GRP78/BiP

minogen activator lacking glycosylation sites (18–20). In contrast, elevated levels of GRP78/BiP decrease the secretion of von Willebrand factor (vWF) and factor VIII (18–20). Although these studies provide convincing evidence that alterations in GRP78/BiP levels lead to selective changes in the processing and secretion of certain coagulation factors, the effect of GRP78/BiP on the prothrombotic potential of cells was not investigated.

The major physiological initiator of the extrinsic coagulation cascade is tissue factor (TF), a 47-kDa transmembrane glycoprotein (21–24). TF initiates the coagulation cascade by complexing with factor VII/VIIa on the surface of cells, leading to the activation of factors X and X and the subsequent generation of thrombin. TF expression is increased in atherosclerotic plaques (25–31) and in vascular smooth muscle cells following balloon injury (32). Infection with adenovirus, as well as other respiratory viruses, increases TF expression and procoagulant activity (33). Growth factors (34) and cytokines (35, 36), endotoxin (37, 38), hypoxia (39), cell injury (40), reactive oxygen species (ROS) (41–43), oxidized LDL (44–46), and increases in intracellular free Ca2+ (47–50) are also known to induce TF expression in a variety of cell types, including vascular endothelial cells, smooth muscle cells, and circulating monocytes. Furthermore, the majority of tumors express TF (51–54), and the prothrombotic state observed in cancer patients has been largely attributed to this expression (55).

Given that TF transits the ER prior to its localization in the plasma membrane, we have postulated that GRP78/BiP overexpression could potentially alter the prothrombotic potential of cells. In this study, GRP78/BiP was stably overexpressed in a human bladder cancer cell line T24/83 having prothrombotic characteristics. Our findings indicate that overexpression of GRP78/BiP significantly decreases TF-mediated cell surface thrombin generation. The finding that GRP78/BiP overexpression fails to decrease cell surface levels of TF suggests that the inhibition of TF PCA by GRP78/BiP does not result from the retention of TF in the ER. The ability of GRP78/BiP overexpression to attenuate TF PCA induced by ionomycin, hydrogen peroxide, or adenovirus supports a mechanism involving cell surface TF protein was immunoprecipitated from total cell protein by the appropriate primary and horseradish peroxidase-labeled secondary antibodies (Affinity Biologicals, Hamilton, ON), the membranes were developed using the Renaissance chemiluminescence reagent kit (PerkinElmer Life Sciences). Images were subsequently captured and analyzed using Northern Exposure image analysis/archival software (Misisiuaga, ON).

Indirect Immunofluorescence and Image Analysis—Polycacylic antibodies to GRP78/BiP (sc-1050) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunofluorescence for GRP78/BiP was performed as described previously (58, 59). For detection of cell surface levels of TF, cells were washed with ice-cold 1× PBS for 2 min, blocked with 10% BSA in 1× PBS for 30 min at 4°C and incubated with human TF monoclonal antibody (5 μg/ml) for 1 h at 4°C. After three washes with 1× PBS containing 0.5% BSA, cells were incubated with the appropriate Alexa-labeled secondary antibody (BioLynx, Brockville, ON) for 1 h at 4°C, washed again, and fixed with 1% paraformaldehyde. Images were subsequently captured and analyzed using Northern Exposure image analysis/archival software (Misisiuaga, ON).

Immunoprecipitation of Cell Surface TF—Cell surface TF was immunoprecipitated essentially as described previously (42). Briefly, anti-human TF polyclonal antibodies at 0.5 μg/ml in PBS-free M199 medium were added to cell monolayers at 4°C for 2 h. After washing twice with ice-cold 1× PBS, cells were lysed in ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% (w/v) containing protease inhibitors (Roche Diagnostics, Laval, QC), and cell surface TF protein was immunoprecipitated from total cell protein by overnight incubation with protein A-Sepharose-linked beads (Pierce). After washing with RIPA buffer, the beads were boiled in Laemmli sample buffer, and the immunoprecipitates were separated on 10% SDS-polyacrylamide gels under reducing conditions. Gels were transferred to nitrocellulose membranes and immunoblotted with anti-human TF polyclonal antibodies, as described above.

Biotinylation of Cell Surface Proteins—Cell surface biotinylation was performed as described previously (60, 61). Briefly, cell monolayers were washed once with ice-cold PBS-free M199 medium and three times with warm 1× PBS to remove residual PBS and any unbound protein from the culture medium. PBS containing 1 mg/ml EZ Link NHS-SS-Biotin (Pierce) was added to the monolayers, and the biotinylation reaction was carried out at room temperature for 30 min with gentle shaking. Following three washes with ice-cold 1× PBS, cells were solubilized in Laemmli sample buffer. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. To detect biotinylated cell surface proteins, immunoblot analysis was performed using HRP-conjugated ExtrAvidin (Sigma).

Thrombin Generation—Thrombin generation studies were conducted with either control pooled plasma from healthy adults or factor VII-deficient plasma (Affinity Biologicals). Factor VII levels in the deficient plasma were <1%, according to the manufacturer's instructions. In the thrombin generation assay, plasmas were defibrinated using arvin and total amidolytic activity of thrombin generated on cell surfaces was measured as previously described (62, 63). Cell monolayers in 24-well plates were placed on a Thermolyne dri-bath set at 37°C. After washing twice with 1 ml of aceta/berb/saline (ABS) buffer (0.036 mM sodium acetate, 0.036 mM sodium diethylbarbiturate, 0.145 mM NaCl, pH 7.4), monolayers were incubated for 3 min with 100 μl of ABS buffer and 200 μl of defibrinated plasma, in the absence or presence of 10% activated partial thromboplastin time (APTT) reagent (Reagen Teknika Corp., Durham, NC). At time periods up to 30 min following the addition of 100 μl of 40 μM CaCl2 in ABS buffer, 25-μl aliquots of the reaction mixture were removed and mixed with 475 μl of 5 μM S-2238. EDTA samples were also used to measure the concentrations of thrombin, thrombin-antithrombin (TAT) complexes and thrombin-heparin cofactor II complexes. Prothrombin, TAT, and thrombin-heparin cofactor II complexes were assayed using commercially available ELISA kits (Affinity Biologicals). Because thrombin bound to α2-macroglobulin (α2M) retains its enzymatic activity (64), the contribution of thrombin-α2M to total thrombin activity was measured as previously described (65). Briefly, the amidolytic activity of total thrombin was measured as described above, except that the 25-μl reaction mixture taken at each time point was incubated with 3.5 μl of...
Prevention of TF PCA by GRP78/BiP

0.15 M NaCl containing 0.25 units of standard heparin and 0.042 units of antithrombin (to inhibit any free thrombin) for 1 min on ice, followed by the addition of 475 μl of 5 mM EDTA. αM-dependent thrombin activity was then subtracted from the total thrombin activity to give the amount of physiologically active free thrombin generated by the cell surface.

Prothrombin Consumption—EDTA samples were used to determine prothrombin consumption during the experiments. Prothrombin concentra-
tions were determined for each time point during the thrombin generation experiments using a commercially available ELISA kit. Control plasma with a known concentration of prothrombin was used as a standard.

Factor VII Activation Assay—Factor VIIa generation on the surface of T24/83 cells was performed by a one-step assay, which directly measures the conversion of factor VII to factor VIIa, using the chromogenic substrate S-2288 (65). Cell monolayers were washed twice in ABS buffer and incubated for time periods up to 30 min in the absence (blank control) or presence of 108 nM recombinant human factor VII (Enzyme Research Laboratories, South Bend, IN) and 5 mM Ca2+ in ABS buffer. The generation of factor VIIa was assessed by subsampling cell surface supernatant into S-2288, followed by incubation at 37 °C for 30 min. After termination with 50% acetic acid, absorbance at 405 nm was determined for each sample. The rates of factor VIIa formation in the various samples were calculated based on a standard curve using known amounts of human factor VIIa (Enzyme Research Laboratories) and TF (Thromborel S, Dade Behring, Newark, DE).

Cell Surface Tissue Factor Activity Assay—Cell surface TF activity, measured as the amount of factor Xa generated, was performed as described previously (66, 67). Briefly, cells were seeded onto 24-well tissue culture plates and upon reaching confluence, the culture medium was removed, and the cells were washed once with 1× PBS and incubated in FBS-free medium for 1 h at 37 °C. Cells in FBS-free medium were untreated or treated with ionomycin (5–50 μM) for 5 min at 37°C. After termination with 50% acetic acid, and the absorbance at 405 nm was measured using a microplate reader. Standards containing the reaction mixture and varying amounts of rabbit brain thromboplastin (Thromboplastin C Plus, Dade Behring) were also prepared and incubated with S-2285 as described above. The absorbance at 405 nm was determined and used to generate a standard curve where 100 units of TF activity was defined as the amount of activity in 1 μl of rabbit brain thromboplastin. Cells in the 24-well plates were lysed in RIPA buffer, and the amount of total TF activity was measured using the 1C Protein Assay (Bio-Rad) according to the manufacturer’s instructions. Absorbances were measured at 750 nm and the amount of total protein calculated by comparing the OD750 with reference standards of Bio-Rad Protein Assay Standard II. Cell surface TF PCA was calculated as the amount of factor Xa generated per μg of total protein (units/μg).

Adenoviral Infection—The construction and large scale preparation of recombinant adenoviral vectors expressing GRP78/BiP (AdV-GRP78) or β-galactosidase (AdV-β-Gal) were performed essentially as previously described (68). Wild-type T24/83 cells or HASMC were grown in appropriate medium until reaching 70% confluence. Cells were washed once with 1× PBS and infected with either AdV-GRP78 or AdV-β-Gal at multiplicities of infection (m.o.i.) of 100, 300, or 500 pfu/cell, in FBS-free medium for 30 min at 37 °C. Cells were incubated in medium containing FBS for 72 h at 37 °C. Media was removed, and cells were washed once in 1× PBS prior to cell surface TF PCA assay or lysis preparation for immunoblotting, as described above.

Statistical Analysis—Data represent the mean ± S.E. Significance of differences between control and GRP78/BiP-overexpressing cells was determined by analysis of variance (ANOVA). On finding significance with ANOVA, unpaired Student’s t test was performed. For all analyses, p < 0.05 was considered significant.

RESULTS

Previous studies have demonstrated that non-stimulated T24/83 cells constitutively express both TF mRNA and antigen, which correlate with an increase in cell surface TF levels and activity (69–71). TF PCA in unstimulated T24/83 cells is significantly greater than other cells lines, including human umbilical vein endothelial cells and unstimulated monocytes (69, 70). Furthermore, TF expression in T24/83 cells was increased by phorbol myristate acetate, a known inducer of TF mRNA and protein in a wide variety of cultured cells (69). Based on these findings and given the important role of TF in blood coagulation and thrombosis, T24/83 cells were selected as our model system to study the effect of GRP78/BiP overexpression on TF-dependent thrombin generation.

Stable Overexpression of GRP78/BiP in T24/83 Cells—T24/83 cells were transfected with either the pcDNA3.1(+)–GRP78/BiP expression vector or pcDNA3.1(+) alone and stable transfectants selected in complete medium containing G418. Total cell lysates isolated from wild-type or stably transfected T24/83 cells were analyzed for GRP78/BiP protein by immunoblot analysis using an anti-KDEL monoclonal antibody, which recognizes both human GRP78/BiP and GRP94. As shown in Fig. 1A, two independently isolated G418-resistant cell lines, c1 and c2 (designated T24/83-GRP78c1 and c2, respectively) exhibited a 3.3-fold increase in GRP78/BiP protein levels, compared with wild-type or vector-transfected cells. In contrast, GRP94 was unchanged in all cell lines, suggesting that alterations in GRP78/BiP protein levels do not alter endogenous GRP94 protein levels. To compare the cellular levels and distribution of GRP78/BiP protein, wild-type, vector-transfected, or GRP78/BiP-overexpressing cells cultured on coverslips were fixed, permeabilized, and examined by indirect immunofluorescence using anti-GRP78/BiP polyclonal antibodies. In both wild-type and GRP78/BiP-overexpressing cells, GRP78/BiP was concentrated in the perinuclear region, consistent with its location in the ER (Fig. 1B). However, the fluorescence intensity was much greater in the GRP78/BiP-overexpressing cells, compared with wild-type and vector-transfected cells. Nonspecific immunostaining was not detected in cells in which the primary antibody had been omitted or in which preimmune mouse IgG was substituted for the primary antibody (data not shown). In terms of cellular function, overexpression of GRP78/BiP suppressed the induction of endogenous GRP78/BiP mRNA levels and increased survival of T24/83 cells exposed to the ER stress-inducing agent, A23187 (data not shown), findings consistent with those observed in GRP78/BiP-overexpressing CHO cells (7).

GRP78/BiP protein levels present in the stable GRP78/BiP-overexpressing cell lines were comparable to levels observed in wild-type cells treated with known ER stress-inducing agents, including homocysteine, dithiothreitol, and tunicamycin (Fig. 1C). This observation suggests that the levels of GRP78/BiP observed in these stable cell lines are within the range that can be attained under physiological conditions of ER stress. However, unlike the GRP78/BiP-overexpressing cell lines, cells treated with ER stress-inducing agents had an expected increase in GRP94 protein levels.

Overexpression of GRP78/BiP Decreases Thrombin Generation, Prothrombin Consumption, and the Formation of Thrombin-Inhibitor Complexes on the Surface of T24/83 Cells—Thrombin generation, prothrombin consumption, and formation of thrombin-inhibitor complexes were determined using normal defibrinated human plasma. In control plasma, after the addition of Ca2+, the concentration of physiologically active free thrombin generated on the surface of wild-type or vector-transfected cells increased significantly between 2 and 22 min (p < 0.001), with peak concentrations reaching 110 ± 16 and 131 ± 2 nM, respectively, by 4 min (Fig. 2A). In contrast, the concentration of free thrombin generated on the surface of the stable GRP78/BiP-overexpressing cell lines was negligible for all time points examined, up to 25 min. Consistent with these findings, both prothrombin consumption (Fig. 2B) and formation of thrombin-inhibitor complexes (Table I) were signifi-
cantly reduced in the stable GRP78/BiP-overexpressing cells, compared with wild-type or vector-transfected cells ($p < 0.001$).

**Overexpression of GRP78/BiP Inhibits TF-dependent Factor VII and Factor X Activation**—Factor VII-depleted plasma was used to initially determine whether GRP78/BiP overexpression may have selectively impaired a component of the extrinsic pathway such as TF. Thus, in the presence of factor VII-depleted plasma, free thrombin generation and prothrombin consumption were negligible for all cell lines over the 25-min time period (data not shown), a result consistent with the requirement of factor VIIa for TF-dependent thrombin generation (62–65). To specifically examine TF PCA on cell surfaces, wild-type, vector-transfected, or GRP78/BiP-overexpressing cells were grown to confluency and factor Xa generation was measured over 30 min using the chromogenic substrate S-2765. As shown in Fig. 3A, the rate of factor Xa generation was significantly decreased in the GRP78/BiP-overexpressing cells, compared with wild-type or vector-transfected cells ($p < 0.001$)
pared with wild-type and vector-transfected cells ($p < 0.005$).

To determine whether GRP78/BiP overexpression directly impaired the conversion of factor VII to VIIa at the cell surface by TF, factor VIIa generation was measured over 30 min using the chromogenic substrate S-2288. As shown in Fig. 3B, the rate of factor VIIa generation on the surface of GRP78/BiP-overexpressing cells was significantly decreased, compared with wild-type and vector-transfected cells ($p < 0.005$). The observation that the amidolysis of S-2288 was negligible in the absence of factor VII by 30 min indicates that amidolysis of the substrate correlates with the generation of factor VIIa and is not due to the presence of other cellular factors known to hydrolyze the substrate (i.e. tissue plasminogen activator, kallikrein).

To ensure that the PCA measured was mediated by TF, control experiments measuring the amount of factor Xa generated were performed on wild-type T24/83 cells. Negligible factor Xa was generated on the cell surface upon exclusion of factor VIIa from the reaction, indicating that the generation of factor Xa (which corresponds to PCA) was factor VIIa-TF dependent (Fig. 4A). Furthermore, the addition of increasing concentrations of a neutralizing anti-human TF antibody prior to measuring PCA decreased the amount of factor Xa generated in a dose-dependent manner, with complete inhibition occurring at 10 μg/ml (Fig. 4B). No inhibition was observed using rabbit IgG (data not shown). These findings are consistent with previous published data that PCA on the surface of T24/83 cells is TF-dependent (69, 70) and provides strong evidence that the generation of factor Xa was mediated exclusively by TF.

**Localization of TF on the Cell Surface of T24/83 Cells—**To determine whether alterations in TF PCA were the result of changes in the cellular levels of TF, both non-permeabilized (cell surface) and permeabilized (total) T24/83 cells were examined by indirect immunofluorescence using an anti-human TF monoclonal antibody (Fig. 5). Non-permeabilized cells were characterized by clusters of TF in defined patches distributed over the cell surface (Fig. 5, A–C). The intensity of TF staining was not decreased in the GRP78/BiP-overexpressing cells (Fig. 5C), compared with wild-type (Fig. 5A) or vector-transfected (Fig. 5B) cells. Permeabilized cells exhibited both cell surface and perinuclear staining of TF, a finding consistent with previous studies (72). Again, the intensity of staining was not decreased in the GRP78/BiP-overexpressing cells (Fig. 5F), compared with wild-type (Fig. 5D) or vector-transfected (Fig. 5E) cells. Nonspecific immunostaining was not detected in cells in which the primary antibody had been omitted or in which preimmune mouse IgG was substituted for the primary antibody (data not shown). Consistent with these findings, immunoprecipitation experiments did not show a decrease in cell surface TF on GRP78/BiP-overexpressing cells, compared with wild-type or vector-transfected cells (Fig. 6A).

**Effects of GRP78/BiP Overexpression on Cell Surface Protein Levels—**To determine whether GRP78/BiP overexpression mediates the levels of other cell surface proteins, intact wild-type, vector-transfected, or GRP78/BiP-overexpressing cells were labeled using NHS-SS-Biotin, a membrane impermeable form of biotin. Following biotinylation, cells were washed in 1× PBS, and total cell lysates were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and biotinylated proteins detected using HRP-conjugated ExtrAvidin. As shown in Fig. 6B, there was no significant difference in the migration pattern or intensity of staining of cell surface biotinylated proteins among the different cell types (lanes 4–8). No staining was observed in the lysates from cells that were not pretreated with NHS-SS-Biotin (lanes 1–4). Consistent with these findings, the levels of insulin receptor, caveolin-1, and Fas were not altered among the different cell lines (data not shown).

**Overexpression of GRP78/BiP Attenuates Ionomycin- or Hydrogen Peroxide-induced TF PCA—**Previous studies have demonstrated that increases in cytosolic Ca$^{2+}$ levels by treatment with the Ca$^{2+}$ ionophores, ionomycin or A23187, increases TF PCA, at a rate independent of de novo protein synthesis (47–50). Treatment of cells with hydrogen peroxide also increases TF PCA, which is independent of an increase in TF protein levels (42, 46). Given that GRP78/BiP is a Ca$^{2+}$-binding protein and protects cells from oxidative stress (5–7), the effects of ionomycin and hydrogen peroxide on cell surface TF PCA were examined. Factor Xa generation was significantly increased in all cell lines exposed to ionomycin or hydrogen peroxide, compared with untreated cells (Fig. 7). However, the absolute in-

![Fig. 3](https://www.jbc.org/content/17442/4/2692/F3.large.jpg)
crease in TF PCA upon ionomycin (Fig. 7A) or hydrogen peroxide (Fig. 7B) treatment was significantly lower ($p < 0.05$) in the GRP78/BiP-overexpressing cells, compared with wild-type and vector-transfected control cells. These findings provide evidence that GRP78/BiP overexpression inhibits the stimulation of TF PCA by agents that are known to increase intracellular Ca$^{2+}$ or oxidative stress.

Adenovirus-mediated GRP78/BiP Overexpression Inhibits TF PCA Induced by Viral Infection—To confirm the effect of stable overexpression of GRP78/BiP on TF PCA, an additional approach using adenovirus to overexpress GRP78/BiP was performed. A recombinant adenovirus containing the complete human GRP78/BiP open reading frame under the control of the constitutively expressed CMV promoter (AdV-GRP78) was constructed. As shown in Fig. 8A, GRP78/BiP protein levels were increased $\sim 3$-fold in T24/83 cells and HASMC following infection with AdV-GRP78, compared with infection with the control virus expressing β-galactosidase (AdV-β-Gal) or uninfected (no virus) cells. In addition, adenovirus-mediated overexpression of GRP78/BiP was not associated with a discernable change in cell surface TF protein levels, compared with uninfected cells or cells infected with AdV-β-Gal (Fig. 8B). Adenovirus infection did not significantly affect cell proliferation or cell death (data not shown).

Given that adenovirus infection has been shown to increase TF PCA (33), the effect of GRP78/BiP overexpression on adenovirus-mediated TF PCA was examined. Wild-type T24/83 cells or HASMC were infected with AdV-GRP78 or AdV-β-Gal and TF PCA was determined. T24/83 cells (Fig. 9A) and HASMC (Fig. 9B) infected with AdV-β-Gal exhibited an increase in TF PCA, compared with uninfected cells, a finding consistent with previous studies showing that adenoviral infection increases TF PCA (33). In contrast, TF PCA was not increased in cells infected with AdV-GRP78. Therefore, in addition to attenuating TF PCA stimulated by ionomycin or hydrogen peroxide, GRP78/BiP overexpression also decreases adenovirus-mediated TF PCA.

**FIG. 4.** Procoagulant activity on T24/83 cells is specific for TF. Wild-type T24/83 cells were incubated in the absence of factor VIIa (panel A) or with increasing concentrations of TF neutralizing antibody (panel B) prior to the determination of amount of factor Xa generated, using the chromogenic substrate S-2765. Cell surface TF PCA was measured as the amount of factor Xa generated per microgram of total protein (units/μg). Data represent mean ± S.E. of three separate experiments. *, $p < 0.05$ versus wild-type cells incubated in the presence of factor VIIa (panel A) or the absence of TF neutralizing antibody (panel B) (0 μg/ml).

**FIG. 5.** Indirect immunofluorescence detection of TF in T24/83 cells. Wild-type (A and D), vector-transfected (B and E) or GRP78/BiP-overexpressing T24/83 cells (C and F) were grown on glass coverslips and immunostained with an anti-human TF monoclonal antibody without (panels A–C) or with (panels D–F) permeabilization using Triton X-100. Primary antibody was detected with a goat Alexa-conjugated anti-mouse IgG. Original magnification, ×1000.
overexpression on TF PCA in these different cell lines provides evidence for a general cellular mechanism.

**DISCUSSION**

TF-dependent thrombin generation plays a critical role in hemostasis after tissue injury and also in the pathogenesis of multiple thrombotic disorders associated with a wide range of diseases, including cardiovascular disease, sepsis and cancer (21–24,73). Despite its importance in coagulation and human disease, the cellular factors that regulate TF expression and/or activity are relatively unknown. In this report, we provide novel evidence that overexpression of GRP78/BiP in the prothrombotic cell line T24/83 prevents cell surface thrombin generation by inhibiting TF PCA. The observation that GRP78/BiP overexpression does not decrease cell surface TF suggests that the inhibition of TF PCA is not due to an impairment in the trafficking of TF through the ER. However, the ability of GRP78/BiP overexpression to attenuate TF PCA induced by ionomycin, hydrogen peroxide or viral infection supports a mechanism involving cellular Ca^{2+} and/or oxidative stress. These findings demonstrate that alterations in the expression of GRP78/BiP can have profound effects on the prothrombotic characteristics of cells.

Stable overexpression of GRP78/BiP does not cause a decrease in cell surface levels of TF in T24/83 cells. In fact, TF levels are somewhat increased in GRP78/BiP-overexpressing cells, compared with wild-type or vector-transfected cells. This finding is consistent with previous studies demonstrating that GRP78/BiP overexpression increases macrophage colony-stimulating factor (MCSF) expression and secretion in CHO cells (7, 20). Since overexpression of GRP78/BiP can inhibit the secretion of factor VIII and vWF (7, 20), structural differences among proteins which affect their association with GRP78/BiP are likely to occur. Indeed, both factor VIII and vWF, but not MCSF, have been shown to stably associate with GRP78/BiP in the ER lumen (7, 20). Several lines of evidence suggest that GRP78/BiP does not stably associate with TF or directly alter its activity. First, immunoprecipitation experiments have failed to identify stable complexes between TF and GRP78/BiP in...
Prevention of TF PCA by GRP78/BiP

Fig. 8. Effect of AdV-GRP78 or AdV-β-Gal infection on GRP78/BiP and TF expression. A, wild-type T24/83 cells or HASMC were infected with adenovirus expressing GRP78/BiP (AdV-GRP78) or β-galactosidase (AdV-β-Gal) at 100, 300, or 500 pfu/cell for 72 h. Uninfected wild-type cells (T24/83 or HASMC) were used as a control. Total protein lysates were separated by SDS-PAGE under reducing conditions and immunoblotted with an anti-KDEL monoclonal antibody (lower panel). To control for protein loading, total protein lysates separated by SDS-PAGE were stained with Coomassie Blue (upper panel). The migration position of β-galactosidase in cells infected with AdV-β-Gal is denoted by the asterisk (upper panel). B, wild-type cells were infected with adenovirus expressing GRP78/BiP (AdV-GRP78) or β-galactosidase (AdV-β-Gal) at 300 pfu/cell for 72 h. Uninfected wild-type cells (T24/83 or HASMC) were used as a control. Cell surface TF was immunoprecipitated from wild-type or adenovirus infected cells using a rabbit anti-human TF polyclonal antibody, as described under Experimental Procedures.‘’ Immunoprecipitation of wild-type cells with preimmune rabbit IgG was used to control for nonspecific binding (rabbit IgG). The migration position of TF is shown by the arrowhead.

T24/83 cells. Second, the BiP Score program (13), which has been successfully used to predict BiP binding sites within antibodies and HIV gp160 (13–15), indicates a low probability of TF-GRP78/BiP association. Third, TF PCA in rabbit brain thromboplastin is not inhibited by recombinant GRP78/BiP. Taken together, these findings demonstrate that GRP78/BiP chaperone function is not required for the folding-processing of all proteins that transit the ER. However, given that GRP78/BiP has been shown to bind a number of proteins, GRP78/BiP could potentially regulate TF PCA through its interactions with other proteins/factors, the identities of which are as yet unknown.

The observation that GRP78/BiP overexpression decreases TF PCA without a corresponding decrease in cell surface TF levels may be partly explained given that TF expression does not necessarily correlate with activity. Tissue factor expression and activity are considered to be independently regulated by a two-step activation pathway in which certain cellular factors (such as lipoproteins) regulate synthesis of latent TF while other factors (such as ROS) mediate post-translational modifications of existing cell surface TF to an active form (42, 46). Based on our findings, it is unlikely that a mechanism involving increased TF synthesis or impaired trafficking of TF through the ER is responsible for a decrease in TF PCA. This

would imply that GRP78/BiP overexpression indirectly alters TF PCA through a mechanism independent of its chaperone activity. In support of this concept, GRP78/BiP overexpression attenuates TF PCA induced by ionomycin, suggesting that alterations in ER Ca2+ stores play an important role in the regulation of TF PCA by GRP78/BiP. Given that GRP78/BiP is a major Ca2+-binding protein (10, 11) and that TF PCA is mediated by changes in intracellular levels of free Ca2+ (47–50), overexpression of GRP78/BiP could potentially inhibit TF PCA indirectly by sequestering intracellular Ca2+. Although it is not completely understood as to how alterations in intracellular Ca2+ increase TF PCA, it may involve the production of ROS. It is well established that efflux of Ca2+ from the ER enhances the peroxidase activity of cyclooxygenases and lipoxygenases, thereby leading to the production of ROS (74). Furthermore, elevated levels of intracellular Ca2+ lead to mitochondrial Ca2+ uptake, mitochondrial dysfunction and the generation of ROS (5, 6, 74). Oxidative stress has been shown to increase TF PCA (42, 46), suggesting that changes in intracellular Ca2+ could indirectly alter TF PCA through the generation of ROS. This mechanism is also supported by findings that in contrast to ionomycin, thapsigargin, an inhibitor of the ER Ca2+-ATPase, does not significantly increase TF PCA and

2 L. M. Watson and R. C. Austin, unpublished results.
induces a lower intracellular Ca\textsuperscript{2+} response than ionomycin.\textsuperscript{3} The observation that GRP78/BiP overexpression attenuates TF PCA induced by both ionomycin and hydrogen peroxide also suggests a link between intracellular Ca\textsuperscript{2+} and oxidative stress. In addition, GRP78/BiP overexpression could potentially limit the accessibility of anionic phospholipids essential for TF PCA. Exposure of anionic phospholipids on the outer plasma membrane increases TF PCA (47, 66, 75, 76) and is regulated by Ca\textsuperscript{2+} levels (47, 75, 77). Therefore, modulation of any component/cofactors involved in this pathway by GRP78/BiP could ultimately attenuate TF PCA.

Infection of cultured human vascular endothelial cells with adenovirus, as well as other respiratory viruses, increases TF PCA and expression (33). Consistent with these findings, we demonstrated that infection of T24/83 cells and HASMC with a recombinant adenovirus expressing β-galactosidase increases TF PCA in a dose-dependent manner. However, TF PCA did not increase in T24/83 cells or HASMC infected with a recombinant adenovirus expressing GRP78/BiP. Given that viral infection could potentially increase intracellular Ca\textsuperscript{2+} concentration (74), which could lead to the production of ROS, the inhibitory effect of GRP78/BiP on TF PCA induced by viral infection further supports a mechanism involving Ca\textsuperscript{2+} and oxidative stress. This mechanism is also suggested by the observation that adenovirus-mediated delivery of GRP78/BiP inhibits a rise in intracellular Ca\textsuperscript{2+} caused by hydrogen peroxide, thereby protecting neuronal cells from hydrogen peroxide-mediated cell death (78).

On the basis of our findings, is there a plausible biological link between GRP78/BiP overexpression and TF PCA? Numer-

\textsuperscript{3} L. M. Watson, J. G. Dickhout, and R. C. Austin, unpublished results.
ous agents and/or conditions, including viral infection, Ca\textsuperscript{2+} ionophores and homocysteine, that activate TF expression and activity can also induce ER stress (9). The ability of ER stress to increase TF expression and activity could involve the activation of NFκB. It has been reported that ER stress activates NFκB through a mechanism involving release of Ca\textsuperscript{2+} from the ER and the subsequent generation of ROS (74). This is consistent with previous studies demonstrating that TF PCA is increased by elevations in intracellular Ca\textsuperscript{2+} and oxidative stress (42, 46–50). However, the induction of GRP78/BiP, as well as other ER-resident molecular chaperones, is considered to be a protective mechanism elicited by cells to alleviate the adverse effects of ER stress (58, 59). Based on our findings, GRP78/BiP overexpression may thus impair TF PCA in an attempt to reduce the thrombotic potential often associated with ER stress agents/conditions.

In summary, we have provided novel evidence that overexpression of GRP78/BiP can inhibit TF-mediated thrombin generation at the cell surface. Because TF is a primary determinant of the thrombogenicity of human atherosclerotic plaques, the ability to inhibit TF PCA by modulating GRP78/BiP protein levels could potentially alleviate many TF-dependent pathological conditions, including myocardial infarction and acute arterial injury.

Acknowledgments—We thank Drs. Jack Hirsh and Jeffrey Weitz for valuable comments throughout the course of this study and during preparation of the article. We also thank Duc Ngo for the production and purification of recombinant adenoviral stocks.

REFERENCES