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Transcription Factor TFII-I Causes Transcriptional Upregulation of GRP78 Synthesis in Prostate Cancer Cells

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ABSTRACT

Receptor-recognized forms of α_2-macroglobulin (α_2M^*) bind to cell surface-associated GRP78 and induce proliferative and survival signaling in prostate cancer cells. As part of the cellular response to α_2M^*, GRP78 expression is itself upregulated. In response to other stimuli, the transcription factor TFII-I upregulates GRP78 by binding to its gene promoter. We have, therefore, studied the role of TFII-I in transcriptional upregulation of GRP78 in 1-LN human prostate cancer cells stimulated with α_2M^*. This treatment caused a two- to threefold increase in TFII-I and GRP78 synthesis from [^{35}S]-labeled precursor amino acids. Synthesis of both TFII-I and GRP78 were significantly reduced by silencing *TFII-I* gene expression or pretreatment of cells with genistein or actinomycin D. Confocal microscopy was employed to demonstrate relocation of TFII-I to the nucleus. In α_2M^*-stimulated cells, moreover, TFII-I bound to the *GRP78* promoter as determined by CHIP assay. We also demonstrate binding of TFII-I to the *c-fos* promoter, consistent with its role in upregulating *c-fos* gene expression. In non-lymphoid cells, phosphorylated c-Src is an activator of TFII-I. Ligation of GRP78 on 1-LN cells with α_2M^* was followed by tyrosine phosphorylation of c-Src as well as TFII-I. We conclude that α_2M^*-induced increase in GRP78 synthesis is caused by transcriptional upregulation of TFII-I which binds to the *GRP78* promoter and thus potentiates its cell survival and antipoptotic functions in 1-LN prostate cancer cells. J. Cell. Biochem. 106: 381–389, 2009.

KEY WORDS: A_2-MACROGLOBULIN AND SIGNAL TRANSDUCTION; TFII-I AND GRP78 REGULATION; CELL SURFACE-ASSOCIATED GRP78; CHIP ASSAYS; TRANSCRIPTIONAL REGULATION; GENE SILENCING

The glucose-regulated protein of 78 kDa (GRP78) is a biological sensor of endoplasmic reticulum (ER) stress caused by a variety of patho-physiological conditions which trigger the unfolded protein response (UPR) and anti-apoptotic signaling [Hendershot, 2004; Lee, 2007]. GRP78 is involved in translocation of newly synthesized polypeptides across the ER membrane and their subsequent folding, maturation, transport, or retrotranslocation. In addition to its role as an ER chaperone, GRP78 is located on the cell surface of various cell types where it functions as a co-receptor for viruses and the major histocompatibility complex class I antigen presentation system as well as the signaling receptor for the activated forms of the proteinase inhibitor α_2-macroglobulin (α_2M^*) [Chu et al., 1994; Misra et al., 2002; Misra and Pizzo, 2008]. Activation by α_2M^* of a receptor complex consisting of GRP78, MTJ-1, Gαq11, and other accessory factors on the surface of murine peritoneal macrophages, and human 1-LN prostate cancer cells triggers pro-proliferative and anti-apoptotic behavior [Misra et al., 2006; Misra and Pizzo, 2008]. The circulating concentration of α_2M is about 2–5 μM, and its proteinase-activated form may comprise approximately 200–500 nM of this pool [Chu et al., 1994]. This is well above the K_d of 50–100 pM for α_2M^* binding and activation of GRP78 [Misra et al., 2002, 2006]. In addition, prostate specific antigen, a proteinase produced by prostate cancer cells, binds readily to α_2M converting it to α_2M^* [Misra et al., 2006]. Aggressive prostate cancers also produce matrix metalloproteinases, which convert α_2M to α_2M^* and may thus expand the α_2M^* pool. Autoantibodies against GRP78 are present in the sera of prostate cancer patients and they are a biomarker of aggressive behavior and

Abbreviations used: α_2M, α_2-macroglobulin; α_2M^*, the receptor recognized forms derived from either amine or proteinase activator; TFII-I, transcription factor II-I; GRP78, 78 kDa glucose-regulated protein; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-related kinase; CHIP, chromatin immunoprecipitation; HHBSS, Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4, and 3.5 mM $NaHCO_3$; ER, endoplasmic reticulum; UPR, unfolded protein response; ECF, enhanced chemofluorescence.

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poorer survival [Mintz et al., 2003; Gonzalez-Gronow et al., 2006]. These autoantibodies bind to the same site on GRP78 as α_2M^* and they function as receptor agonists triggering signaling cascades comparable to the natural ligand [Gonzalez-Gronow et al., 2006]. The GRP78 promoter contains multiple copies of the ER stress response element, which is the primary regulatory element for the ER stress mediated transcriptional regulated synthesis of GRP78 [Yoshida et al., 1998; Roy and Lee, 1999]. Under ER stress, several transcription factors including transcription factor TFII-I interact with ER stress response elements of the *GRP78* promoter and cause transcriptional upregulation of its synthesis [Parker et al., 2001; Roy, 2001, 2006; Hong et al., 2005].

Transcription factors serve to direct signal transduction pathways to cell-type specific genes and thereby provide a molecular link between signal transduction and growth, proliferation, or the developmental program in a given cell. Many extracellular signals mediated through cell surface receptors, enhance tyrosine phosphorylation and increase the transcriptional activity of TFII-I [Parker et al., 2001; Roy, 2001, 2006; Hong et al., 2005]. In non-lymphoid cells, TFII-I is phosphorylated by c-Src [Roy, 2001, 2006; Cheriyath et al., 2002]. A variety of sequence-specific DNA transcriptional activators are also substrates for MAPKs [Roy, 2001, 2006; Cheriyath et al., 2002]. Thus TFII-I functions as a signaling molecule for the activation of the *c-fos* promoter by integrating signals from both tyrosine kinases and the MAPK pathway [Kim et al., 1998; Parker et al., 2001; Roy, 2001, 2006; Cheriyath et al., 2002; Hong et al., 2005]. Recently, we have reported that binding of α_2M^* to cell surface-associated GRP78 in human 1-LN prostate cancer cells upregulates the levels of GRP78 and various components of UPR signaling [Misra et al., 2006]. To understand the mechanism(s) by which α_2M^* increases GPR78 levels in 1-LN prostate cancer cells, we have examined the role of TFII-I, which causes transcriptional upregulation of GRP78 under ER stress conditions. In addition, since TFII-I binds to the *c-fos* promoter, we also examined the upregulation of this gene. We show that treatment of 1-LN prostate cancer cells with α_2M^* causes induction of TFII-I, its activation, and its binding to the GRP78 and the *c-fos* promoters.

EXPERIMENTAL PROCEDURES

MATERIALS

α_2M^* was prepared as previously described [Misra et al., 2006]. Culture media were purchased from Invitrogen (Carlsbad, CA). Antibodies against the rabbit COOH-terminal domain of GRP78 were from Nventa Biopharmaceuticals Corp. (San Diego, CA). Antibodies against TFII-I, c-fos, and c-Src were purchased from Santa Cruz Biotechnology, Inc. (San Diego, CA). Antibodies against phosphorylated and unphosphorylated ERK1/2, phosphorylated c-Src (Ty 527), and phosphorylated tyrosine were from Cell Signaling Technologies. Antibodies against phosphorylated c-fos (T325) were from Abcom (Cambridge, MA). Chromatin immunoprecipitation (CHIP) assay kit (Cat # 17–295) was from Upstate Cell Signaling Solutions (Charlottesville, VA). [S^{35}]-labeled protein ExpreS35S35 was purchased from Perkin Elmer (Boston, MA).

WESTERN BLOTTING OF GRP78 AND TFII-I IN α_2M^*-STIMULATED 1-LN PROSTATE CANCER CELLS

The highly metastatic prostate cancer cell line 1-LN was a kind gift from Dr. Philip Walther (Duke University Medical Center, Durham, NC). 1-LN cells in six well plates (500×10^3 cells/well) were allowed to grow to confluence in RPMI 1640 medium containing 10% FBS, penicillin (12.5 U/ml), streptomycin (6.5 μg/ml), 2 mM glutamine, and 10 nM insulin at 37°C in a humidified CO_2 incubator (5%). At about 90% confluence, the medium was aspirated, the monolayers washed with ice-cold Hepes-buffered Hanks' basic salt solution, pH 7.4, and a fresh volume of medium was added to the monolayers. The cells were treated with varying concentration of α_2M^* and incubated for varying time periods as above. The reactions were stopped by aspirating the medium and a volume of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 1% Nonidet p-40, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and leupeptin (20 μg/ml) was added. The cells were lysed for 10 min over ice, scraped into tubes, and centrifuged at 800*g* for 5 min at 4°C. Supernatants were collected, their protein contents determined [Bradford, 1976] and equal amount of protein electrophoresed according to Laemmli (10% gel). The immunoblotting of membranes with antibodies specific for GRP78 and TFII-I was performed according to manufacturer's instruction. The specificity of antibodies used was assessed by reactivity of the blotted antigen with non-immune serum. Under this condition, no reactivity was observed. The detection and quantification of immunoblots were performed by ECF and phosphorimaging. The membranes were reprobed for protein loading control actin [Misra et al., 2006].

GRP78 AND TFII-I SYNTHESIS FROM [^{35}S]-LABELED PRECURSOR AMINO ACIDS IN α_2M^*-STIMULATED 1-LN PROSTATE CANCER CELLS

Confluent 1-LN cells incubated as above in six well plates (4×10^6 cells/well) were labeled with ExpreS35S35 protein labeling mixture (Perkin Elmer) (200 μCi/ml) for 2 h at 37°C in a humidified CO_2 incubator (5%). The labeling was stopped by aspirating the medium, monolayers washed four times with cold HHBSS buffer, a volume of RPMI medium added, and cells incubated at 37°C for 5 min for temperature equilibration. To the respective wells, α_2M^* (50 pM or 100 pM) was added and the cells incubated as above for 20 or 30 min for ^{35}S incorporation into GRP78 and TFII-I respectively for the desired time periods. The reaction was stopped by aspirating the medium, a volume of the above described buffer added to each well, cells lysed for 10 min over ice, scraped into tubes, centrifuged for 5 min at 800*g* at 4°C, and protein contents of lysates determined [Bradford, 1976]. Equal amounts of lysate proteins were immunoprecipitated with either anti-GRP78 (1:50) or anti-TFII-I (1:50) antibodies at 4°C overnight with gentle rotation. The GRP78 and TFII-I immunoprecipitates were washed thrice with above cold lysis buffer by centrifugation at 3,000 rpm for 5 min at 4°C. Thirty microliters of 4× sample buffer was added to each immunoprecipitate, each sample boiled for 5 min, centrifuged for 4 min, and equal volume of supernatant electrophoresed (10% gel), transferred to membranes, and the membranes autoradiographed in an phosphorimager and quantified.

EFFECTS OF TFII-I GENE SILENCING BY SIRNA ON A α_2M^*-INDUCED GRP78 AND TFII-I SYNTHESIS FROM [^{35}S]-LABELED AMINO ACIDS PRECURSORS IN 1-LN CELLS

The sense (5′-UUG AAA GGA AAU AUG CUC AAC-3′) and the antisense (5′-UGA GCA UAU UUC CUU UCA AAC-3′) oligonucleotides against the target homologous gene sequence nucleotides (5′-GTT TGA AAG GAA ATA TGC TCA-3′) (Swiss Prot Entry Name GTF2I_Human Primary accession number P78347) were chemically synthesized and annealed by Ambion (Austin, TX). Other details of transfection of 1-LN cells with dsTFII-I dsRNA (50 nmol/48 h) and evaluation of the magnitude of transfection by estimating the mRNA levels of TFII-I by qualitative PCR and the protein levels of TFII-I and GRP78 by Western blotting were the same as described previously [Misra and Pizzo, 2007]. Protein levels of TFII-I in dsTFII-I RNA and GRP78 in transfected 1-LN cells were determined by Western blotting of cell lysates as described previously [Misra and Pizzo, 2007]. The details of determining the synthesis of GRP78 and TFII-I protein from [^{35}S]-labeled amino acid precursors in 1-LN cells transfected with dsTFII-I RNA were the same as described in the preceding sections. In experiments where the effects of inhibiting tyrosine kinases (genistein, 50 μM/3 h) or transcription inhibitor (actinomycin D, 10 μg/ml/10 min) was studied, these inhibitors were added before α_2M^* and protein synthesis was examined as described above.

TFII-I BINDING TO THE GRP78 AND C-FOS PROMOTERS BY CHIP ASSAY IN 1-LN CELLS TREATED WITH α_2M^*

The binding of TFII-I to the *GRP78* and *c-fos* promoters in α_2M^*-stimulated 1-LN prostate cancer cells was determined by CHIP assay as described previously [Misra and Pizzo, 2007. Briefly, 1-LN prostate cancer cells in six well plates (3×10^6 cells/well) were stimulated with α_2M^* (50 pM) in quadruplicate and incubated as above. The reaction was stopped by aspirating the medium, a volume of RPMI buffer added, and cells processed for determining the binding of TFII-I to the GRP78 and *c-fos* promoters by CHIP assay using a CHIP assay kit (Cat #17.295, Upstate Signaling Solutions). The sequence of the primers used for the GRP78 promoters were 5′-CAT TGG TGG CCG TTA GAA TGA CCAG-3′ (forward) and 5′-AGT ATC GAG CGC GCC GTC GC-3′ (reverse) yielding a 220-bp product. To assess the specificity of TFII-I binding to the *GRP78* promoter, a second scrambled sequence 5′-AAC CCT TCA CTG GTT TCT CT CATCC-3′ (forward) and 5′-TGC TGG AAT ATC CCA CAA TCA CAGG-3′ (reverse) was also used as the primer. The sequence of the primers used for the *c-fos* promoter were 5′-TTC TCT GTT CCG CTC ATG ACGT-3′ (forward) and 5′-CTT CTC AGT TGC TAG CTG CAA TCG-3′ (reverse). Amplified PCR products were resolved by 2.5% agarose gel electrophoresis and visualized by ethidium dibromide and photographed [Misra and Pizzo, 2007].

TYROSINE PHOSPHORYLATION OF TFII-I AND C-SRC IN α_2M^*-STIMULATED 1-LN PROSTATE CANCER CELLS

To determine the tyrosine phosphorylation of TFII-I and c-Src in 1-LN prostate cancer cells stimulated with α_2M^*, 1-LN cells (3×10^6 cell/swell) were stimulated with α_2M^* as above. The reaction was stopped by aspirating the medium. The cells were lysed in above described lysis buffer and their protein content determined [Bradford, 1976]. Equal amounts of cell lysate proteins (50 μg) were immunoprecipitated with anti-TFII-I antibodies (1:50) and anti-c-Src antibodies (1:50) respectively in presence of protein A-agarose and samples incubated at 4°C overnight with gentle rotation. The respective immunoprecipitates were washed in lysis buffer and processed for electrophoresis and immunoblotting for TFII-I and c-Src as described above. These immunoblots were reprobed for phosphotyrosine residues in TFII-I and c-Src proteins according to the instructions provided by the manufacturer (Amersham). These immunoblots were then reversed immunoblotted for phosphorylated tyrosine residues in TFII-I and c-Src.

NUCLEAR LOCALIZATION OF TFII-I, GRP78, PHOSPHORYLATED ERK1/2, AND C-FOS IN α_2M^*-STIMULATED 1-LN PROSTATE CANCER CELLS

To determine the nuclear localization of α_2M^*-induced activated TFII-I, ERK1/2, c-fos, and GRP78, 1-LN cells (3×10^6 cells/well in six well plates) were stimulated with α_2M^* (50 pM/15 min) as described above. The reaction was terminated by aspirating the medium and a volume of chilled homogenizing buffer containing 10 μM Tris–HCl (pH 7.3, 10 mM NaCl, 1 mM PMSF, 10 mM benzamidine, and leupeptin (20 μg/ml) was added to the cells on ice. After 10 min, the cells were scraped into tubes and homogenized in a Dounce homogenizer with 15 up-down strokes over ice. The homogenates were transferred into clean tubes and centrifuged at 800*g* for 10 min at 4°C. The pellet was suspended in HHBSS and layered over a 200 μl cushion of 50% sucrose in HHBSS and centrifuged at 14,000*g* in an Eppendorf microcentrifuge for 3 min at 4°C. The nuclear pellet was collected and lysed in a volume of buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 1% NP40, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and 10 μg/ml leupeptin [Misra et al., 2006]. The protein contents of nuclear lysates were determined [Bradford, 1976]. Purity of isolated nuclear fractions was evaluated by electron microscopy and enzyme assays as previously described and showed that nuclear fraction demonstrated no more than 3–7% contamination with other subcellular fractions [Misra et al., 2006]. Equal amounts of lysate proteins were electrophoresed, and immunoblotted for GRP78, tyrosine phosphorylated TFII-I, phosphorylated ERK1/2, and phosphorylated c-fos. Statistical analyses where indicated were done by student's *t*-test.

DETERMINATION OF NUCLEAR LOCALIZATION OF TFII-I IN 1-LN PROSTATE CANCER CELLS STIMULATED WITH α_2M^* BY CONFOCAL MICROSCOPY

1-LN prostate cancer cells ($2–3 \times 10^4$) in RPMI 1640 medium containing the additions listed above were pipetted onto glass coverslips in 35 mm dish and incubated overnight. The cells were then washed once with medium, a volume of the medium added, and the respective monolayers incubated with buffer or α_2M^* (100 pM/30 min). The reactions were terminated by aspirating the medium and monolayers washed thrice with chilled PBS containing 0.1% Triton X 100 (PBST) for 25 min at 37°C. The cells were fixed with formaldehyde (4%) in PBS for 25 min at 37°C. The fixed cells on coverslips were permeabilized with 0.5% Triton X 100 in PBS for 5 min at room temperature and washed thrice with chilled PBST. The

permeabilized cells were incubated with 0.1% BSA in PBST for blocking non-specific binding for 2 h at room temperature with rotation. The incubations were terminated by aspirating the medium and incubating the cells with rabbit polyclonal anti-TFII-I antibodies in 0.1% goat serum (1:100) overnight at 4°C with rotation. The incubations were terminated by washing the cells with chilled PBST thrice and the cells were then incubated with AlexFluor 568-labelled goat anti-rabbit IgG (1:100) for 2 h at room temperature. The cells were washed thrice with chilled PBST (10 min each time) with rotation and coverslips mounted on glass slides and sealed with nail polish for confocal microscopy. For controls, buffer treated, fixed, and permeabilized cells were incubated with non-specific rabbit polyclonal antibodies as above and cells processed for confocal microsocpy. Samples were analyzed on a Zeiss LSM 410 confocal microscope. Images were then processed in AdobePhotoshop (S) [Misra et al., 2008].

RESULTS

α_2M^*-INDUCES TFII-I PROTEIN EXPRESSION IN 1-LN CANCER CELLS

α_2M^* binds to cell surface-associated GRP78 causing activation of the Ras/MAPK and PI-3 kinase signaling which promotes cellular proliferation and cell survival [Misra et al., 2006]. α_2M^*-stimulated 1-LN cells also demonstrate increased GRP78 protein expression which is significantly reduced by prior treatment with inhibitors of tyrosine kinases and the Ras/MAPK kinase signaling pathway [Misra et al., 2006]. Recent studies demonstrate that TFII-I is an important regulator of the stress response pathway [Kim et al., 1998; Parker et al., 2001; Roy, 2001, 2006; Cheriyath et al., 2002; Hong et al., 2005]. We first studied the induction of TFII-I protein expression and its synthesis from [^{35}S]-labeled amino acid precursors in α_2M^*-stimulated 1-LN cells and then its role in α_2M^*-induced increase in GRP78 expression. Stimulation of cells induced a two- to threefold increase in TFII-I and GRP78 protein expression on treatment with α_2M^* in a concentration- and time-dependent fashion (Fig. 1A,B). The maximum increase in protein levels of both TFII-I and GRP78 occurred at 15–20 and 30–60 min of incubation with 50–100 pM of α_2M^*, respectively (Fig. 1A,B).

INDUCTION OF TFII-I AND GRP78 SYNTHESIS IN α_2M^*-STIMULATED 1-LN CELLS AND THE EFFECT OF SILENCING TFII-I GENE EXPRESSION BY RNAI

To understand the mechanism of α_2M^*-induced increase in GRP78 and TFII-I proteins we quantified by Western blotting the synthesis of GRP78 and TFII-I from [^{35}S]-labeled amino acids in 1-LN cells stimulated with α_2M^* (Fig. 1C). Ligation of cell surface GRP78 with α_2M^* caused about a twofold increase in [^{35}S] incorporation into TFII-I and about a 1.5-fold increase in [^{35}S] incorporation into GRP78 compared to buffer stimulated cells (Fig. 1C). α_2M^*-induced synthesis of TFII-I and GRP78 proteins, as determined by the [^{35}S] incorporation, was greatly reduced by silencing *TFII-I* gene expression while α_2M^*-treatment caused negligible effects in cells transfected with scrambled dsRNA (Fig. 1C). Using the stable cell lines NIH3T3, SWISS3T3, and 293 T with suppressed TFII-I levels, Hong et al. [2005] have also reported a requirement of TFII-I for

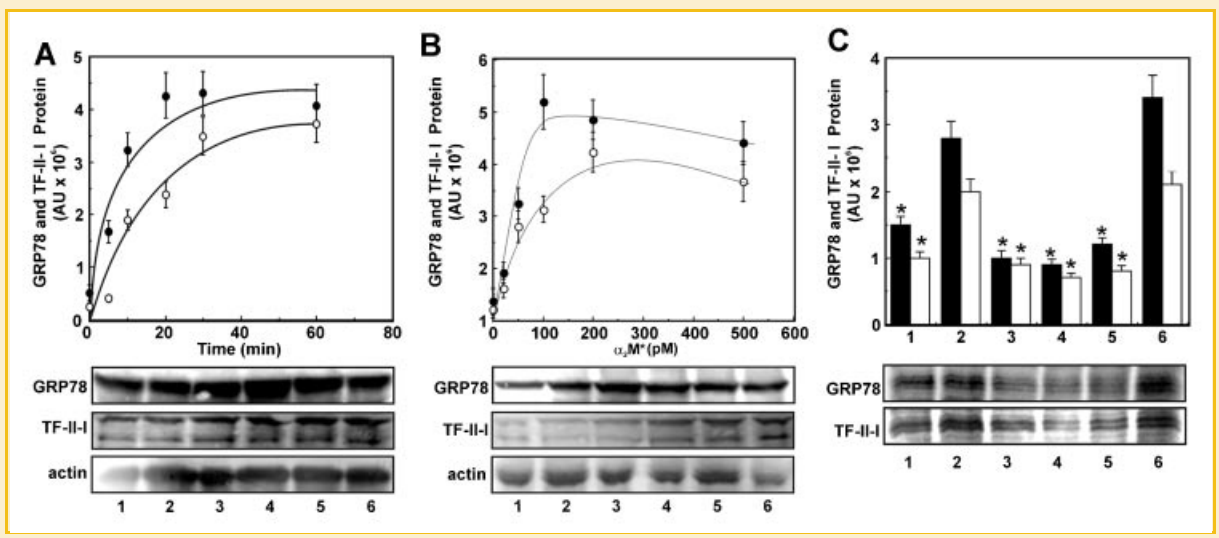

Fig. 1. α_2M^*-induced upregulated expression of TFII-I and GRP78 protein in 1-LN cells. Panel A: Effect of time of incubation of 1-LN cells with α_2M^* (50 pM) on expression of TFII-I (○) and GRP78 (●) protein. Changes in protein levels are expressed in arbitrary units ($\times 10^6$) and are the mean ± SE from three experiments in each group. Representative immunoblots of TFII-I and GRP78 protein and the protein loading control actin are shown below the graph. Panel B: Effect of incubating 1-LN cells with varying concentration of α_2M^* on expression of TFII-I (○) and GRP78 (●) protein. Changes in protein levels are expressed in arbitrary units ($\times 10^6$) and are mean ± SE from three experiments in each group. Representative immunoblots of TFII-I and GRP78 as well as of protein loading control actin is shown below the bar graph. Panel C: TFII-I (□) and GRP78 (■) synthesis and its modulation in 1-LN cells treated with: (1) buffer; (2) α_2M^* (50 pM); (3) genistein (50 μm/3 h) then α_2M^*; (4) dsTFII-I RNA then α_2M^*; (5) actinomycin D (10 μg/ml/10 min) then α_2M^*; and (6) scrambled dsRNA then α_2M^*. Changes in [S^{35}] incorporation into respective protein is expressed as percent change over buffer treated cells (which is considered 100%), and are expressed as the mean ± SE from three experiments performed in duplicate. Representative autoradiograph is shown below the bar diagram. *Significantly different from α_2M^* treated controls at the 5% level.

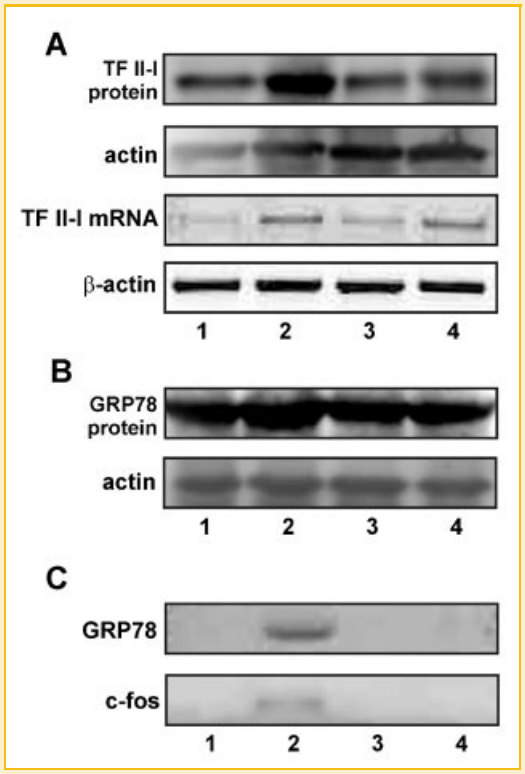

Fig. 2. TFII-I mRNA and protein levels and GRP78 protein levels in 1-LN cells transfected with dsTFII-I RNA. Panel A: TFII-I mRNA and protein levels. The lanes are: (1) buffer; (2) α_2M^*; (3) dsTFII-RNA then α_2M^*; and (4) scrambled RNA then α_2M^*. The loading controls are β-actin (mRNA) and actin (protein). Panel B: GRP78 protein levels in 1-LN cells treated as in Panel A. Panel C: CHIP assay showing binding of TFII-I to the *GRP78* and *c-fos* promoters in 1-LN cells. The lanes in the blot show the treatment with: (1) buffer; (2) α_2M^*; (3) dsTFII-I RNA then α_2M^*; and (4) scrambled DNA then α_2M^*.

optimal induction of GRP78 during thapsigargin-induced ER stress. Silencing *TFII-I* gene expression by RNAi caused about a 60% reduction in both its mRNA and protein levels (Fig. 2A). This treatment also reduced the protein levels of GRP78 by about 60% (Fig. 2B). These data suggest the dependence of GRP78 protein synthesis on TFII-I expression under these experimental conditions. α_2M^*-induced synthesis of TFII-I and GRP78 proteins was also significantly inhibited in cells pretreated with genistein or actinomycin D (Fig. 1C). We have observed similar induction of GRP78 and TFII-I synthesis in mouse peritoneal macrophages stimulated with α_2M^* (data not shown). Taken together these data demonstrate the requirement of tyrosine phosphorylation for transcriptional upregulation of GRP78 by TFII-I. Pretreatment of cells with genistein has been shown to suppress ER-induced TFII-I phosphorylation and inhibit transcriptional upregulation of GRP78 synthesis [Hong et al., 2005].

α_2M^*-INDUCED BINDING OF TFII-I TO THE GRP78 AND C-FOS PROMOTERS IN 1-LN CELLS

Silencing *TFII-I* gene expression by RNAi caused a marked inhibition of α_2M^*-induced synthesis of GRP78 which suggests a transcriptional downregulation of GRP78 promoter (Fig. 1C). Therefore, we next studied the binding of TFII-I to the *GRP78* gene promoter by CHIP assay (Fig. 2). Significant binding of TFII-I to the *GRP78* promoter occurred in α_2M^*-stimulated cells, which was abrogated in cells transfected with dsTFII-I RNA (Fig. 2C). These results support the involvement of TFII-I in transcriptional activation of GRP78 in 1-LN prostate cancer cells.

α_2M^* UPREGULATES PHOSPHORYLATED C-FOS LEVELS IN 1-LN CELLS

c-fos, a member of the B-zip family of transcription factors, heterodimerizes with c-Jun to form the AP-1 (activating protein-1) transcription factor that regulates expression of genes involved in cell growth, differentiation, and transformation. c-fos is involved in the signaling pathway that mediates GRP78 induction in response to ER stress [He et al., 2000]. A variety of growth promoting and mitogenic stimuli enhance tyrosine phosphorylation of TFII-I and subsequent activation of the *c-fos* promoter, which has many TFII-I binding sites [Kim et al., 1998; He et al., 2000; Roy, 2001]. Tyrosine phosphorylation at Y248 of TFII-I is essential for its transcriptional activity at several promoters as well as for its physical interaction with MAPKs through the conserved D-box [Kim et al., 1998; Roy, 2001]. TFII-I utilizes the Ras pathway for transcriptional activation of c-fos [Kim et al., 1998; Roy, 2001]. We have previously reported that in macrophages, α_2M^* upregulates the activation of Ras, MAPK and *c-fos* [Misra et al., 2004] and upregulates the synthesis of GRP78 (Fig. 1). We next studied the involvement of TFII-I in c-fos regulation by assaying its binding to the *c-fos* promoter by CHIP assay (Fig. 2) and cellular phospho-c-fos levels (Fig. 3). A significant binding of TFII-I occurred to the *c-fos* promoter in α_2M^*-stimulated cells compared to unstimulated cells. Binding of TFII-I to the *c-fos* promoter was completely abrogated in cells transfected with dsTFII-I RNA (Fig. 2C). Stimulation of 1-LN prostate cancer cells with α_2M^* (50 pM) caused phosphorylation of c-fos within 5–10 min which plateaued thereafter (Fig. 3). The results show that TFII-I not only causes transcriptional upregulation of GRP78, but also causes activation of the early response gene, *c-fos*, involved in cellular proliferation in α_2M^*-stimulated prostate cancer cells. Indeed, as determined by Western blotting, there was about a 1.5-fold increase over the basal state in phosphorylated c-fos in 1-LN cells stimulated with α_2M^* (Fig. 3), supporting TFII-I mediated activation of *c-fos* by α_2M^* in 1-LN cancer cells.

TYROSINE PHOSPHORYLATION OF C-SRC AND TFII-I IN 1-LN CELLS STIMULATED WITH α_2M^*

In B-cells, a large proportion of TFII-I is constitutively associated with Bruton tyrosine kinase (Btk), which catalyzes its tyrosine phosphorylation in the cytoplasm, upon immunoglobulin receptor cross-linking. Phosphorylation of TFII-I causes its dissociation from Btk and its translocation to nuclei. In non-lymphoid cells, the non-receptor tyrosine kinase c-Src phosphorylates TFII-I on tyrosine residues Tyr^{248} and Tyr^{611} and its translocation to nuclei in response to growth factor signaling. We have shown previously that, α_2M^* functions like a growth factor and upon binding to its receptor, GRP78 it promotes cellular growth and proliferation as well as synthesis of GRP78. As demonstrated above this synthesis is TFII-I-dependent (Fig. 1). To assess the role of non-receptor tyrosine

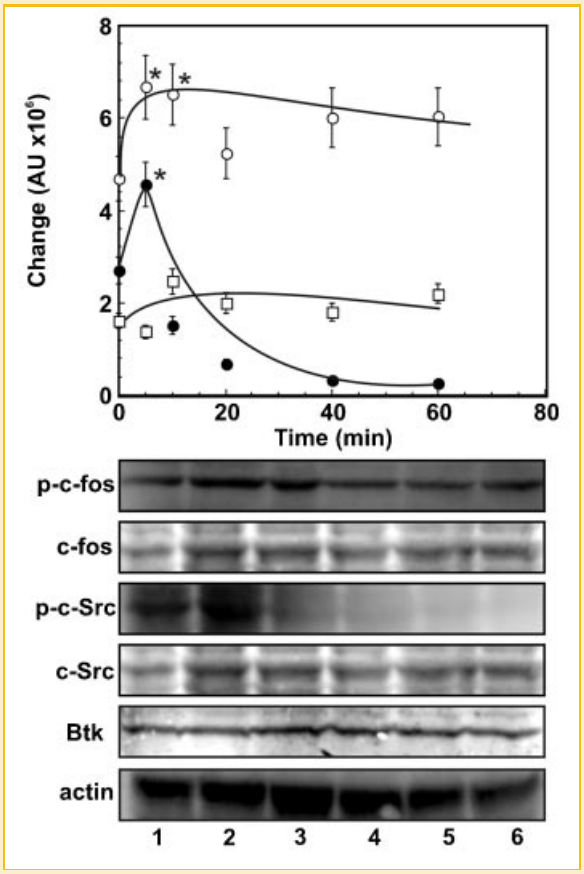

Fig. 3. Effect of time of incubation of 1-LN cells with α_2M^* for varying periods of time on levels of phosphorylated c-fos (p-c-fos, ○), phosphorylated c-Src (p-c-Src, ●) and Bruton tyrosine kinase (Btk, □). Representative immunoblots of phosphorylated and the control unphosphorylated c-fos and c-Src are shown below respective immunoblots. The Btk protein loading control, actin is also shown below the graph. Changes in levels of p-c-fos, p-c-Src, and Btk are the mean ± SE from three individual experiments. *Significantly different from buffer treated controls at the 5% level.

kinases in α_2M^*-induced GRP78 upregulation, we quantified phosphorylation of c-Src and protein levels of Btk in 1-LN cells stimulated with α_2M^* for varying periods of time (Fig. 3). α_2M^* caused ~ a twofold increase in phosphorylated-c-Src at about 5–10 min of incubation. Phosphorylation declined to basal values at longer periods of incubation (Fig. 3). In contrast, the protein levels of Btk in 1-LN prostate cancer cells were fairly low and these were ineligibly affected by α_2M^* treatment compared to c-Src (Fig. 3). These results suggest that Btk plays a minor role in TFII-I activation in α_2M^*-stimulated 1-LN prostate cancer cells. To examine the activation of tyrosine phosphorylated c-Src kinase and of TFII-I by tyrosine phosphorylation, the cell lysates from stimulated and unstimulated cells were immunoprecipitated with anti-TFII-I and c-Src antibodies, respectively and the immunoprecipitates first probed for c-Src and TFII-I and vice versa and then reprobed for phosphotyrosine residues by immunoblotting (Fig. 4). Significant tyrosine phosphorylation of both c-Src and TFII-I was observed in α_2M^*-stimulated cells compared to unstimulated cells (Fig. 4) and phosphorylated TFII-I and c-Src co-immunoprecipitated, indicating

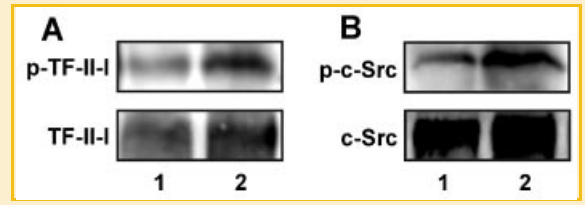

Fig. 4. α_2M^*-induced tyrosine phosphorylation of TFII-I and c-Src observed in 1-LN cells. Immunoblots shown are representative of two experiments performed in duplicate. See Experimental Procedures Section for details. The lanes in the immunoblot show the treatment of cells with: (1) buffer and (2) α_2M^*.

thereby a physical interaction of TFII-I and c-Src. A similar complex formation between TFII-I and c-Src has been postulated in ER-induced induction of TFII-I phosphorylation [Hong et al., 2005].

NUCLEAR LOCALIZATION OF GRP78, PHOSPHORYLATED-TFII-I, C-FOS, AND ERK1/2 IN α_2M^*-STIMULATED CELLS

Growth factor-induced tyrosine phosphorylation of TFII-I in the cytoplasm promotes its binding to activated ERK1/2 and this complex translocates to nuclei via a nuclear localization signaling motif on TFII-I [Kim et al., 1998]. The c-fos promoter is the best-studied immediate early gene promoter which responds to a variety of extracellular ligands [Kim et al., 1998; Roy, 2001]. Tyrosine phosphorylated nuclear TFII-I activates a chromosomally integrated *c-fos* reporter has been demonstrated in vivo [Parker et al., 2001; Roy, 2001, 2006; Cheriyath et al., 2002; Hong et al., 2005]. TFII-I enhances transcriptional activity of the c-fos promoter by binding to sites on *c-fos* promoter. In the next series of experiments we determined the nuclear translocation of TFII-I. A significant presence of GRP78, TFII-I, phosphorylated-TFII-I, phosphorylated c-fos, and phosphorylated ERK1/2 was observed in the nuclei of α_2M^*-stimulated 1-LN cells (Fig. 5A). We also determined the nuclear localization of TFII-I in 1-LN prostate cancer cells stimulated with buffer, control IgG, or α_2M^* by confocal microscopy (Fig. 5B). In α_2M^*-stimulated cells, a very pronounced localization of TFII-I is seen in the nuclei as compared to buffer or IgG-treated cells. These results suggest that in α_2M^*-stimulated 1-LN prostate cancer cells, TFII-I upregulation and activation regulates GRP78 synthesis.

DISCUSSION

GRP78, a member of HSP70 family, is involved in many cellular processes including translocation of newly synthesized proteins across the ER membrane, facilitating the folding and assembly of newly synthesized proteins, preventing intra- or inter-molecular aggregation, targeting misfolded proteins for degradation, regulating calcium homeostasis [Hendershot, 2004; Lee, 2007]. GRP78 is constitutively expressed, but its expression is enhanced manyfolds by a variety of environmental and patho-physiological conditions that perturb ER functions and homeostasis [Hendershot, 2004; Lee, 2007]. Pharmacological agents that affect protein folding, glycosylation, or the ER calcium pool also upregulate GRP78

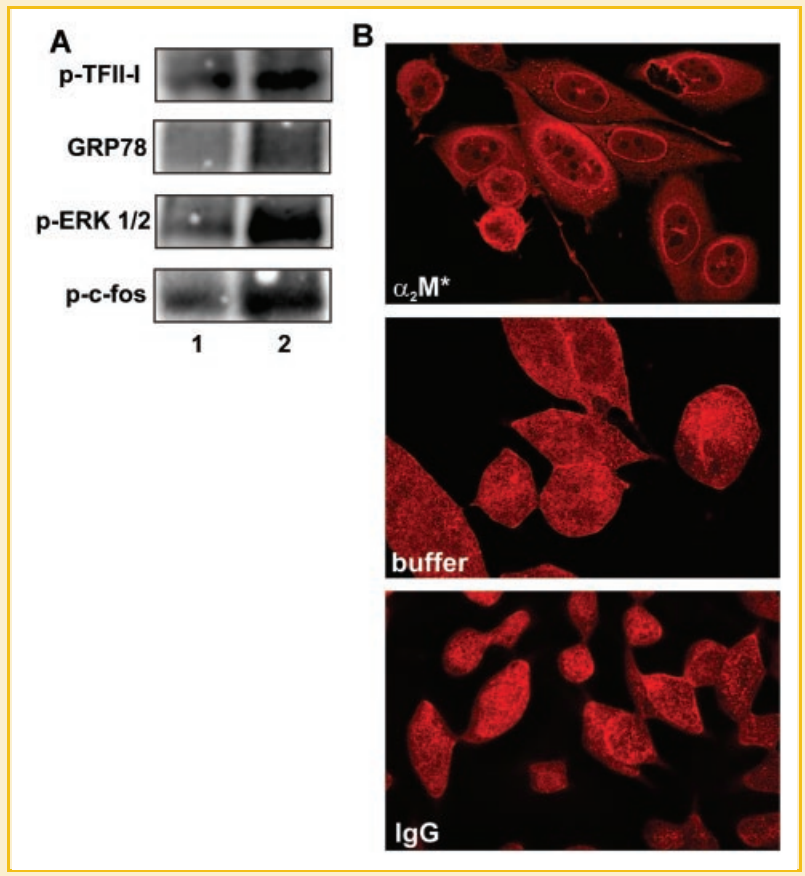

Fig. 5. Nuclear co-localization of activated TFII-I with GRP78, phosphorylated c-fos and phosphorylated ERK1/2 in 1-LN cells stimulated with α_2M*. Panel A: Co-immunoprecipitation of activated TFII with GRP78, phosphorylated c-fos and GRP78 in nuclear lysates of 1-LN cells stimulated with: (1) buffer or (2) α_2M*. Immunoblots shown are representative of three independent experiments. See Experimental Procedure Section for details. Panel B: Nuclear localization of TFII-I in 1-LN prostate cancer cells stimulated with α_2M* by confocal microscopy. Nuclear localization of TFII-I in buffer or IgG-treated controls are also shown. The immunofluorescence pattern in 1-LN cells shown is representative of two experiments performed in duplicate. Experimental details are described under Experimental Procedures Section.

expression [Hendershot, 2004; Lee, 2007]. Under these conditions the cells respond by activating UPR to protect cell survival and cellular proliferation. The onset of UPR results in translational arrest, induction of cell death pathway, and transcriptional activation of cellular genes that promote cell survival [Hendershot, 2004; Lee, 2007]. A major cellular target of UPR is the ER resident GRP78 protein, which demonstrates anti-apoptotic properties. GRP78 binds to unfolded proteins and regulates the activation of ER stress signal transducers. In addition, cell surface-associated GRP78 functions as a receptor for α_2M* [Misra et al., 2006; Lee, 2007]. The binding of α_2M* to GRP78 causes enhanced expression of GRP78, and triggers IP_3/calcium, Ras/MAPK, PI-3 kinase, and NFκB signaling which culminated in cellular proliferation and cell survival [Misra et al., 2006]. Recent studies on chemically induced ER stress in cell cultures demonstrate the induction of TFII-I which transcriptionally upregulates GRP78 under these conditions [Parker et al., 2001; Hong et al., 2005]. Most of these studies have used over-expression and reporter gene assays. We have examined the role of endogenous TFII-I in 1-LN prostate cancer cells in transcriptional upregulation of GRP78 in α_2M*-stimulated cells. The salient features of this study are: α_2M* induces expression and synthesis of TFII-I and GRP78 and promotes the binding of TFII-I to the *GRP78* and *c-fos* promoters, silencing TFII-I gene expression by RNAi or pretreatment with actinimycin D or genistein profoundly inhibited synthesis of both TFII-I and GRP78; transfection of cells with dsTFII-I RNA abrogated the binding of TFII-I to the *GRP78* and *c-fos* promoters; α_2M* treatment upregulated the tyrosine phosphorylation of c-Src and TFII-I and caused its nuclear translocation with GRP78, phosphorylated c-Src, and phosphorylated ERK1/2.

TFII-I was initially identified as the general transcription factor that binds to initiator (Inr) elements and also activates transcription of various genes either through an Inr element or through other upstream elements [Roy, 2001]. TFII-I activates ER stress elements through binding to its GC-rich motifs. The levels of TFII-I mRNA and protein increase in response to a variety of ER stresses [Parker et al., 2001; Cheriyath et al., 2002; Hong et al., 2005]. Antisense RNA targeted against TFII-I substantially reduced steady state levels of TFII-I and these levels remained low after ER stress. TFII-I is also a co-activator for ATF6 [Parker et al., 2001; Hong et al., 2005] and the presence of TFII-I stabilizes the binding of ATF6 to ERSE [Parker et al., 2001; Hong et al., 2005]. c-Src is activated by ER stress and over expression of c-Src can activate the *GRP78* promoter, since

a dominant mutant of c-Src partially suppressed thapsagargin-induced ER stress as well as TFII-I-mediated activation of the *GRP78* promoter [Cheriyath et al., 2002; Roy, 2007]. The *c-fos* promoter, a downstream target of tyrosine phosphorylated TFII-I, is also activated by ER stress, which leads to increase in c-fos protein and through complex formation with the GRP78 transcriptional machinery contributes to ER stress-induced activation of the *GRP78* promoter leading to increase in GRP78 protein [Parker et al., 2001; Roy, 2001, 2006, 2007; Cheriyath et al., 2002; Hong et al., 2005].

In addition to its role as a transcriptional upregulator of GPR78, TFII-I also modulates the transcription of other genes. Under normal growth conditions TFII-I is recruited to the cyclin D1 promoter and transcriptionally activates this gene [Desgranges et al., 2005]. Elevated TFII-I levels enhance cycling of cells. Degradation of TFII-I occurred following γ-irradiation, in an p53-dependent manner, because in p53 null cells no degradation of TFII-I was observed [Desgranges et al., 2005]. Thus TFII-I not only transcriptionally upregulates the anti-apoptotic protein GRP78, but also promotes cell cycle progression, and this synergy may potentiate the survival and proliferative effects of cells during ER stress, induced in a variety of patho-physiological environment. The ER is the site for synthesis, folding and trafficking of secretory and cell surface proteins. The molecular chaperone GRP78 was originally discovered as a protein inducible by glucose starvation and it serves many functions in maintaining cellular homeostasis. GRP78 participates in ER protein translocation, chaperoning, protein ''quality control'', ER-associated protein degradation, ER stress sensing and regulation, and ER calcium binding. In addition, GRP78 promotes tumor proliferation, metastasis, and drug resistance, which have major clinical implications in the prognosis and treatment of cancer. Additionally, the localization of GRP78 on the cell surface of certain cell types suggests that GRP78 serves novel functions as a cell surface receptor for signaling. In cancer cells elevated glucose metabolism, increased glycolytic activity, and poor vascularization in fast growing tumors leads to glucose starvation, low pH, and hypoxia which induce ER stress and activation of GRP78 transcription. ER stress-independent activation of the GRP78 promoter has also been reported. Thus in addition to ER stress, GRP78 transcription may be upregulated through other mechanisms during tumor growth and confer survival advantage. In addition to protection against ER stress, cell surface GRP78 directly promotes tumor cell proliferation in 1-LN prostate cancer cells by transducing extracellular stimuli to intracellular signals to promote cancer development.

In a recent report we showed that 1-LN cells treated with α_2M^* elevated GRP78 protein expression and triggered UPR signaling, a response which protects cells from ER stress [Misra et al., 2006]. In this report we show that α_2M^* treatment of these cancer cells results in induction and activation of TFII-I which binds to the *GRP78* promoter thereby enhancing transcriptional upregulation of GRP78. The transcription-activating competency of TFII-I was rendered by activated c-Src-mediated tyrosine phosphorylation of TFII-I. α_2M^*-induced elevated TFII-I also regulated c-fos levels and activation. The data presented in this communication suggests an important role of TFII-I in cell survival and proliferation via GRP78 in cancer growth. Thus cells exposed to α_2M^* are more competent to resist ER stress such as is typical in the tumor millieu where oxygen levels are diminished, pH is below neutrality, and glucose supplies restricted.

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