Activated $\alpha_2$-Macroglobulin Regulates Transcriptional Activation of c-MYC Target Genes through Cell Surface GRP78 Protein*

Received for publication, December 3, 2015, and in revised form, March 18, 2016 Published, JBC Papers in Press, March 21, 2016, DOI 10.1074/jbc.M115.708131

Udhayakumar Gopal, Mario Gonzalez-Gronow, and Salvatore Vincent Pizzo

From the Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

Activated $\alpha_2$-macroglobulin ($\alpha_2$M*) signals predominantly through cell surface GRP78 (CS-GRP78) to promote proliferation and survival of cancer cells; however, the molecular mechanism remains obscure. c-MYC is an essential transcriptional regulator that controls cell proliferation. We hypothesize that $\alpha_2$M*/CS-GRP78-evoked key signaling events are required for transcriptional activation of c-MYC target genes. Activation of CS-GRP78 by $\alpha_2$M* requires ligation of the GRP78 primary amino acid sequence (Leu98–Leu145). After stimulation with $\alpha_2$M*, CS-GRP78 signaling activates 3-phosphoinositide-dependent protein kinase-1 (PDK1) to induce phosphorylation of PLK1, which in turn induces c-MYC transcription. We demonstrate that PLK1 binds directly to c-MYC and promotes its transcriptional activity by phosphorylating Ser62. Moreover, activated c-MYC is recruited to the E-boxes of target genes FOsi1 and ID2 by phosphorylating histone H3 at Ser10. In addition, targeting the carboxyl-terminal domain of CS-GRP78 with a mAb suppresses transcriptional activation of c-MYC target genes and impairs cell proliferation. This work demonstrates that $\alpha_2$M*/CS-GRP78 acts as an upstream regulator of the PDK1/PLK1 signaling axis to modulate c-MYC transcription and its target genes, suggesting a therapeutic strategy for targeting c-MYC-associated malignant progression.

$\alpha_2$-Macroglobulin ($\alpha_2$M)2 is a plasma protein that interacts with and entraps virtually all proteins, thereby blocking access to their substrates (1). In prostate cancer patients, $\alpha_2$M is proteolytically activated ($\alpha_2$M*) and signals predominantly through interaction with cell surface GRP78 ($K_d \approx 50–100$ pm), promoting proliferation and survival of cancer cells (2, 3). GRP78 is a stress-inducible, prosurvival, endoplasmic reticulum chaperone belonging to the HSP70 family. It is composed of an ATPase domain, a peptide binding domain, and a COOH-terminal domain of unknown function (4–6). Several different cell types, including proliferating endothelial cells and tumor cells, express GRP78 on their surface (7–15). GRP78 expression at the cell surface and its ligation by $\alpha_2$M* are clearly implicated in the development of metastatic prostate cancer (2, 9, 16–19). However, the mechanism by which $\alpha_2$M*/cell surface GRP78 (CS-GRP78) signaling regulates gene transcription and their responses in cell proliferation is unknown.

CS-GRP78 is a multifunctional receptor that forms complexes with phosphatidylinositol 3-kinase (PI3K) and enhances phosphatidylinositol 3,4,5-trisphosphate production, consistent with its novel role as a regulator of the PI3K/Akt signaling pathway. Thus it promotes cell proliferation, survival, metastasis, and chemoresistance (9, 20–22). CS-GRP78, through its NH2-terminal domain, drives PI3K/Akt activity (2), whereas targeting the COOH-terminal domain with antibody promotes apoptotic signaling (21, 23). Recently, we demonstrated in vivo that targeting the GRP78 COOH-terminal domain with monoclonal antibody C38 (C38 mAb) delays tumor growth and prolongs survival (15). We also demonstrated that $\alpha_2$M*/CS-GRP78 signaling is required for mechanistic target of rapamycin (mTOR) complex-mediated phosphorylation of Akt by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (22). PDK1 regulates a diversity of substrates and targets that induce aberrant signaling in human malignancy (24). Indeed, recent studies show that PDK1 is required for c-MYC accumulation, and it regulates c-MYC activity through the downstream target PLK1 (25), indicating a potential functional link of $\alpha_2$M*/CS-GRP78 signaling and c-MYC in proliferation of cancer cells. $\alpha_2$M*/CS-GRP78-mediated PI3K/Akt signaling is well documented; however, its role in cancer-associated gene regulation by transcription factors has yet to be identified.

The oncogene c-MYC globally reprograms cells and drives proliferation by regulating an estimated 15% of the genes in the human genome (26). Recent work suggests that rather than acting as a general amplifier of transcription (27, 28) c-MYC activates and represses transcription of discrete gene sets, leading to changes in cell proliferation, tumor progression, and maintenance (29). Moreover, phosphorylation of c-MYC at certain sites governs its activation and subsequent biological functions through transcriptional activation of target genes that are necessary for cell proliferation. Specifically, Ser62 phosphorylation is necessary for its oncogenic activity (30). A key question is whether $\alpha_2$M*/CS-GRP78 signaling is required for activation of c-MYC and its downstream target genes.

In the present study, we demonstrate that $\alpha_2$M*/CS-GRP78-mediated PDK1/PLK1 signaling contributes to the transcriptional activation of c-MYC target genes and proliferation. We further demonstrate that PLK1 can directly bind to c-MYC and promote its transcriptional activity by phosphorylating at his-
tone H3 Ser\(^{10}\) (H3S10). These findings suggest that \(\alpha_2M^{*}\)/CS-GRP78 signaling drives c-MYC target gene expression in human cancers and provide a therapeutic approach for targeting c-MYC-driven tumors.

**Experimental Procedures**

**Cell Culture**—1-LN prostate cancer cells were a kind gift from Dr. Philip Walther, Department of Surgery, Duke University Medical Center. They now reside in our laboratory and are available on request. DU145 prostate cancer cells, A375 melanoma cells, and U373 glioma cells were purchased from the Duke Cell Culture Facility. 1-LN and DU145 cells were maintained in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37 °C in a 5% CO\(_2\)-humidified atmosphere. A375 and U373 cells were maintained in DMEM (high glucose; Gibco, Life Technologies) containing 10% FBS, 1% penicillin/streptomycin at 37 °C in a 5% CO\(_2\)-humidified atmosphere.

**Antibodies and Reagents**—Antibodies recognizing c-MYC, P-c-MYC (Ser\(^{62}\)), PDK1, P-PDK1 (Ser\(^{241}\)), PLK1, P-PLK1 (Thr\(^{210}\)), P-histone H3 (Ser\(^{10}\)), histone H3, cleaved poly(ADP-ribose) polymerase (Asp\(^{214}\)), kinase buffer (10×), ATP, and SignalSilence c-MYC siRNAII were purchased from Cell Signaling Technology. GAPDH antibody was purchased from Genscript. c-MYC recombinant protein was purchased from Novus Biologicals. Cell proliferation kit II (XTT) was purchased from Roche Applied Science. Glycogen, Lipofectamine 2000, and secondary antibodies conjugated with Alexa Fluor 680, Alexa Fluor 790, and Alexa Fluor 647 were purchased from Invitrogen. IRDye 800 CW was purchased from Rockland. Protein G-agarose beads and Proteinase K were purchased from Sigma. Protein A/G magnetic beads were purchased from Thermo Scientific. Bx795 and B12536 were purchased from Selleck Chemicals. \(\alpha_2M^{*}\) was measured as described previously (27). GRP78 murine monoclonal antibody (C38) was produced in our laboratory (32).

**Peptides**—GRP78 wild type (WT) peptide CLIGRTWDNPS-VQQDIKFL (Leu\(^{98}\)–Leu\(^{115}\)), mutant peptide LIGRTWDNPS-VQQDIVFL (K\(^{113}\)V), and scrambled peptide GTNKSDL-WIPQLRDF1 were purchased from Genemed Synthesis, Inc.

**Small Interfering RNA (siRNA) Interference and Lentiviral Transfections**—siRNAs targeting c-MYC and nonspecific siRNA were transfected into 1-LN cells with Lipofectamine 2000 reagent according to the manufacturer’s instructions. sh-c-MYC lentiviral particles (Clone ID TRCN000000-39642) were obtained from Sigma and transfected into 1-LN cells according to the manufacturer’s instructions. After transfection with sh-c-MYC vector, 1-LN cells were selected with 2 mg/ml puromycin.

**Flow Cytometry**—CS-GRP78 was analyzed by flow cytometry as described previously (15). The mean fluorescence intensity of the signal was calculated by Flow Jo® software, and signal obtained from GRP78 was normalized with that obtained from isotype controls.

**Cell Proliferation Assays**—Cells were plated in 96-well plates at 10,000 cells/well in 0.1 ml of growth medium containing inhibitor BX795, B12536, or C38 mAb for 72 h. Cell viability was measured according to the manufacturer’s instruction protocol by using the XTT assay. Absorbance was read at \(\lambda = 450\) nm.

**Soft Agar Colony Formation Assay**—Soft agar assays were conducted in 6-well plates in triplicate. For each well, 10,000 cells were mixed in growth medium containing 1.2% agarose and inhibitor BX795, B12536, or C38 mAb. Cells were then layered over 2% agarose in regular medium. Medium containing BX795, B12536, or C38 mAb was added to each well every 3 days. The assays were terminated after 21 days, colonies were counted under a microscope or stained with crystal violet, and pictures were taken.

**Immunoblotting and Immunoprecipitation**—Protein extracts, immunoblotting, and immunoprecipitate analysis were performed as described previously (27), and all blots are representative of a minimum of two independent experiments.

**Immunoprecipitation and In Vitro Kinase Assays**—PLK1 immunoprecipitation–kinase assays were performed as described previously (27) and analyzed by immunoblotting using P-c-MYC (Ser\(^{62}\)) and c-MYC antibodies.

**Quantitative Real Time PCR and PCR Array**—Total RNA was prepared from cells using the Quick-RNA Mini Prep kit (Zymo Research), and cDNAs were generated using the iScript cDNA synthesis kit (Bio-Rad). SYBR Green reactions were done using a Bio-Rad CFX96 quantitative real time PCR system. For data analysis, raw counts were normalized to the housekeeping gene averaged for the same time point and condition (ΔC\(_{\text{t}}\)). Counts are reported as -fold change relative to the untreated control (2\(^{-\Delta\Delta C_{\text{t}}}\)). All primers were designed and synthesized by Integrated DNA Technologies or Eurofins MWG Operon. Primers are listed in Table 1. Array samples were prepared according to the manufacturer’s instructions by using RNeasy Plus Mini kits (Qiagen). Samples were analyzed using a human MYC target gene PCR array (Qiagen PAHS-17772A). Genes were considered differentially expressed if they demonstrated a significant p value ≤0.05 and were at least 2-fold or greater up-regulated. An average of biological triplicates was used to generate heat maps by using Qiagen data analysis center software.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assays were done in 1-LN prostate cancer cells as described previously (33). ChIP enrichment for a primer set was evaluated by quantitative PCR as percentage of input and normalized to a negative primer set. FOSL1 and ID2 gene probes used for ChIP analysis were designed as described previously (33) and are listed in Table 2.

**Statistical Analysis**—Data are presented as mean ± S.D. unless otherwise stated. A Student’s t test was used to compare two groups for statistical significance analysis. p values ≤0.05 were considered as significant.

### Table 1

| Gene   | Primer Sequence | Sequence |
|--------|-----------------|----------|
| FOSL1  | Forward         | 5′-CAG GCC GAG ACT GAC AAA CTG-3′ |
| ID2    | Forward         | 5′-TCC TCC GGG TAT TCA TTT GCA GAT-3′ |
| c-MYC  | Forward         | 5′-GCC TCC TGG CAA AAG GTC A-3′ |
|        | Reverse         | 5′-AGT CTG TCA GTG ATG GCA CAG-3′ |

CS-GRP78 Signaling Promotes c-MYC Activation
CS-GRP78 Signaling Promotes c-MYC Activation

RESULTS

αM*-induced c-MYC Expression Promotes Tumor Growth in Soft Agar—We first determined the ability of αM* to promote proliferation by using an in vitro assay that measures anchor-age-independent growth in soft agar. This assay demonstrates that αM* promotes a significant increase in colony formation in a variety of cancer cell lines (Fig. 1A). Furthermore, we analyzed the effect of αM* on cell proliferation in standard cell culture of the same panel of cancer cell lines. Consistent with our previous report, αM* significantly increased cell proliferation in these lines (Fig. 1B) (9). This result confirms the potential role of αM* in cancer cell proliferation.

To determine the mechanism by which αM* is involved in cell proliferation, we examined the expression of c-MYC. αM* stimulation of 1-LN cells induces c-MYC protein expression in a dose- and time-dependent manner (Fig. 1C). Maximal c-MYC expression occurred with 100 pm αM* at 30 min. Of interest, the maximal effective dose of 100 pm is consistent with many previous studies, demonstrating that the maximal proliferative effect of αM* on proliferation occurs at this dose with a falloff in activity at 500 pm (for example, see Refs. 2 and 31). The reason for this dose effect is unknown; however, we used these conditions for subsequent experiments. Next we investigated whether αM* induces c-MYC at a post-translational or transcriptional level. 1-LN cells were treated with either cycloheximide or actinomycin D and then stimulated with αM*). At 500 pm, αM* decreased the c-MYC expression in a dose- and time-dependent manner (Fig. 1D). αM* induced strong phosphorylation of PDK1 (Ser241) and increased phosphorylated PLK1 (Thr210) in a dose- and time-dependent manner (data not shown). Further analyses in multiple cancer cell lines showed that targeting CS-GRP78 with C38 mAb abolished αM*-induced phosphorylation of PDK1 (Ser241) and PLK1 (Thr210) (Fig. 2C, third and fifth lanes). These data confirm that GRP78 is a functional molecular target on cancer cell surfaces.

In our previous studies, we identified the GRP78 primary amino acid sequence LIGRTWNDPSQKDIKL (Leu98–Leu115) as the putative binding site for αM* (9). We next demonstrated the specificity of CS-GRP78 signaling by stimulating multiple cancer cell lines with αM* in the presence of WT (Leu98–Leu115), mutant (K113–V), or scrambled GRP78 peptides. GRP78 WT peptide suppressed c-MYC induction and decreased αM*-dependent phosphorylation of both PDK1 and PLK1. In contrast, GRP78 mutant peptide did not affect αM*-mediated c-MYC induction or phosphorylation of PDK1 and PLK1 (Fig. 2D). These results further demonstrate that αM* signals specifically through the GRP78 (Leu98–Leu115) binding site to induce c-MYC expression.

αM* Promotes PLK1 Interaction with c-MYC to Induce Phosphorylation of c-MYC in a PDK1-dependent Manner—To dissect the PDK1/PLK1 signaling pathway, we treated multiple cancer cell lines with the small molecule PDK1 inhibitor BX795 or PLK1 inhibitor BI2536 and then stimulated with αM*. Indeed, the PDK1 inhibitor BX795 abolished αM*-induced phosphorylation of PDK1 and expression of c-MYC at the pro-
tein as well as transcriptional level (Fig. 3, A (first and fifth lanes) and B). Furthermore, like BX795, the PLK1 inhibitor BI2536 eliminated c-MYC protein expression and affected its transcription level but did not affect P-PDK1 (Fig. 3, A (third and fifth lanes) and B). These findings suggest that PLK1 functions as a downstream kinase to PDK1 in α2M*/CS-GRP78-induced c-MYC expression.

To determine whether PDK1 is required for the PLK1 activation, we stimulated 1-LN cells with α2M* followed by PDK1 immunoprecipitation. We demonstrated enhanced phosphorylation of endogenous PLK1 at Thr210 that was abolished when cells were treated with BX795 or BI2536 (Fig. 3 C). This suggests that PDK1 directly regulates PLK1 in α2M*/CS-GRP78-stimulated cells. Importantly, cells treated with C38 mAb also showed greatly reduced PLK1 phosphorylation. Together these data indicate that PDK1-induced PLK1 phosphorylation requires α2M*/CS-GRP78 signaling.

We next investigated whether PLK1 directly regulates c-MYC activation. Through co-immunoprecipitation assays, we showed that α2M* promoted interaction between PLK1 and c-MYC that is blocked by BX795, BI2536, or C38 mAb (Fig. 3 D). We explored whether PLK1 kinase activity is required for the c-MYC phosphorylation and accumulation. We used in vitro kinase assays using endogenous PLK1 pulled down by antibody from α2M*-stimulated scrambled and c-MYC-silenced 1-LN cells. We observed a robust induction of Ser62 phosphorylation of recombinant c-MYC that was blocked in the presence of BX795 or BI2536 (Fig. 3, E and F). Importantly, PLK1 kinase-dependent c-MYC phosphorylation was strongly abolished in cells treated with C38 mAb (Fig. 3, E and F). Phosphorylation of recombinant c-MYC in c-MYC-silenced cells confirms the specificity of the recombinant in vitro kinase assay (Fig. 3 F). Thus we not only showed direct phosphorylation of c-MYC by PLK1 but also found that PLK1 activity with respect to c-MYC
is crucially dependent on PDK1. Together these data strongly support the hypothesis that α2M*/CS-GRP78 signaling potentiates PDK1/PLK1/c-MYC signaling in cancer cells.

α2M*/CS-GRP78 Signaling Axis Phosphorylates Histone H3 Ser62 in a c-MYC-dependent Manner—To further substantiate the role of c-MYC activation, we stimulated multiple cancer cell lines with α2M* in the absence or presence of C38 mAb. In α2M*-stimulated cells, we observed induction of c-MYC Ser62 phosphorylation that was accompanied by a similar pattern of phosphorylation of histone H3S10, which is known for the transcriptional activation of the gene. We further showed that C38 mAb and GRP78 WT peptide blocked α2M*-mediated phosphorylation of c-MYC and P-histone H3S10, whereas c-MYC-silenced cells and GRP78 mutant peptide did not have any further effect (Fig. 4A, B, and C). Notably, in multiple cancer cell lines, BX795 or BI2536 treatment also inhibited α2M*-mediated c-MYC phosphorylation and c-MYC accumulation (Fig. 4D). This finding demonstrates that α2M*/CS-GRP78-mediated c-MYC activity is required for histone H3S10 phosphorylation and suggests a possible role of transcriptional activation of c-MYC target genes.

Role of α2M*/CS-GRP78 Signaling in c-MYC-dependent Gene Regulation—We next sought to determine whether α2M*-mediated c-MYC induction activates specific sets of target genes that are characteristic of c-MYC transformed cells. To achieve that, we performed human MYC target gene PCR array analysis of α2M*-stimulated 1-LN cells in the presence or absence of C38 mAb. α2M* significantly induced expression of c-MYC, FOSL1, and ID2 genes among those genes that were up-regulated (Fig. 5A). Importantly, C38 mAb suppressed up-regulation of these genes by α2M*, demonstrating a role for CS-GRP78 activation in regulating the c-MYC target genes. We next validated c-MYC target genes FOSL1 and ID2 by quantitative PCR analysis. α2M* induced FOSL1 and ID2 transcript levels in a dose- and time-dependent manner (data not shown). Moreover, silencing of c-MYC strongly inhibited FOSL1 and ID2 expression, and α2M* had no further effect (Fig. 5B). To confirm that α2M* induces the c-MYC target genes FOSL1 and ID2 through its receptor CS-GRP78, we treated the 1-LN cells with 50 μg of C38 mAb for 6 h and then stimulated with α2M* (100 μM) for 30 min. We observed that targeting CS-GRP78 abrogated the α2M*-induced FOSL1 and ID2 transcription level (Fig. 5C). These results demonstrate that α2M*/CS-GRP78 signaling is...
required for the transcriptional activation of c-MYC target genes.

To show the capability of PDK1/PLK1 signaling in regulation of c-MYC target genes, we treated the 1-LN cell line with BX795 or BI2536 inhibitor. Each compound inhibited induction of FOSL1 and ID2 transcripts by \( \alpha_2 M^* \) (Fig. 5D). Together these findings show that \( \alpha_2 M^*/CS-GRP78 \) signaling modulates PDK1/PLK1 signaling to evoke c-MYC target genes.

**\( \alpha_2 M^*/CS-GRP78 \) Signaling Is Required for the Transcriptional Activation of FOSL1 and ID2 Genes**—To demonstrate direct binding of c-MYC to the FOSL1 and ID2 regulatory sequences, we performed a ChIP assay using an antibody against c-MYC followed by RT-quantitative PCR analysis in 1-LN cells. As expected, \( \alpha_2 M^* \)-stimulated 1-LN cells showed increased c-MYC association at the FOSL1 enhancer and the ID2 E-box region, whereas C38 mAb, BX795, or BI2536 abolished c-MYC binding and reduced its expression (Fig. 6A). To further confirm the specificity of c-MYC binding to FOSL1 and ID2, we used a negative primer downstream region within the first intron of FOSL1 and ID2. These results establish that \( \alpha_2 M^*/CS-GRP78 \) signaling recruits c-MYC to E-box elements of the c-MYC-regulated genes FOSL1 and ID2 through its downstream kinase PDK1/PLK1 signaling, thus contributing to malignant progression.

Next we analyzed whether \( \alpha_2 M^*/CS-GRP78 \) signaling contributes to H3S10 phosphorylation at the FOSL1 and ID2 enhancer region. An analysis of the H3S10 phosphorylation signal in \( \alpha_2 M^* \)-stimulated 1-LN cells showed increased phosphorylation of H3S10 at the FOSL1 and ID2 enhancer region, whereas C38 mAb, BX795, or BI2536 inhibited the H3S10 phosphorylation signal and reduced the expression of FOSL1 and ID2 (Fig. 6B). These results demonstrate that \( \alpha_2 M^*/CS-GRP78 \) signaling promotes c-MYC activation, thus contributing to malignant progression.

**FIGURE 3. \( \alpha_2 M^*/CS-GRP78 \) signaling is required for the interaction of PLK1 with c-MYC to induce c-MYC phosphorylation.** A, immunoblotting (IB) analysis of the indicated cancer cell lines treated with BX795 (2.5 \( \mu \)mol/liter) or BI2536 (10 nmol/liter) for 16 h and then stimulated with \( \alpha_2 M^* \) (100 \( \mu \)g) for 30 min. B, c-MYC transcript analysis in 1-LN prostate cancer cell line treated with BX795 (2.5 \( \mu \)mol/liter) or BI2536 (10 nmol/liter) for 16 h and then stimulated with \( \alpha_2 M^* \) (100 \( \mu \)g) for 30 min. C, immunoprecipitation (IP) analysis of endogenous PDK1 and PLK1 in 1-LN prostate cancer cell line with or without BX795 (2.5 \( \mu \)mol/liter) or BI2536 (10 nmol/liter) for 16 h and then stimulated with \( \alpha_2 M^* \) (100 \( \mu \)g) for 30 min. D, immunoprecipitation analysis of endogenous PDK1 and P-PLK1 in 1-LN prostate cancer cell line with or without BX795 (2.5 \( \mu \)mol/liter) or BI2536 (10 nmol/liter) for 16 h and then stimulated with \( \alpha_2 M^* \) (100 \( \mu \)g) for 30 min. E and F, immunoprecipitation and in vitro kinase assay using recombinant c-MYC and immunoprecipitated endogenous PLK1 from 1-LN Scr-siRNA and si-c-MYC cells in the presence or absence of BX795 (2.5 \( \mu \)mol/liter) or BI2536 (10 nmol/liter) for 16 h or C38 mAb (50 \( \mu \)g) for 6 h and then stimulated with \( \alpha_2 M^* \) (100 \( \mu \)g) for 30 min. Error bars represent S.D.
regulates the transcriptional activation of FOSL1 and ID2 genes with an increase of H3S10 phosphorylation at the enhancer region. Because in c-MYC-silenced cells the transcription level of FOSL1 and ID2 decreased (Fig. 5B), we analyzed whether c-MYC association with the FOSL1 and ID2 enhancer is required for H3S10 phosphorylation. Quantitative ChIP analysis demonstrated that 2M* did not induce H3S10 phosphorylation at the FOSL1 and ID2 enhancer in c-MYC-silenced cells (Fig. 6C). These data indicate that H3S10 phosphorylation at the FOSL1 and ID2 enhancer is dependent on c-MYC.

\[ \alpha_2 M^* /CS-GRP78 induces histone H3 phosphorylation through PDK1/PLK1/c-MYC signaling. \]

A, immunoblotting analysis of the indicated cancer cell lines treated with C38 mAb (50 μg) for 6 h and then stimulated with \( \alpha_2 M^* \) (100 pm) for 30 min. B, immunoblotting analysis of the indicated proteins in 1-LN Scr-siRNA and si-c-MYC cells stimulated with \( \alpha_2 M^* \) (100 pm) for 30 min in the absence or presence of Scr peptide (100 pm for 1 h), GRP78 WT peptide (100 pm for 1 h), or GRP78 mutant peptide (100 pm for 1 h) and immunoblotted for the respective proteins. C, various cancer cell lines were stimulated with \( \alpha_2 M^* \) (100 pm) for 30 min in the absence or presence of Scr peptide (100 pm for 1 h), GRP78 WT peptide (100 pm for 1 h), or GRP78 mutant peptide (100 pm for 1 h) and immunoblotted for the respective proteins. D, immunoblotting analysis of the indicated cancer cell lines treated with BX795 (2.5 μmol/liter) or BI2536 (10 nmol/liter) for 16 h and then stimulated with \( \alpha_2 M^* \) (100 pm) for 30 min.

CS-GRP78 Signaling Promotes c-MYC Activation

- Alpha M*/CS-GRP78-induced PDK1/PLK1/c-MYC Signaling Drives Cell Proliferation and Tumorigenesis—To address the functional role of \( \alpha_2 M^*/CS-GRP78-mediated PDK1/PLK1/c-MYC signaling in cancer cells, we examined the transforming capacity of \( \alpha_2 M^* \) in multiple cancer cell lines by using the soft agar assay. \( \alpha_2 M^* \) potentiated increased colony numbers in a panel of cancer cell lines, whereas BX795 or BI2536 treatment significantly decreased colony number. This result further demonstrates that PDK1/PLK1 signaling is required for \( \alpha_2 M^* \)-induced transformation. Moreover, targeting CS-GRP78 by C38 mAb abolished \( \alpha_2 M^* \)-induced colony growth (Fig. 7A). Consistent with our previous studies (9), \( \alpha_2 M^* \) significantly increased cell proliferation of multiple cancer cell lines, and this was reduced by BX795 or BI2536 treatment (Fig. 7B). This finding further demonstrates the role of PDK1/PLK1 signaling in cell growth. Indeed, C38 mAb treatment also resulted in more effective growth inhibition. Together these data support the conclusion that \( \alpha_2 M^* \)-induced transformation depends on CS-GRP78-mediated PDK1/PLK1 signaling in cell proliferation.

Next, we evaluated the role of \( \alpha_2 M^*/CS-GRP78 \) in regulating PDK1/PLK1 signaling and cancer cell survival. As
expected, BX795, BI2536, or C38 mAb induced massive apoptosis as evidenced by strong detection of poly(ADP-ribose) polymerase cleavage (Fig. 7C). As in our previous studies, targeting CS-GRP78 with C38 mAb induces significant apoptosis (23). Taken together these results further support the role of α2M+/CS-GRP78 signaling in cell proliferation and malignant progression.

**Discussion**

This study defines a new role for the α2M+/CS-GRP78 signaling axis as a c-MYC-dependent modifier of chromatin. On the basis of biochemical and functional evidence, we show that α2M+/CS-GRP78-dependent PDK1/PLK1 signaling is required for the transcriptional activation of a subset of c-MYC target genes and cell proliferation.

Cell surface expression GRP78 and its ligation by α2M* are clearly implicated in the development of metastatic prostate cancer (19). Furthermore, CS-GRP78 is differentially expressed in cancer cells and stressed endothelial cells, providing a potential opportunity for highly specific therapeutic intervention (7–13). We and others have shown that cell surface GRP78 through its interaction with α2M* functions as an upstream regulator of PI3K/Akt signaling (3, 23, 37). Recently, our in vivo model suggested that targeting CS-GRP78 by C38 mAb blocks the Akt pathway to prolong the survival of ovarian cancer-bearing mice (15). By using GRP78 WT (Leu98–Leu115) and mutant (K113–V) peptides, we further show that α2M* induced PDK1/PLK1/c-MYC signaling through the GRP78 primary amino acid sequence Leu98–Leu115, thereby demonstrating that this region is essential for signaling and transcriptional activation of c-MYC. Although α2M+/CS-GRP78 signaling is associated with PI3K/Akt signaling in oncogenesis, our study uncovered another arm of signaling that routes to PDK1/PLK1/c-MYC signaling to activate c-MYC target genes and promote malignant progression. Consistent with previous findings that α2M+/CS-GRP78 induces PDK1 activation, we now further show that PDK1 induces PLK1 phosphorylation to maintain the cell growth in cancer cells (22, 25). Importantly, the pathway we identified using a chemical and genetic approach with a GRP78 WT and mutant peptide and C38 mAb treatment shows that α2M*/CS-GRP78 activates PDK1 to function as an upstream regulator of PLK1 for c-MYC induction. This is in contrast to a previous report suggesting that PDK1 induces c-MYC at a post-translational but not transcriptional level (25, 36). Further studies are needed to determine the mechanism by which α2M*/CS-GRP78 induces the c-MYC transcript level.
Recent reports highlight that phosphorylation of c-MYC at Ser62 enhances c-MYC activity through regulation of protein stability in Ras-expressing cells and prostate and breast cancer cells (25, 30, 38, 39). Consistent with previous reports, we showed by immunoprecipitation experiments after stimulation with H92512M* that PLK1 forms a complex with c-MYC and phosphorylates Ser62 (25). Moreover, we have demonstrated that C38 mAb reduces c-MYC protein stability by repressing Ser62 phosphorylation and abrogates transcriptional activity of c-MYC. According to the current view, phosphorylation at the NH2-terminal domain of H3 is required to loosen the interaction between DNA and nucleosome and/or to generate a platform to recruit additional regulatory factors as described in the histone code hypothesis (41). It was shown previously that after treatment with growth factors H3 is phosphorylated rapidly at Ser10 by MDK1/MSK2 (33, 42–44). Our results confirm these previous findings and emphasize that α2M*/CS-GRP78 phosphorylates histone H3S10 as a result of c-MYC activity and that this is required for the transcriptional activation of genes mediated by c-MYC. It has been estimated that about 11% of cellular genes contain a functional E-box with which c-MYC can associate on the genome (45). In agreement with these data, ChIP analysis in H92512M*-stimulated 1-LN prostate cancer cells revealed that c-MYC recruitment to chromatin requires CS-GRP78 signaling. As noted, c-MYC is necessary to phosphorylate histone H3S10 to activate transcription of its target genes FOSL1 and ID2. This is in agreement with previous findings that phosphorylation of H3S10 is necessary for transcriptional activation of FOSL1 and ID2 genes (33). C38 mAb suppressed c-MYC target gene expression that is dependent on H92512M* treatment, suggesting that α2M*/CS-GRP78 participates in transcriptional activation of c-MYC-regulated genes.

C38 mAb strongly inhibited the formation of α2M*-dependent colonies in soft agar. In addition, PDK1 or PLK1 inhibition...
resulted in decreased colony number. Although our experiments do not exclude the possibility that other signaling pathways might cooperate with c-MYC, our data strongly suggest that α2M+/CS-GRP78-dependent PDK1/PLK1/c-MYC signaling induces phosphorylation of histone H3S10 that is necessary to regulate key genes required for c-MYC-dependent cell proliferation. Moreover, the main characteristic of the α2M+/CS-GRP78-induced proliferation is that it is able to induce tumor initiation and progression. This study therefore provides a molecular mechanism for α2M+/CS-GRP78-mediated c-MYC-associated gene regulation and c-MYC-dependent cell proliferation. Because mutations that alter c-MYC expression are among the most common found in human and animal cancers (46), it is conceivable that inhibiting PLK1 association with c-MYC and/or targeting CS-GRP78 by C38 mAb might represent a method for the treatment of c-MYC-driven cancers in human.

An intriguing finding of this study is the identification of α2M+/CS-GRP78-dependent kinase activation upstream to PDK1/PLK1/c-MYC signaling to regulate cell proliferation (Fig. 8). We provide evidence that α2M+/CS-GRP78 signaling activates PDK1 to induce PLK1 phosphorylation for the c-MYC activity. We further show here that PLK1 can directly bind to c-MYC and promote transcriptional activation of FOSL1 and ID2 genes by phosphorylating histone H3S10. Regardless of whether or not PDK1/PLK1 signaling regulates c-MYC stability through a similar or distinct mechanism, the regulation of c-MYC by α2M+/CS-GRP78 signaling immediately suggests a therapeutic approach targeting tumors that are driven by c-MYC. Indeed, our data show a preferential killing by small molecule inhibitors of PDK1 or PLK1, thereby targeting c-MYC in cancer cells. Given that a clinical inhibitor of c-MYC is not available and targeting PLK1 with small molecule inhibitors such as BI2536 had a modest effect in patients with solid tumors (46, 47), this study suggests that therapeutic targeting of CS-GRP78 by C38 mAb may yield a more favorable therapeutic index in c-MYC-associated tumors.

In patients with prostate cancer, the levels of both native and α2M* in serum decrease during disease progression, whereas CS-GRP78 is often found to be overexpressed in human cancer cells when compared with the normal cells (9, 48). Notably, we showed previously that α2M* increases GRP78 expression in cancer cells (40). Prostate cancer cells should readily bind α2M* from serum, thus activating CS-GRP78 to promote metastasis.
CS-GRP78 Signaling Promotes c-MYC Activation

with poor survival. This notion is consistent with a recent report from Mandelin et al. (19) showing in vivo that cell surface occurrence of α2M* and GRP78 is clearly implicated in the development of prostate cancer bone metastasis. In particular, the pathway we identified using multiple approaches in different cancer cell lines validates its relevance in human cancers. Importantly, targeting CS-GRP78 by specific monoclonal antibodies inhibits tumor growth in murine xenograft models of various tumors (12, 15, 37). Collectively, CS-GRP78 has attracted much attention as a potential therapeutic target in cancer. We propose that c-MYC can be an alternative pharmacodynamics marker for the evaluation of C38 mAb under preclinical and clinical development.

Author Contributions—U. G. performed all of the studies contained in this manuscript. M. G.-G. gave advice with respect to experimental design and supplied the peptides used in some of the experiments. S. V. P. and U. G. designed and interpreted the studies reported here. U. G. and S. V. P. wrote the paper. All authors have read and approved the final version of this manuscript.

Acknowledgment—We thank Dr. Cinghu Senthilkumar for providing assistance in designing the ChiP primers.

References
1. Borth, W. (1992) α2-Macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J. 6, 3345–3353
2. Misra, U. K., Deedwania, R., and Pizzo, S. V. (2006) Activation and cross-talk between Akt, NF-κB, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. J. Biol. Chem. 281, 13694–13707
3. Misra, U. K., and Pizzo, S. V. (2012) Receptor-recognized α2-macroglobulin binds to cell surface-associated GRP78 and activates mTORC1 and mTORC2 signaling in prostate cancer cells. PLoS One 7, e51735
4. King, L. S., Berg, M., Chevalier, M., Carey, A., Elguindi, E. C., and Blond, S. Y. (2001) Isolation, expression, and characterization of fully functional nontoxic BiP/GRP78 mutants. Protein Expr. Purif. 22, 148–158
5. Lee, A. S. (2007) GRP78 induction in cancer: therapeutic and prognostic implications. Cancer Res. 67, 3496–3499
6. Luo, S., Mao, C., Lee, B., and Lee, A. S. (2006) GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. Mol. Cell. Biol. 26, 5688–5697
7. Arap, M. A., Lahdenranta, J., Mintz, P. J., Hajitou, A., Sarkis, A. S., Arap, W., and Pasqualini, R. (2004) Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. Cancer Cell 6, 275–284
8. Davidson, D. J., Haskell, C., Majest, S., Kherzai, A., Egan, D. A., Walter, K. A., Schneider, A., Gubbins, E. F., Solomon, L., Chen, Z., Lesniewski, R., and Henkin, J. (2005) K5ringle 5 of human plasminogen induces apoptosis of endothelial and tumor cells through surface-expressed glucose-regulated protein 78. Cancer Res. 65, 4663–4672
9. Gonzalez-Gronow, M., Cuchacovich, M., Llanos, C., Urzua, C., Gawdi, G., and Pizzo, S. V. (2006) Prostate cancer cell proliferation in vitro is modulated by antibodies against glucose-regulated protein 78 isolated from patient serum. Cancer Res. 66, 11424–11431
10. G. S., Folini, M., Pizzo, S. V. (2004) Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. Cancer Cell 6, 275–284
11. Xiao, G., Chung, T. F., Pyun, H. Y., Fine, R. E., and Johnson, R. J. (1999) KDEL proteins are found on the surface of NG108–15 cells. Brain Res. Mol. Brain Res. 72, 121–128
12. Liu, R., Li, X., Gao, W., Zhou, Y., Wey, S., Mitra, S. K., Krasnoperov, V., Dong, D., Liu, S., Li, D., Zhu, G., Louie, S., Conti, P. S., Li, Z., Lee, A. S., and Gill, P. S. (2013) Monoclonal antibody against cell surface GRP78 as a novel agent in suppressing PI3K/AKT signaling, tumor growth, and metastasis. Clin. Cancer Res. 19, 6802–6811
13. Lee, A. S. (2014) Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. Nat. Rev. Cancer 14, 263–276
14. Rasche, L., Duell, J., Morgner, C., Chatterjee, M., Hensel, F., Rosenwald, A., Einsele, H., Topp, M. S., and Brändlein, S. (2013) The natural human IgM antibody PAT-SM6 induces apoptosis in primary human multiple myeloma cells by targeting heat shock protein GRP78. PLoS One 8, e63414
15. Mo, L., Bachelder, R. E., Kennedy, M., Chen, P. H., Chi, J. T., Berchuck, A., Cianciolo, G., and Pizzo, S. V. (2015) Synergic murine ovarian cancer model reveals that ascites enriches for ovarian cancer stem-like cells expressing membrane GRP78. Mol. Cancer Ther. 14, 747–756
16. Rauschert, N., Brändlein, S., Holzinger, E., Hensel, F., Müller-Hermelink, H. K., and Vollmers, H. P. (2008) A new tumor-specific variant of GRP78 as target for antigen-based therapy. Lab. Invest. 88, 375–386
17. Pootrakul, M., Datar, R. H., Shi, S. R., Cai, J., Hawes, D., Groschen, S. G., Lee, A. S., and Cote, R. J. (2006) Expression of stress response protein GRP78 is associated with the development of castration-resistant prostate cancer. Clin. Cancer Res. 12, 5987–5993
18. Roller, C., and Maddalo, D. (2013) The molecular chaperone GRP78/BiP in the development of chemoresistance: mechanism and possible treatment. Front. Pharmacol. 4, 10
19. Mandelin, J., Cardó-Vila, M., Driessen, W. H., Mathew, P., Navone, N. M., Lin, S. H., Logothetis, C. J., Rietz, A. C., Dobroff, A. S., Proneth, B., Sidman, R. L., Pasqualini, R., and Arap, W. (2015) Selection and identification of ligand peptides targeting a model of castrate-resistant osteogenic prostate
cancer and their receptors. Proc. Natl. Acad. Sci. U.S.A. 112, 3776–3781
20. Zhang, Y., Tseng, C. C., Tsai, Y. L., Fu, X., Schiff, R., and Lee, A. S. (2013) Cancer cells resistant to therapy promote cell surface relocalization of GRP78 which complexes with PI3K and enhances PI(3,4,5)P3 production. PLoS One 8, e80071
21. Misra, U. K., Mowery, Y., Kaczowka, S., and Pizzo, S. V. (2009) Ligation of cancer cell surface GRP78 with antibodies directed against its COOH-terminal domain up-regulates p53 activity and promotes apoptosis. Mol. Cancer Ther. 8, 1350–1362
22. Misra, U. K., and Pizzo, S. V. (2014) Activated α₂-macroglobulin binding to cell surface GRP78 induces T-loop phosphorylation of Akt1 by PDK1 in association with Raptor. PLoS One 9, e88373
23. Misra, U. K., and Pizzo, S. V. (2010) Ligation of cell surface GRP78 with antibody directed against the COOH-terminal domain of GRP78 suppresses Ras/MAPK and PI 3-kinase/akt signaling while promoting caspase activation in human prostate cancer cells. Cancer Biol. Ther. 9, 142–152
24. Peifer, C., and Alessi, D. R. (2008) Small-molecule inhibitors of PDK1. ChemMedChem 3, 1810–1838
25. Tan, J., Li, Z., Lee, P. L., Guan, P., Aau, M. Y., Lee, S. T., Feng, M., Lim, C. Z., Lee, E. Y., Wei, Z. N., Lim, Y. C., Karuturi, R. K., and Yu, Q. (2013) PDK1 signaling toward PLK1-MYC activation confers oncogenic transformation, tumor-initiating cell activation, and resistance to mTOR-targeted therapy. Cancer Discov. 3, 1156–1171
26. Dang, C. V., O’Donnell, K. A., Zeller, K. I., Nguyen, T., Osthus, R. C., and Li, F. (2006) The c-Myc target gene network. Semin. Cancer Biol. 16, 253–264
27. Lin, C. Y., Lovén, J., Rahl, P. B., Paranal, R. M., Burge, C. B., Bradner, J. E., Lee, T. I., and Young, R. A. (2012) Transcriptional amplification in tumor cells with elevated c-Myc. Cell 151, 56–67
28. Nie, Z., Hu, G., Wei, G., Cui, K., Yamanee, A., Resch, W., Wang, R., Green, D. R., Tessarollo, L., Casellas, R., Zhao, K., and Levens, D. (2012) c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell 151, 68–79
29. Sabò, A., Kress, T. R., Pelizzola, M., de Pretis, S., Gorski, M. M., Tesi, A., Morelli, M. J., Bora, P., Doni, M., Verrecchia, A., Tonelli, C., Fagì, G., Bianchi, V., Ronchi, A., Low, D. M., Muller, H., Guccione, E., Campanier, S., and Amati, B. (2014) Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. Nature 411, 488–492
30. Hann, S. R. (2006) Role of post-translational modifications in regulating c-Myc proteolysis, transcriptional activity and biological function. Semin. Cancer Biol. 16, 288–302
31. Misra, U. K., Payne, S., and Pizzo, S. V. (2011) Ligation of prostate cancer cell surface GRP78 activates a proproliferative and antiapoptotic feedback loop: a role for secreted prostate-specific antigen. J. Biol. Chem. 286, 1248–1259
32. de Ridder, G. G., Ray, R., and Pizzo, S. V. (2012) A murine monoclonal antibody directed against the carboxyl-terminal domain of GRP78 suppresses melanoma growth in mice. Melanoma Res. 22, 225–235
33. Zippo, A., De Robertis, A., Serafini, R., and Oliviero, S. (2007) PIM1-dependent phosphorylation of histone H3 at serine 10 is required for MYC-dependent transcriptional activation and oncogenic transformation. Nat. Cell Biol. 9, 932–944
34. Cole, M. D., and Cowling, W. H. (2008) Transcription-independent func-

tions of MYC: regulation of translation and DNA replication. Nat. Rev. Mol. Cell Biol. 9, 810–815
35. Cannell, I. G., Kong, Y. W., Johnston, S. J., Chen, M. L., Collins, H. M., Doblyn, H. C., Elia, A., Kress, T. R., Dickens, M., Clemens, M. J., Heery, D. M., Gaestel, M., Eilers, M., Willis, A. E., and Bushell, M. (2010) p38 MAPK/MAPK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. Proc. Natl. Acad. Sci. U.S.A. 107, 5375–5380
36. Tan, J., Lee, P. L., Li, Z., Jiang, X., Lim, Y. C., Hooi, S. C., and Yu, Q. (2010) B55β-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer. Cancer Cell 18, 459–471
37. Lin, Y. G., Shen, J., Yoo, E., Liu, R., Yen, H. Y., Mehta, A., Rajaei, A., Yang, W., Mhawech-Fauceglia, P., DeMayo, F. J., Lydon, J., Gill, P., and Lee, A. S. (2015) Targeting the glucose-regulated protein-78 abrogates Pten-null driven AKT activation and endometrioid tumorigenesis. Oncogene 34, 5418–5426
38. Zhang, C., Zhang, S., Zhang, Z., He, J., Xu, Y., and Liu, S. (2014) ROCK has a crucial role in regulating prostate tumor growth through interaction with c-Myc. Oncogene 33, 5582–5591
39. Liu, S., Goldstein, R. H., Scepansky, E. M., and Rosenblatt, M. (2009) Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone. Cancer Res. 69, 8742–8751
40. Misra, U. K., Wang, F., and Pizzo, S. V. (2009) Transcription factor TFII-I causes transcriptional upregulation of GRP78 synthesis in prostate cancer cells. J. Cell. Biochem. 106, 381–389
41. Strahl, B. D., and Allis, C. D. (2000) The language of covalent histone modifications. Nature 403, 41–45
42. Nowak, S. J., and Corces, V. G. (2004) Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. Trends Genet. 20, 214–220
43. Solano, A., Thomson, S., Wiggan, G. R., Rampersaud, N., Dyson, M. H., Hazzali, C. A., Mahadevan, L. C., and Arthur, J. S. (2003) MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. EMBO J. 22, 2788–2797
44. Sassone-Corsi, P., Mizzen, C. A., Cheung, P., Croso, C., Monaco, L., Jacquot, S., Hanauer, A., and Allis, C. D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. Science 285, 886–891
45. Fernandez, P. C., Frank, S. R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003) Genomic targets of the human c-Myc protein. Genes Dev. 17, 1115–1129
46. Cole, M. D., and McMahon, S. B. (1999) The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation. Oncogene 18, 2916–2924
47. Hofheinz, R. D., Al-Batran, S. E., Hochhaus, A., Jäger, E., Reichardt, V. L., Fritsch, H., Trommshausen, D., and Munzert, G. (2010) An open-label, phase I study of the polo-like kinase-1 inhibitor, BI 2536, in patients with advanced solid tumors. Clin. Cancer Res. 16, 4666–4674
48. Sinnreich, O., Kratzsch, J., Reichenbach, A., Gläser, C., Huse, K., and Birkenmeier, G. (2004) Plasma levels of transforming growth factor-1 are elevated in patients with prostate cancer and their receptors. Proc. Natl. Acad. Sci. U.S.A. 112, 3776–3781

CS-GRP78 Signaling Promotes c-MYC Activation

JOURNAL OF BIOLOGICAL CHEMISTRY

MAY 13, 2016 • VOLUME 291 • NUMBER 20

10915

Downloaded from http://www.jbc.org/ by guest on July 26, 2018
Activated α2-Macroglubulin Regulates Transcriptional Activation of c-MYC Target Genes through Cell Surface GRP78 Protein

Udhayakumar Gopal, Mario Gonzalez-Gronow and Salvatore Vincent Pizzo

*J. Biol. Chem.* 2016, 291:10904-10915. doi: 10.1074/jbc.M115.708131 originally published online March 21, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M115.708131

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 19 of which can be accessed free at http://www.jbc.org/content/291/20/10904.full.html#ref-list-1