Binding of activated forms of the proteinase inhibitor α2-macroglobulin (α2M*) to cell-surface-associated GRP78 on 1-LN human prostate cancer cells causes their proliferation. We have now examined the interplay between Akt activation, regulation of apoptosis, the unfolded protein response, and activation of NF-κB in α2M*-induced proliferation of 1-LN cells. Exposure of cells to α2M* (50 μM) induced phosphorylation of NF-κB B inhibitor IκB, inducing translocation of NF-κB to the nucleus, where it up-regulates the transcription of anti-apoptotic genes, such as the inhibitors of apoptosis proteins (IAPs) and Bcl-2 (8–13). The molecular chaperone GRP78 (glucose-regulated protein of 78 kDa) is important in the folding, maturation, and transport of proteins out of the cell. GRP78 is critical for the unfolded protein response (UPR) required to alleviate ER “stress,” maintain ER function, and protect cells against death (14–17). Whereas GRP78 is constitutively expressed, its synthesis is induced by stressful conditions that perturb protein folding and assembly within the ER (14–17). A small pool of this newly synthesized GRP78 translocates to the cell surface from the ER in association with MTJ-1 (18), where it functions as a co-receptor for viruses and major histocompatibility complex Class I antigen presentation and as a receptor for activated forms of the plasma proteinase inhibitor α2-macroglobulin (α2M*) (see Refs. 18–21 and references therein). Activation of this receptor on the surface of 1-LN human prostate cancer cells by α2M* triggers proliferative and antiapoptotic behavior; therefore, it could be argued that up-regulation of cell-surface-associated GRP78 is part of the aggressive phenotype in prostate cancer (22). Consistent with this hypothesis, autoantibodies against GRP78 appear in the sera of prostate cancer patients, and they are a biomarker of aggressive behavior (23, 24). The circulating concentration of α2M is about 2–5 μM, and its proteinase activated form, α2M*, may comprise approximately a 200–500 nm concentration of this pool (25). Prostate cancer cells themselves may produce prostate cancer-specific antigen, a proteinase that binds readily to α2M, converting it to α2M* (26, 27). Aggressive prostate cancers also produce matrix metalloproteinases, which readily convert α2M to α2M*. Therefore, it could be envisaged that under the conditions that exist in patients harboring prostate cancer, a substantial amount of α2M* is available to bind to cell-surface-associated GRP78, thus triggering the activation of mitogenic signaling and promoting cellular proliferation.

The activation of all three MAPK cascades (namely extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK) occurs in cells undergoing ER stress (28, 28–33, 35–39). ASK1 apoptosis; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase (JNK), and p38 MAPK) occurs in cells undergoing ER stress (28, 28–33, 35–39). ASK1...
(apoptosis signal-regulating kinase 1) is a Ser/Thr kinase that activates both the p38 MAPK and JNK pathways by directly phosphorylating MKK3/MKK6 and MKK4/MKK7, respectively (28, 39–42). ASK1 is activated by a variety of stimuli, including TNFα, ER stress, and calcium signaling consequent to ligation of G-protein-coupled receptors (39–42). The antiapoptotic activity of Bcl-2 is reduced when it is phosphorylated by activated ASK1 (28, 41). On the other hand, overexpression of ASK1 may induce not only apoptosis but cell differentiation and survival, depending on the cell type and cellular context (28, 41, 42). TNFα is a potent activator of ASK1, and this activation is regulated by TRAF2 (TNF receptor-associated factor 2) (28, 40–42). TRAF2 is an adaptor protein that couples TNFα receptor ligation to activation of the ASK1/JNK/p38 MAPK cascades (28, 40–42).

Prolonged ER stress can activate apoptosis both by mitochondria-dependent and -independent pathways (14–17). Bad, a proapoptotic member of the Bcl-2 family, triggers release of cytochrome c from mitochondria (see Ref. 43 and references therein), and this activates cytosolic apoptotic proteinase-activating factor 1, thus activating the downstream caspase-dependent proapoptotic cascade (43, 44). UPR-induced free calcium elevation activates calpains, which cleaves procaspase-12, an ER stress-specific caspase (14–17, 45, 50). Once activated during ER stress, the catalytic subunit of procaspase-12 is released into the cytosol, where it activates the caspase-9 cascade in a cytochrome c-independent manner. Procaspase-12 can also be activated by caspase-7 or through IRE1/TRAF2-dependent pathways (16, 29, 49–51).

NF-κB regulates the transcription of many genes involved in stress remediation, cell growth, and apoptosis (52–55). In the inert state, NF-κB is present in the cytoplasm in association with the inhibitory protein, IκB. Activation of NF-κB occurs in response to a variety of stressful conditions, where impaired protein folding in the ER is characteristic (52–55). These stimuli activate the IκB kinase (IKK) β subunit, which phosphorylates NF-κB-bound IκB and targets it for ubiquitin-dependent degradation, allowing liberated NF-κB dimers to translocate to the nucleus (53). In an alternative pathway, the IKK α subunit of the IKK complex processes NF-κB2 precursor protein, which preferentially binds Reib in the cytoplasm, resulting in the release of Reib-p52 dimers (55). Persistent NF-κB activation is detected in many solid tumors, and its inhibition increases their sensitivity to chemotherapeutic agents and radiation (54). Overexpression of NF-κB suppresses apoptosis, whereas IκB-α mutants, which can no longer be phosphorylated, are resistant to ubiquitin-mediated degradation and suppress NF-κB activation, thus sensitizing cells to apoptotic insults (16, 52–55). IRE1 is also involved in the activation of NF-κB induced by ER stress (52–55). Activation of NF-κB by ER stress-inducing agents is inhibited by dominant negative IRE1 (50) or TRAF2 mutants (56). A recent report demonstrates that UV light-induced activation of NF-κB is due to the PERK-induced phosphorylation of eIF2α, which causes translational inhibition of new IκB synthesis (57).

In the present report, we have examined the role of Akt activation, regulation of apoptosis, and the unfolded protein response in αM* induced growth of 1-LN prostate cancer cells. We report an increase in Akt Thr-308 and Akt Ser-473 kinase activities, up-regulation of UPR components, including IRE1α, XBP-1, ATF6, GRP78, p-PERK, p-eIF2α, GADD34 (growth arrest and DNA damage-inducible 34), and ATF4, as well as up-regulation of components of the anti-apoptotic machinery Bcl-2, p-FOXO1, NF-κB, GADD45β, and XIAP in 1-LN prostate cancer cells treated with αM* (58). We have also studied the role of GRP78 in αM*-induced activation of cell survival and antiapoptotic signaling by silencing GRP78 gene expression employing RNAi.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture media were purchased from Invitrogen. αM* was prepared as described previously (18, 19). Antibodies against Akt protein, p-AktThr-308, p-AktSer-473, phosphorylated and unphosphorylated ERK1/2, p38 MAPK, JNK, ASK1, MKK3/6, MKK4, MKK7, eIF2α, FOXO, GSK3β, p-PERK, TRAF2, NF-κB (p65), NF-κBp2 (p52), IκKα/β, IκB-α, IκB-β, NIK, Bcl-2, XIAP, p27Kip1, cyclin D1, cleaved caspase-3, procaspase-9, and procaspase-12, used in this study were procured from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against IRE1, XBP1, ATF6, ATF4, GADD153, GADD34, GADD45β, 14–3–3, and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GRP78 antibodies and glyceraldehyde-3-phosphate dehydrogenase antibodies were from Stressgen (Victoria, Canada). [γ-32P]ATP (specific activity 3000 Ci mmol) was purchased from PerkinElmer Life Sciences. The peptide substrates for AktSer-473 (NH2-RRPHQPFSASYA-COOH) and for AktThr-308 kinase (NH2-KTFCGTPEYLAPEVRR-COOH) were synthesized by Genemed (South San Francisco, CA). Control substrate peptide Zak3tide and glutathione S-transferase-IκB-α substrate (amino acids 1–54) were purchased from Upstate Cell Signaling Solutions (Charlottesville, VA). Other reagents were of the highest available grade.

**Western Blotting of Activated Akt in αM*-stimulated 1-LN Prostate Cancer Cells**—The highly metastatic prostate carcinoma cell line 1-LN, derived from less metastatic PC-3 cells, was a kind gift from Dr. Philip Nalther (Duke University Medical Center, Durham, NC). 1-LN cells in 6-well plates (500 × 103 cells/well) were allowed to grow to confluence in RPMI 1640 medium containing 10% FBS, penicillin (12.5 units/ml), streptomycin (6.5 μg/ml), 2 mM glutamine, and 10 nM insulin at 37 °C in a humidified CO2 (5%) incubator. At about 90% confluence, the medium was aspirated, the monolayers were washed with ice-cold Hapes-buffered Hanks’ basic salt solution, pH 7.4, and a fresh volume of medium was added to the monolayers. The cells were exposed to αM* (50 pm) for varying periods of time. The reaction was stopped by aspirating the medium, and a volume of lysis buffer containing 50 mM Tris HCl (pH 7.5), 120 mM NaCl, 1% (v/v) 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 8000 × g for 10 min at 4 °C, and their protein contents were determined (58). In each case an equal amount of protein was used for electrophoresis. The immunoblotting of membranes with antibodies specific for phosphorylation of Akt in Thr-308 or Ser-473 was performed according to the manufacturer’s instructions. The detection and quantification of immunoblots were performed by ECF and phosphorimaging. The membranes were reprobed for the protein loading control actin.

**Measurement of Phosphorylation of Akt at Thr-308 and Ser-473 and Their Kinase Activities in Cells Stimulated with αM**—The p-AktThr-308 and p-AktSer-473 kinase activities in Akt immunoprecipitates of 1-LN prostate cancer cells stimulated with αM* (50 pm/15 min) were assayed essentially according to Hill and Jennings (59). Briefly, 1-LN cells (in two 6-well plates, 4 × 106 cells/well) were incubated as above until confluence. The monolayers were washed in Hapes-buffered Hanks’ basic salt solution, pH 7.4, twice, and a fresh volume of incubation medium was added to each well. The monolayers were stimulated with buffer or αM* (50 pm/15 min) in triplicate. 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% (v/v) 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 to each incubation. The cells were lysed for...
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10 min over ice, scraped into tubes, and centrifuged at 8000 × g for 10 min at 4 °C, and their protein contents were determined (58). Equal amounts of lysate proteins were immunoprecipitated with Akt antibodies (1:50) at 4 °C overnight with gentle rotations, according to the manufacturer’s instruction. Akt immunoprecipitates were washed sequentially with lysis buffer supplemented with 0.5 M NaCl, lysis buffer, and 50 mM Tris-HCl (pH 7.4) supplemented with 1 mM dithiothreitol, 1 mM PMSF, and 1 mM benzamidine with centrifugation at 8000 × g for 5 min at 4 °C employed between each wash. To each immunoprecipitate, 40 μl of cold kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 20 μg/ml leupeptin was added, followed by the addition of 30 μM AktThr-328 kinase substrate peptide (NH2-RRHPFQFSYSA-COOH) in respective tubes. Zak3tide (NH2-GGEEEEYELKKK-COOH) was used as an unrelated peptide.

The reaction was initiated by adding 50 μM ATP and 2 μCi of [γ-32P]ATP in each tube, and the tubes were incubated for 30 min at 30 °C with shaking. The reaction was stopped by adding 5 μl of 0.5 M EDTA to each tube, the tubes were centrifuged at 3000 rpm for 3 min, and 40 μl of each supernatant was applied on p81 phosphocellulose paper (Whatman). Supernatants were allowed to dry, and the papers were washed four times each by immersing them in a liter of 1 n phosphoric acid for 3 min. The papers were rinsed with acetone, and their radioactivity was counted in a liquid scintillation counter (22).

Western Blotting of p-ERK1/2Thr-202/Tyr-204, p-MKK3/6, p-MKK4/7Thr-218/Tyr-202, p-MAPK, p-JNK, p-ASKThr-83, and TRAF2 in aspirating the medium, and a volume of lysis buffer containing 50 mM PMSF, 1 mM benzamidine, and leupeptin was added, followed by the addition of 30 μM AktThr-308 kinase substrate peptide (NH2-RRHPFQFSYSA-COOH) in respective tubes. Zak3tide (NH2-GGEEEEYELKKK-COOH) was used as an unrelated peptide.

The reaction was initiated by adding 50 μM ATP and 2 μCi of [γ-32P]ATP in each tube, and the tubes were incubated for 30 min at 30 °C with shaking. The reaction was stopped by adding 5 μl of 0.5 M EDTA to each tube, the tubes were centrifuged at 3000 rpm for 3 min, and 40 μl of each supernatant was applied on p81 phosphocellulose paper (Whatman). Supernatants were allowed to dry, and the papers were washed four times each by immersing them in a liter of 1 n phosphoric acid for 3 min. The papers were rinsed with acetone, and their radioactivity was counted in a liquid scintillation counter (22).

Measurement of the Effects of Treatment of 1-LN Prostate Cancer Cells with αM* on Lip-regulation of Bcl-2, p-BadSer-136, p-FOXO1Ser-256, p-ASKThr-83, and Cyclin D1 by Western Blotting—The experimental details of incubating 1-LN prostate cancer cells with αM*, their lysis, and electrophoresis were identical to those described above. The respective membranes were immunoblotted with antibodies specific for Bcl-2, p-BadSer-136, p-FOXO1, p-ASKThr-83, and Cyclin D1, respectively. The membranes were reprobed for FOXO1, Bad, GSK3βSer-9, and actin as the protein loading control. The detection and quantification of immunoblots were performed by ECF and phosphorimaging.

Measurement of Activation of NF-κB (p65), NF-κB (p52), p-IKKα/βSer-176/180, p-IκBαSer-32, and NIK in 1-LN Cells Stimulated with αM* by Western Blotting—In an experiment identical to the preceding one, cell lysates from buffer and αM*-stimulated 1-LN cells were electrophoresed and transferred to membranes, and the respective membranes were probed for NF-κB (p65), NF-κB (p52), p-IKKα/β, IκB-α, p-IκB-β, p-RelA, and NIK, respectively by Western blotting.

Measurement of the Effects of Treatment of 1-LN Prostate Cancer Cells with αM* on Activation of Caspases by Western Blotting—The experimental details of incubating 1-LN prostate cancer cells with αM*, their lysis, and electrophoresis were identical to those described above. The respective membranes were immunoblotted with antibodies specific for procaspase-12, procaspase-9, and cleaved caspase-3, respectively. Since the antibody is specific for mouse procaspase-12, we performed a study to demonstrate that this antibody cross-reacts with the human protein. For these studies, murine macrophages were compared with human 1-LN and PC-3 prostate cancer cells to demonstrate that the anti-murine antibody recognizes human procaspase-12. This study is shown as a control in Fig. 3. The membranes were reprobed for actin, a protein loading control. The detection of immunoblots was performed by ECF and phosphorimaging.
Measurement of the Effects of α,M arb treatment of 1-LN prostate cancer cells on up-regulation of the components of the UPR signaling pathway by Western Blotting—The experimental details of incubating 1-LN prostate cancer cells with α,M arb, their lysate, and electrophoresis were identical to those described above. The respective membranes were immunoblotted with antibodies specific for GRP78, IRE1α, XBP-1, ATF6, p-ERK1/2Thr-202/Tyr-204, p-AktSer-473; p-eIF2αSer-51, ATF4, GADD34, and GADD153, respectively (37). The membranes were reprobed for eIF2α and actin as the protein loading control. The detection of immunoblots was performed by ECF and phosphorimaging.

Measurement of the Effects of PI 3-Kinase Inhibitor LY294002 on α,M arb-induced up-regulation of p-Akt, BadSer-136, p-FOXO1, p-GSK3β, NF-κB (p65), NF-κB2 (p52), p-IKKα/β, p-IκB-α, NIK, and p-ASK1 in 1-LN Stimulated Cells by Western Blotting—The experimental details of incubating 1-LN prostate cancer cells with α,M arb were as described above except that the cells were preincubated with the PI 3-kinase inhibitor LY294002 (20 μM/20 min) before adding α,M arb. The reaction was terminated by aspirating the medium. The details of cell lysis and lystate electrophoresis were as described above. The respective membranes were immunoblotted with antibodies specific for p-AktThr-308, p-AktSer-473, p-BadSer-136, p-FOXO1, p-ASK1, NF-κB (p65), NF-κB2 (p52), p-IKKα/β, p-IκB-α, NIK, and p-GSK3β, respectively.

Chemical Synthesis of dsRNA Homologous in Sequence to the Target GRP78 Gene—The chemical synthesis of dsRNA homologous in sequence to the target GRP78 peptide sequence KIQQLVK376, mRNA sequence 5'-AAA ATA CAG CAA TTA GTA AAG-3' (Swiss-Prot GRP primary sequence accession number P11022), was performed by Ambion (Austin, TX). For making dsRNA of the sense 5'-AAU UAC UAA UUG CUG UAU UTT-3' and antisense 5'-CUU UAC UAA UUG CUG UAU UTT-3', oligonucleotides were annealed according to the manufacturer’s instructions. Throughout the studies, handling of reagents was performed in an RNase-free environment. The protocol is described in detail elsewhere (20, 22). Briefly, equal amounts of sense and antisense oligonucleotides were mixed in annealing buffer and heated at 90 °C for 2 min and then maintained for 1 h at 37 °C in an incubator. The dsRNA preparation was stored at −20 °C before use.

Transfection of 1-LN Cells with dsRNA Homologous in Sequence to the GRP78 Gene—Silencing of GRP78 gene expression was performed as previously described (18–22). We have used the following protocol for silencing the expression of the GRP78 gene in macrophages and 1-LN cells. This protocol results in 60–70% suppression of GRP78 gene expression as quantified by GRP78 mRNA and protein levels. This suppression of GRP78 gene expression also causes a concomitant loss of cell surface-anchored GRP78 as measured by receptor binding, as well as
abrogation of αM* -induced L,4,5-trisphosphate synthesis, elevation of [Ca^{2+}]], DNA synthesis, and mitogenic signaling (19–22). Confluent 1-LN cell monolayers (1.5 × 10^6/well in 6-well plates) in quadruplicate, incubated as described above, were washed twice with Hank's balanced salt solution, and 2 ml of DMEM containing 10% of FBS and antibiotics was added. The cells were incubated as above for 16 h. Just before each transfection, 25 μg of GRP78 dsRNA was diluted to 100 μl of serum- and antibiotic-free DMEM in a tube. In another tube, 10 μl of Lipofectamine was diluted into 100 μl of serum- and antibiotic-free medium. The two solutions were combined, mixed gently, and incubated for 45 min at room temperature, followed by the addition of 800 μl of serum- and antibiotic-free medium to each tube. The monolayers were washed twice with serum-antibiotic-free DMEM, layered in each well with 1 ml of Lipofectamine-DMEM or lipid dsRNA mixtures containing 25 μg of dsRNA of GRP78 target mRNA gently mixed, and incubated for 5 h at 37 °C in a humidified CO2 incubator in separate experiments. At the end of the incubation, 1 ml of antibiotic-free DMEM containing 10% FBS was added to each well, and the cells were incubated for 16 h. The medium was replaced with DMEM containing antibiotics and 10% FBS 24 h following the start of the transfection. The monolayers were incubated for a further 24 h as above. At the end of the incubation, the medium was aspirated, and the monolayers were washed with the above medium once. A volume of the same medium was added, and the cells were used for the experiment outlined below. To demonstrate that the transfection of 1-LN prostate cancer cells with dsRNA homologous in sequence to the target GRP78 gene does not produce any nonspecific effects on target gene expression, the 1-LN cells were transfected with equimolar concentrations of scrambled small interference RNA (Silencer<sup>TM</sup> negative control, catalog number 4610; Ambion) under identical conditions as described above for transfection with GRP78 dsRNA. At the end of the transfection period (48 h), the medium was aspirated, and a volume of DMEM was added. The cells were stimulated either with buffer or αM* (50 pm) for 15 min. The reaction was stopped by aspirating the medium and adding a volume of lysis buffer as described above. As stated above, this protocol suppresses the expression of GRP78 gene expression by 60–70% as measured by GRP78 mRNA and protein levels (19–22). Equal amounts of lysate protein as determined by Bradford (58) were employed for the Akt kinase assay and IKK<sub>e</sub> kinase assays as well as electrophoresis. We have previously demonstrated that this protocol does not significantly alter cell viability (20, 22).

Assay of Akt and IKK<sub>e</sub> Kinase Activities in 1-LN Cells Transfected with GRP78 dsRNA and Stimulated with αM*—p-Akt<sup>Thr-308</sup>, p-Akt<sup>Ser-473</sup>, and IKK<sub>e</sub> kinase activities were determined in Akt or IKK<sub>e</sub> immunoprecipitates from Lipofectamine plus buffer, Lipofectamine plus αM* (50 pm/15 min), GRP78 dsRNA-transfected plus αM*, or scrambled dsRNA + αM*-treated cells as described.

Measurement of the Effects of Transfecting 1-LN Cells with GRP78 dsRNA on Activation of NF-κB1, NF-κB2, pIKK-α, and pIKB-α after αM* Stimulation—Equal amounts of lysate protein from 1-LN cells treated with Lipofectamine plus buffer, Lipofectamine plus αM* (50 pm/15 min), GRP78 dsRNA plus αM* (50 pm/15 min), or GRP78 dsRNA plus αM* were electrophoresed, and protein bands were transferred to membranes. After immunoblotting with the respective antibodies, the immunoblots were visualized and quantified by ECF and phosphorimaging. The immunoblots were reprobed for actin as a protein loading control.

Measurements of the Effects of Transfecting 1-LN Cells with GRP78 dsRNA on UPR Signaling Components after αM*—Equal amounts of lysate protein from 1-LN cells treated with Lipofectamine plus buffer, Lipofectamine plus αM* (50 pm/15 min), GRP78 dsRNA + αM*, or scrambled dsRNA + αM* were electrophoresed, and protein bands were transferred to membranes. The membranes were immunoblotted with antibodies againstIRE1α, XBP-1, ATF6, p-PERK, p-eIF2α, ATF4, GADD34, or GADD153, respectively. The immunoblots were visualized and quantified by ECF and phosphorimaging as described above. The immunoblots were reprobed for actin as a protein loading control.

RESULTS

αM* Up-regulates Activation of Akt in 1-LN Cells in a PI 3-Kinase-dependent Manner—Activation of Akt is dependent upon PI 3-kinase activation, and disregulation of Akt activation is implicated in the behavior of malignant tumors (5–7). Upon stimulation, cytosolic Akt is recruited to the plasma membrane through the binding of its NH<sub>2</sub>-terminal plekstrin homology domain to the lipid product of PI 3-kinase activation (5–7). Akt is then activated by phosphorylation on two residues, namely Thr-308 in the activation loop and Ser-473 in the hydrophobic motif of the COOH-terminal tail. Phosphorylation of both residues is required for its full activation (60–62). 3'-Phosphatidylinositol-dependent kinase 1 is a plekstrin homology domain-containing kinase that phosphorylates Akt at Thr-308 (5, 6); however, the identity of the kinase responsible for phosphorylating Akt at Ser-473 is disputed (60–62). We have previously demonstrated that expression of GRP78 on the cell surface is essential for αM*-dependent signal transduction in both macrophages and human prostate cancer (18–22). We have characterized the identity of cell surface synthesis, cellular proliferation, and mitogenic signaling upon binding of αM* to GRP78 in a variety of prostate cancer cells of differing metastatic potential as well as in macrophages (18–22). These postreceptor events are drastically inhibited by prior treatment of cells with antibodies against GRP78 or silencing GRP78 gene expression by RNA interference (18–22). Human prostate cancer cells lacking GRP78 on their cell surface do not demonstrate αM*-dependent signal transduction (18, 20). This includes PC-3 cells, the parent line for 1-LN cells. Thus, PC-3 cells do not express GRP78 on the cell surface and do not demonstrate αM*-dependent activation of signaling cascades. We therefore focused the present studies on the 1-LN cell line. Treatment of 1-LN prostate cancer cells with αM* (50 pm) elevated both p-Akt<sup>Thr-308</sup> and p-Akt<sup>Ser-473</sup> at about 5 min, and these levels remained elevated during 60 min of incubation (Fig. 1A). The kinetics of activation of both p-Akt<sup>Thr-308</sup> and p-Akt<sup>Ser-473</sup> were comparable in αM*-treated 1-LN prostate cancer cells (Fig. 1A). αM*-induced phosphorylation of Akt at Thr-308 and Ser-473 was greatly reduced by prior treatment of cells with LY294002, a specific inhibitor of PI3-kinase (Fig. 1B), demonstrating that activation of Akt is PI3-kinase dependent. We further demonstrated αM*-induced phosphorylation of Akt at Thr-308 and Ser-473 in 1-LN cells by assaying their kinase activities using specific kinase peptide substrates (Fig. 1C). αM* treatment caused a nearly equal activation of both p-Akt<sup>Thr-308</sup> kinase and p-Akt<sup>Ser-473</sup> kinase which corroborated the Western blotting data (Fig. 1A). The results presented show that αM*, like growth factors,
induces mitogenic and cell survival signaling in 1-LN prostate cancer cells.

Suppression of GRP78 Gene Expression Inhibits $\alpha_M^*$-induced Activation of Akt$^{\text{Thr}-308}$ and Akt$^{\text{Ser}-473}$ Kinases in 1-LN Cells—In the next series of experiments, we examined the role of ligating 1-LN prostate cancer cell surface-associated GRP78 with $\alpha_M^*$ on activation of Akt kinase activities and downstream signaling by silencing GRP78 gene expression. We have shown that silencing GRP78 gene expression reduces its mRNA and protein levels by about 50–60% in macrophages and 1-LN prostate cancer cells (20, 22). This level of suppression disproportionately reduces signal transduction events in these cells (20–22), consistent with our observation that there is a threshold level of GRP78 that must be present on the cell surface before the cells can respond to $\alpha_M^*$ (63). Silencing of GRP78 gene expression inhibited the activities of Akt$^{\text{Thr}-308}$ and Akt$^{\text{Ser}-473}$ kinases in $\alpha_M^*$-treated 1-LN cells to nearly basal levels (Fig. 2). Under identical experimental conditions, transfection of 1-LN cells with scrambled dsRNA showed negligible effects on $\alpha_M^*$-induced Akt kinase activities (Fig. 2). These studies demonstrate that binding of $\alpha_M^*$ to cell surface-associated GRP78 is involved in activating downstream cell survival signaling in 1-LN prostate cancer cells.

$\alpha_M^*$ Up-regulates Activation of ERK1/2 in 1-LN Prostate Cancer Cells—The activation of surface receptors leads to the activation of Ras, a membrane-resident GTPase, which recruits Raf kinase from the cytosol to the cell membrane. Most tumors demonstrate sustained and elevated activation of the Raf-MEK-ERK pathway. This pathway promotes cell survival by activating antiapoptotic mechanisms. In the MEK-dependent pathway, activated ERK activates 90-kDa ribosomal S6 kinase, which phosphorylates and inactivates proapoptotic Bad (16, 33, 35, 64–70). 90-kDa ribosomal S6 kinase also activates the transcription factor CREB, which promotes cell survival by up-regulating Bcl-2 (20). In 1-LN prostate cancer cells, $\alpha_M^*$ treatment promotes cell proliferation as evidenced by increased DNA synthesis and increased cell numbers (20). To assess the role of the MEK-ERK pathway in 1-LN cancer cells, we determined the effect of $\alpha_M^*$ treatment on activation of ERK1/2, Akt kinases, and IKK kinases (Fig. 3). Shown are p-IKK$\alpha/\beta$ (white bars) and p-ERK (black bars) from cells treated as above. The results are expressed in arbitrary units and are the mean ± S.E. from triplicate experiments. A representative immunoblot is shown below the graph. * values significantly different from corresponding buffer-treated and GRP78 dsRNA-transfected cells and cells treated with 2M*. Seven 3A, 7B, 7C, and 7D represent the effect of transfection of 1-LN cells with GRP78 dsRNA on $\alpha_M^*$-induced activation of Akt kinases and IKK kinase and up-regulation of NF-κB activation. For experimental details, see “Experimental Procedures.” A, Akt$^{\text{Thr}-308}$ kinase (white) and Akt$^{\text{Ser}-473}$ kinase (black) activities in 1-LN cells treated with Lipofectamine plus buffer (1), Lipofectamine plus $\alpha_M^*$ (50 ps/15 min) (2), GRP78 dsRNA plus $\alpha_M^*$ (3), and scrambled dsRNA and $\alpha_M^*$ (4). The kinase activities are shown as the mean ± S.E. from two experiments performed in triplicate and are expressed as pmol of [γ-32P]ATP incorporated into substrate peptides/mg of protein. B, IKK$\alpha$ kinase activity as measured by the phosphorylation of IκB-α in 1-LN cells treated as above. The respective autoradiograph is shown below the graph. IKK$\alpha$ kinase activity is in arbitrary units and is expressed as the mean ± S.E. from two experiments performed in duplicate. C, effect of silencing GRP78 gene expression on $\alpha_M^*$-induced activation of NF-κB1 and NF-κB2 in 1-LN prostate cancer cells. Shown are NF-κB1 (white bars) and NF-κB2 (black bars) from 1-LN cells treated as above. A representative immunoblot is shown below the graph. The results are expressed in arbitrary units and are the mean ± S.E. from triplicate experiments. A representative immunoblot is shown below the graph. * values significantly different from corresponding buffer-treated and GRP78 dsRNA-transfected cells and cells treated with $\alpha_M^*$ at the 5% level. Actin was used as a protein loading control in all experiments, but only a representative actin immunoblot is shown.
ER stress, the catalytic subunit of caspase-12 is released into the cytosol, where it activates the caspase-9 cascade (73). To assess the role of pro-apoptotic caspases in $\alpha_M^+$-induced cell proliferation in 1-LN prostate cancer cells, we quantified levels of caspase-12, caspase 9, and cleaved caspase-3 (Fig. 3) by Western blotting. Under our experimental conditions, $\alpha_M^+$ did not activate the activation of caspase-12, caspase-9, and caspase-3.

**Up-regulation of Bcl-2 and p-Bad$_{\text{Ser-136}}$ in 1-LN Prostate Cancer Cells Treated with $\alpha_M^+$**—In the next series of experiments, we examined the effects of treating 1-LN prostate cancer cells with $\alpha_M^+$ on levels of Bcl-2 and Bad phosphorylated at Ser-136 by Western blotting (Fig. 4). Bcl-2 is the prototype for a large family of structurally related proteins that regulate cell death in mammalian cells. Bcl-2 family members bind to apoptotic proteinase-activating factor 1 and inhibit caspase-9, and this binding is antagonized by Bax (43). Bad binds to Bcl-2, neutralizing the antiapoptotic effects of Bcl-2 (43); however, Akt phosphorylates Bad at Ser-136, which causes its sequestration with 14-3-3 protein (5). In $\alpha_M^+$-stimulated 1-LN cells, an approximately 2-fold increase in Bcl-2 was noted at about 10–20 min of incubation, which declined at longer periods of incubation compared with controls (Fig. 4A). This was accompanied by an increase in protein 14-3-3 (Fig. 4A). A similar but sustained increase in p-Bad$_{\text{Ser-136}}$ was observed at about 10–20 min of incubation of 1-LN prostate cancer cells with $\alpha_M^+$ (Fig. 4B). That Akt is the mediator of Bad phosphorylation at Ser-136 in 1-LN cells is demonstrated by their treatment with the PI 3-kinase inhibitor LY2904002 (20 $\mu$M/20 min) before $\alpha_M^+$ stimulation (Fig. 4C).

**FIGURE 3.** Immunoblots showing that $\alpha_M^+$ does not induce activation of caspase-12, caspase-9, and caspase-3 in 1-LN cells. Immunoblots shown are representative of two or three independent experiments. The protein loading control glyceraldehyde-3-phosphate dehydrogenase (GADPH) is also shown. As a control, the figure also demonstrates that the anti-murine procaspase-12 antibody reacts with not only murine procaspase-12 (lane 1) but also human procaspase-12 derived from human 1-LN cells (lane 2), MDA-MB231 breast cancer cells (lane 3), PC-3 prostate cancer cells (lane 4), and DM-6 melanoma cells (lane 5), respectively.

**FIGURE 4.** Induction of antiapoptotic signaling in 1-LN cells treated with $\alpha_M^+$. A, up-regulation of Bcl-2 (white), XIAP (gray), and 14-3-3 (black) in 1-LN cells treated with $\alpha_M^+$. Values are expressed in arbitrary units and are the mean ± S.E. from three or four experiments. Representative corresponding immunoblots along with the protein loading control actin are shown below each graph. B, p-Bad$_{\text{Ser-136}}$ (white bars), p-FOXO1$_{\text{Ser-256}}$ (dark gray bars), p-GSk3$_{\text{Ser-9}}$ (black bars), and cyclin D (light gray bars). Values are expressed in arbitrary units and are the mean ± S.E. from three or four experiments. Representative corresponding immunoblots along with protein loading controls are shown below the graph. C, regulation of p-FOXO1$_{\text{Ser-256}}$ (white), p-GSk3$_{\text{Ser-9}}$ (dark gray), p-Bad$_{\text{Ser-136}}$ (black), and XIAP (light gray) by Akt. Values are expressed in arbitrary units and are the mean ± S.E. from two or three experiments. Representative corresponding immunoblots and protein loading controls are shown below the graph. Bars 1, buffer; bars 2, $\alpha_M^+$ (50 pM/15 min); bars 3, LY2904002 (20 $\mu$M/20 min) and then $\alpha_M^+$. *, p values significantly different from buffer and inhibitor-treated cells at the 5% level.
α3M* Up-regulates the Phosphorylation of FOXO1 in 1-LN Cancer Cells—Akt activation positively regulates G1/S cell cycle progression through inactivation of GSK3, leading to increased cyclin D1, inhibition of forkhead transcription factors including the FOXO subfamily, and the subsequent reduction of p27\(^{kip}\) (5, 74, 75). Phosphorylation of FOXO1 by activated Akt promotes its export from the nucleus to the cytosol, thus preventing FOXO1 interaction with DNA, which otherwise would up-regulate transcription factors involved in the apoptotic pathway. FOXO1 also interacts with the 14-3-3 protein, which serves to localize the p-FOXO1 in the cytoplasm, and it also facilitates nuclear export of FOXO1 (5, 74, 75). Treatment of 1-LN cancer cells with α3M* (50 pM/15 min) elevated p-FOXO1 by about 50–70% at about 10 min of incubation, and these levels remained elevated up to 60 min of incubation as compared with control 1-LN cells (Fig. 4B). Treatment of 1-LN cells with LY294002 (20 μM/20 min), before α3M* addition markedly reduced p-FOXO1 levels (Fig. 4C). An increased up-regulation of 14-3-3 parallel to that of p-FOXO1 was also observed in 1-LN cells treated with α3M* (Fig. 4A), which suggests Akt-mediated inactivation of proapoptotic FOXO1.

Inactivation of GSK3β in 1-LN Cells Treated with α3M*—Activation of the PI 3-kinase pathway promotes GSK3β phosphorylation at Ser-9, thereby inhibiting its activity as an apoptosis-inducing kinase (76, 77). Treatment of 1-LN cells with α3M* (50 pM) elevated p-GSK3β\(^{Ser-9}\) by 2–3-fold at 20 min and longer periods of incubation (Fig. 4B). Pretreatment of cells with the PI 3-kinase inhibitor LY294002 significantly decreased α3M*-induced inactivation of GSK3β (Fig. 4C). This demonstrates that PI 3-kinase-dependent activation of Akt in 1-LN cells causes inactivation of GSK3β and thus affords protection against GSK3β-induced apoptotic signaling.

Activation of NF-κB1 and NF-κB2 in 1-LN Prostate Cancer Cells Treated with α3M*—NF-κB is constitutively activated in many cancers where ER stress is a common occurrence. Treatment of 1-LN cancer cells with α3M* (50 pM) caused a severafold increase in the NF-κB1 as determined by Western blotting (Fig. 5A). The upstream activators of NF-κB1 (namely IκKα/β and IκB-α) also exhibited a 1.5–2-fold transitory activation at about 10–20 min of incubation (Fig. 5A), subsequently returning to the basal state. The Western blotting data with respect to p-IκKα are also corroborated by the IκKα kinase activity studies, which
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demonstrate a nearly parallel increase in $\alpha_M^*$-stimulated 1-LN cells, as compared with buffer-treated cells (Fig. 5B). Under these experimental conditions, the levels of phosphorylated IkB-Î² were not affected (data not shown). Activation of NF-Î³B is also affected by NIK in the cytoplasm as well as in the modulation of its transactivation potential in the nucleus (55). NIK associates with TRAF2 and promotes the phosphorylation of IKKÎ± and IKKÎ². Enhanced activity of NIK occurs in various tumors, which suggests its association with enhanced levels of p52 in breast cancer (53, 54). We therefore studied the effect of $\alpha_M^*$ in 1-LN cancer cells with respect to activation of NF-Î²B by the noncanonical pathway (Fig. 5C). Exposure of 1-LN cells to $\alpha_M^*$ caused a transient increase in both TRAF2 (Fig. 5A) and NIK (Fig. 5C) at about 10–20 min of incubation. $\alpha_M^*$ treatment of 1-LN cancer cells also caused a 50–60% sustained increase in NF-Î²B2 (Fig. 5C). Furthermore, whereas activation of NF-Î²B1 via the canonical pathway was PI3-kinase-dependent, activation of NF-Î²B2 via the noncanonical pathway was PI3-kinase-independent (Fig. 5D). Incubation of 1-LN cells with LY294002 (20 Î¼M/20 min) before the addition of $\alpha_M^*$ inhibited the increase in the protein levels of NF-Î²B1 and p-IkB-Î± but had no effect on NF-Î²B2 and NIK protein levels compared with $\alpha_M^*$-treated cells (Fig. 5D). That activated NF-Î²B and NF-Î²B2 translocate to nuclei in $\alpha_M^*$-stimulated 1-LN cells is shown in Fig. 4E, where these proteins were present at concentrations about 2-fold higher than the buffer-treated controls.

Attenuation of $\alpha_M^*$-induced Activation of IKKÎ± Kinase and NF-Î³B in 1-LN Cells Transfected with GRP78 dsRNA—Above, we have shown that $\alpha_M^*$ treatment of 1-LN prostate cancer cells with $\alpha_M^*$ increases activation of IKKÎ± and up-regulates both NF-Î²B1 and NF-Î²B2 in an Akt-dependent manner (Fig. 5). Transfection of 1-LN prostate cancer cells with GRP78 dsRNA nearly abolished $\alpha_M^*$-induced activation of IKKÎ± compared with cells treated with Lipofectamine and $\alpha_M^*$ or cells treated with scrambled dsRNA and $\alpha_M^*$ (Fig. 2). Since silencing GRP78 gene expression profoundly inhibited the activation of p-AktThr308 and p-AktSer473 kinases caused by $\alpha_M^*$ stimulation (Fig. 2), we suggest that activation of IKKÎ±, hence NF-Î³B activation, is mediated by activated Akt. These observations are further supported by the inhibition of p-IKKÎ±/Î², p-IkBÎ±, NF-Î³B1, and NF-Î³B2 in 1-LN cells transfected with GRP78 dsRNA and stimulated with $\alpha_M^*$ as compared with cells treated with Lipofectamine and $\alpha_M^*$ or cells transfected with scrambled dsRNA and treated with $\alpha_M^*$ (Fig. 2).

$\alpha_M^*$ Treatment of 1-LN Cancer Cells Up-regulates the Expression of GADD45B—GADD45B is a pivotal mediator of the cell-protective effects of NF-Î³B against TNFÎ± and Fas-induced apoptosis (78, 79). NF-Î³B induces GADD45B promoter activity, which down-regulates prosapoptotic JNK. GADD45B associates tightly with MKK7, a selective activator of JNK, and inhibits its activation and apoptosis (80, 81). Treatment of 1-LN cancer cells within $\alpha_M^*$ showed little or negligible activation of either MKK7 or JNK phosphorylation as determined by Western blotting (Fig. 6). Under our experimental conditions, JNK activation appears to be suppressed by Akt-dependent phosphorylation of ASK1 (Fig. 1B) and/or NF-Î³B activation (Fig. 5, A and C). In the next series of experiments, we evaluated the role of GADD45B in JNK activation in 1-LN cancer cells treated with $\alpha_M^*$ by Western blotting (Fig. 5C). $\alpha_M^*$ treatment of 1-LN cells elevated the expression of GADD45B by about 2-fold at 10–20 min of incubation but returned to basal levels at 60 min (Fig. 5C). The results indicate that the very modest effect of JNK in $\alpha_M^*$-treated 1-LN cells occurs at the levels of Akt-dependent inhibition of ASK1 and GADD45B inhibition of MKKK7.

Up-regulation of Cyclin D1 and Down-regulation of p27kip1 in 1-LN Cells Exposed to $\alpha_M^*$—During the G1 to S transition, p27kip1 is phosphorylated by kinases, including Akt, resulting in its nuclear exclusion and dissociation from E-CDK2 complexes and processing for degradation (82, 83). As noted above, FOXO-induced cell cycle arrest is in part due to up-regulation of p27kip1. Increased levels of p27kip1 cause G1/S arrest by inhibiting cyclin-cyclin-dependent kinase complexes necessary for S-phase entry and progression (82, 83). FOXO factors directly up-regulate p27kip1 transcription and also prolong its half-life. The subcellular localization of p27kip1 is also tightly regulated. Through the cell cycle, p27kip1 shuttles between nucleus and cytoplasm. During the G0 phase, p27kip1 localization is completely nuclear, and mitogenic stimulation causes cytoplasmic redistribution, where it probably fails to inhibit events involved in the G1/S transition (82, 83).
Thus, in proliferating cells, the levels of p27kip should be low, and that of cyclin D1 should be high, and the reverse should be true in cells undergoing cell arrest and apoptosis. 1-LN prostate cancer cells exposed to αM* showed low and unchanged levels of p27kip protein in contrast to the levels of cyclin D1 protein, which were elevated at early periods of incubation (Fig. 4B). These results indeed support the growth-promoting effect of αM* on 1 LN cells.

αM* Up-regulates the Expression of XIAP in 1-LN Prostate Cancer Cells—The IAPs are a family of intracellular antiapoptotic proteins that play a key role in cell survival by modulating death signaling pathways at a postmitochondrial level (84). These proteins characteristically contain a caspase recruitment domain, an NH2 terminus repeat motif, which is necessary for its biological activity, and a RING finger domain at their COOH terminus, which is responsible for ubiquitination and degradation of IAP after an apoptosis stimulus (84). Among IAPs, XIAP is the most potent inhibitor of caspases and apoptosis. XIAP directly inhibits caspase-9 (84). XIAP is a physiological substrate of Akt, which phosphorylates it on Ser-87 (85), thereby inhibiting ubiquitination. These effects reduce XIAP degradation, and the increased levels of XIAP are associated with decreased caspase activity and programmed cell death (84). αM* induced a severalfold increase in XIAP (Fig. 4A), and treatment of the cells with PI 3-kinase inhibitor LY294002 before αM* exposure significantly reduced levels of XIAP (Fig. 4C). It is therefore suggested that in addition to down-regulating the activities of several proapoptotic components of programmed cell death, Akt also aids in the attenuation of cell death by preventing XIAP degradation, thus increasing its effective concentration.

Silencing of GRP78 Gene Expression Inhibits the Expression of αM*-induced Proliferative Factors in 1-LN Prostate Cancer Cells—Since both Akt and NF-κB promote cell survival by up-regulating the expression of antiapoptotic factors, one would expect that in 1-LN cells transfected with GRP78 dsRNA, the expression of these antiapoptotic factors would be down-regulated and those of proapoptotic factors would be up-regulated. Indeed, this is correct (Fig. 6). Silencing GRP78 gene expression down-regulated levels of p-FOXO, p-GSK3β, XIAP, and cyclin D1, whereas levels of p27kip were up-regulated in 1-LN prostate cancer cells stimulated with αM* (Fig. 6).

Activation of MKK3/6, ASK1, p38 MAPK, JNK, and TRAF2 in 1-LN Prostate Cancer Cells Exposed to αM*—The ability of cells to react to environmental changes is dependent on the cooperation of intracellular signal transduction pathways to coordinate cellular responses. In most cases, p38 MAPK is simultaneously activated with Akt. Akt activates both the p38 MAPK and JNK pathways by directly phosphorylating MKK4/MKK7 and MKK3/MKK6. In the next series of experiments, we examined the effect of αM* on the activation of stress-activated MAPKs, namely p38 MAPK and JNK in 1-LN prostate cancer cells. Exposure of 1-LN cells to αM* (50 pM) elevated levels of TRAF2 (Fig. 5A) by about 1.5–2-fold transiently and elevated the level of ASK1 at about 10 min of incubation, which remained elevated until the time of incubation (Fig. 5A). Stimulation of 1-LN cells with αM* elevated p-MKK3/6 and p-MKK4 at about 10 min of incubation but showed no effect on the phosphorylation of MKK7, the specific kinase, which activates JNK (Fig. 7B). The results show that αM*-induced receptor activation in 1-LN cells up-regulates ASK1 activity, which activates kinases upstream of p38 MAPK and JNK activation. Akt-mediated phosphorylation of ASK1 inhibits its activity (8). To understand the possible role of ASK1 phosphorylation in suppression of JNK activation in 1-LN cells stimulated with αM*, we determined levels of p-ASK1 (Fig. 7B). Levels of p-ASK were profoundly elevated under our experimental conditions (Fig. 7B). Incubation of cells with the PI 3-kinase inhibitor LY294002 before αM* addition profoundly reduced levels of p-ASK1 compared with controls (Fig. 1B). This indicates a possible role of Akt in suppressing JNK activation (Fig. 7B). An increase in the levels of activated p38 MAPK was observed at about 20–40 min of incubation (Fig. 7A). In contrast, αM* treatment of 1-LN prostate cancer cells only slightly affected levels of p-JNK (Fig. 7B). αM* showed a differential effect on the activation of p38 MAPK and JNK as assessed by Western blotting. An increase in the levels of activated p38 MAPK could be observed at about 20–40 min of incubation (Fig. 7A). In contrast, αM* treatment
of 1-LN prostate cancer cells only slightly affected the levels of p-JNK (Fig. 7B).

Up-regulation of UPR Signaling Cascade in 1-LN Cells Exposed to \( \alpha_2M^* \)—An increased expression of GRP78 protein is part of the UPR required to alleviate ER stress, maintain ER functions, and protect cells against cell death (14–17). GRP78 binds to three mammalian UPR transducers (namely, IRE1, PERK, and ATF6) and maintains them in an inactive state in the absence of ER stress (14–17). Prolonged activation of IRE1 promotes binding to TRAF2 and subsequent recruitment of caspase-12, an ER-specific inducer of apoptosis (16, 29, 49–51). The transient inhibition of protein synthesis during UPR is achieved by the activation of PKR-like ER kinase (PERK), which phosphorylates the eukaryotic translation initiator factor-2a. The transient inhibition of protein synthesis during UPR allows the transcription of ATF4 transcription factor. ATF4 induces GADD34, which in turn dephosphorylates eIF2a, allowing protein synthesis to continue (14–17). The block in protein synthesis that limits the accumulation of unfolded proteins in the ER also induces a G1 cell cycle arrest, activation of NF-κB, and induction of the apoptotic transcription factor GADD153 (14–17). In view of several reports on the up-regulation of UPR signaling in a variety of cancer cells, we have examined the expression of various components of UPR in 1-LN cells treated with \( \alpha_2M^* \) for varying periods of time by Western blotting in the following parameters: GRP78, IRE1, ATF6, ATF4, PERK, eIF2α, GADD153, and GADD34 (Figs. 8 and 9). \( \alpha_2M^* \)-induced expression of the ER stress biomarker, GRP78, showed a steady increase up to 60 min of incubation (Fig. 8). The increase in GRP78 protein was \( \alpha_2M^* \) concentration-dependent, and the maximal increase occurred at 50 pM of \( \alpha_2M^* \) (data not shown). \( \alpha_2M^* \) treatment of 1-LN cancer cells also caused a sustained increase in IRE1-α and XBP-1 (Fig. 8). Under these experimental conditions, levels of ATF6, which have been reported to increase the transcription of GRP78 and XBP1, also showed a 2-fold increase at about 20 min of incubation (Fig. 8). Thus, activation of ATF6 signaling provides a positive feedback for UPR activation.

ER stresses that activate the transcriptional components of UPR also transiently attenuate protein synthesis, a response that is coupled to phosphorylation of PERK and eIF2α (14–17). We next determined the levels of p-PERK, p-eIF2α, and eIF2α in 1-LN cells exposed to \( \alpha_2M^* \) by Western blotting (Fig. 9). 1-LN cancer cells exposed to \( \alpha_2M^* \) (50 pM) showed an increase in p-PERK and p-eIF2α at about 20 min of incubation, which remained elevated up to the time of incubation (Fig. 9A). \( \alpha_2M^* \) treatment of 1-LN cancer cells elevated the protein levels of both ATF4 and GADD34 at about 10–20 min of incubation, and these levels remained elevated until the time of incubation (Fig. 9B). The prime function of UPR expression is to protect cells against ER stress-induced damage to the cells; therefore, one would expect that under these conditions the cell will exhibit maximally the antiapoptotic and minimally the proapoptotic signaling. The block in protein synthesis, which limits the accumulation of unfolded proteins in the ER, also induces a G1 cell cycle arrest, activation of the antiapoptotic protein NF-κB, and induc-
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FIGURE 10. Silencing of GRP78 gene expression attenuates αM4-induced increased activation of UPR transducers in 1-LN prostate cancer cells. For experimental details, see "Experimental Procedures." A, IRE1α (white), XBP1 (gray), and ATF6 (black) from 1-LN cells treated with Lipofectamine plus buffer (1), Lipofectamine plus αM4 (2), GRP78 dsRNA plus αM4 (3), and scrambled dsRNA plus αM4 (4). A representative immunoblot is shown below the graph. The results are expressed in arbitrary units and are the mean ± S.E. from triplicate experiments. B, p-PERK (white), p-eIF2α (light gray), ATF4 (black), and GADD153 (dark gray). The results are expressed in arbitrary units and are the mean ± S.E. from triplicate experiments. A representative immunoblot is shown below the graph. *, values significantly different from corresponding buffer-treated cells and GRP78 dsRNA-transfected and αM4-treated cells; **, significantly different from buffer-treated controls at the 5% level. Actin was used as a protein loading control in all experiments, but only a representative actin immunoblot is being shown.

Attenuation of αM4-induced UPR Signaling in 1-LN Prostate Cancer Cells Transfected with GRP78 dsRNA—Above, we have shown that αM4 stimulation of 1-LN prostate cancer cells up-regulates UPR signaling. Since GRP78 is critically involved in the regulation of UPR signaling during ER stress, we have modulated levels of GRP78 by silencing GRP78 gene expression by RNAi in 1-LN prostate cancer cells and studying its effects on the expression of various components of the UPR signaling cascade dependent on αM4 stimulation (Fig. 10). As expected, the limitations imposed on the availability of intracellular GRP78 by inhibiting its new synthesis resulted in the down-regulation of IRE1α, XBP-1, ATF6, p-PERK, and p-eIF2α compared with control cells treated with Lipofectamine and αM4 (Fig. 10). However, levels of GADD153, an apoptosis-inducing protein, were elevated by 2–3-fold, and those of ATF4 remained nearly unaffected in cells transfected with GRP78 dsRNA compared with control cells treated with Lipofectamine and αM4 (Fig. 10). The results demonstrate that restrictions imposed on the availability of GRP78 cause impairments of cell proliferative and cell survival signaling triggered by the binding of αM4 to cell surface-associated GRP78 and UPR signaling resulting in the induction of proapoptotic signaling.

DISCUSSION

Highly metastatic 1-LN prostate cancer cells were derived from less metastatic PC3 prostate cancer cells. 1-LN prostate cancer cells differ from PC-3 cancer cells in one very important aspect; the former express GRP78 on their cell surface (19–22). GRP78 is a member of the HSP70 family, and its expression is greatly induced upon pharmacologically and/or pathophysiologically induced ER stress, which causes the elicitation of UPR signaling to promote cell survival against the adverse effects of ER stress (see Ref. 14 and references therein). αM4 binds to cell surface-associated GRP78 as well as to the low density lipoprotein receptor-related protein (23). However, the number of binding sites, binding affinities, and signaling events elicited after receptor binding of αM4 to GRP78 and low density lipoprotein receptor-related protein are very different (87, 88). In contrast to low density lipoprotein receptor-related protein, the binding of αM4 to GRP78 elicits mitogenic signaling and promotes cell proliferation in murine peritoneal macrophages (89–92). αM4 binding to 1-LN cells, but not PC-3 cells, also promotes such events (20–22). One might also expect that in 1-LN prostate cancer cells, the ER-resident pool and cell surface resident pool of GRP78 would protect and promote the cell survival and proliferation of these cells by both up-regulation and cross talk between UPR signaling and receptor-activated mitogenic and proliferative signaling, respectively. In this paper, we report that this indeed happens. The major points of this study are as follows. 1) Stimulation of 1-LN prostate cancer cells with αM4 up-regulates the activation of proproliferative and antiapoptotic signaling mediated by ERK1/2 and PI 3-kinase. As a consequence, p-AktThr-308 and p-AkSer-473 kinases are activated, as evidenced by the inhibition of caspase-12, caspase-9, and caspase-3 activation; phosphorylation of BAD at residue Ser-136; suppression of ASK1 activity by its PI 3-kinase-dependent phosphorylation; up-regulation of Bcl-2, XIAP, p-FOXO1, p-GSK3β, and cyclin D1; and down-regulation of p27kip1. 2) αM4 treatment of 1-LN cells up-regulates ASK1 and transient activation of MKK3/6, MKK4, and p38 MAPK with little or no effect on the activation of MKK7 and JNK. 3) αM4 treatment caused up-regulation of TRAF2 and GADD45β. αM4 expression activated NF-κB by both the canonical (NF-κB1) and noncanonical (NF-κB2) pathways, as evidenced by the increased activation of IKKα/β, increased phosphorylation of IκB-α in a PI 3-kinase-dependent manner, and activation of NIK in a PI 3-kinase-independent manner. 4) αM4 treatment of 1-LN cells activated the unfolded protein response by up-regulating the expression of GRP78, IRE1α, XBP-1, ATF6, p-PERK, p-eIF2α, ATF4, and GADD34 and down-regulating the expression of GADD153 (5). Silencing GRP78 gene expression by RNAi suppressed cell survival signaling triggered by αM4 binding to cell surface-associated GRP78 as well as cell survival-promoting UPR signaling regulated by ER-associated GRP78. The molecular chaperone GRP78 regulates the IRE1, ATF6, and PERK-transduced signaling pathways of UPR; however, these signaling path-
ways may have distinctive sensitivities to fluctuations of the free GRP78 pool (34, 72).

Taken together, these studies demonstrate that the effect of $\alpha_2M^*$ on prostate cancers that express high levels of GRP78 on their cell surface is to activate multiple mechanisms that directly promote cellular proliferation but also to block mechanisms triggering programmed cell death. As noted earlier in this report, the occurrence of antibodies against GRP78 in the plasma of patients with prostate cancer correlates with a very poor prognosis (23, 24). Moreover, histologic examination of human prostate cancers clearly demonstrates that GRP78 is dramatically up-regulated both intracellularly and on the cell surface of aggressive tumors (24). In the current report, we provide a mechanistic basis for these clinical observations. It is of interest that anti-GRP78 antibodies may appear in the plasma of patients with prostate cancer (24). Clearly, these are antibodies against “self.” Why such autoantibodies should appear is unclear, although it is a frequent observation in patients bearing malignant tumors that altered immune states exist and may contribute to tumor progression. Whether the antibodies have any effect on the behavior of prostate cancer is as yet unknown. Such antibodies might themselves function as an $\alpha_2M^*$ agonist, or they might antagonize the function of $\alpha_2M^*$ by blocking binding of this ligand to the cell surface. In any event, ultimately the presence of a high concentration of these antibodies in the plasma is a harbinger of a poor outcome.

In summary, here we show that treatment of 1-LN cells with $\alpha_2M^*$ promotes their proliferation by activating ERK1/2, p38 MAPK, and PI 3-kinase signaling cascades and their cell survival by activating Akt and NF-κB signaling. To overcome the ER stress induced by their aggressive growth, these cells trigger UPR. There is an extensive cross-talk between these signaling cascades that contributes to their growth and survival. We have schematically presented these events in Fig. 11.

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FIGURE 11. A schematic representation of signaling cascades and cross-talk among them, involved in the cell proliferation and cell survival of 1-LN prostate cancer cells stimulated with $\alpha_2M^*$.
