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The Monomeric Receptor Binding Domain of Tetrameric α_2-Macroglobulin Binds to Cell Surface GRP78 Triggering Equivalent Activation of Signaling Cascades

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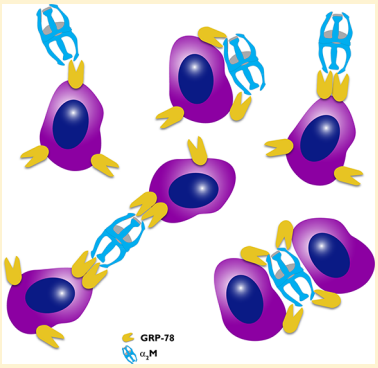
ABSTRACT: α_2-Macroglobulin (α_2M) is a broad spectrum proteinase inhibitor that when activated by proteinases (α_2M*) undergoes a major conformational change exposing receptor recognition sites in each of its four subunits. These complexes bind to two distinct receptors, namely, the low-density lipoprotein receptor-related protein (LRP) and cell surface glucose-regulated protein [$M_r \sim 78000$ (GRP78)]. The latter is a very high affinity receptor (K_d = 50−100 pM) whose ligation triggers pro-proliferative and anti-apoptotic signaling cascades. Despite its four binding sites, Scatchard analysis of binding of α_2M* to cells does not yield a cooperative plot. We, therefore, hypothesize that a monomeric cloned and expressed α_2M receptor binding domain (RBD) should trigger comparable signaling events. Indeed, RBD or its K1370A mutant that binds to GRP78 but cannot bind to LRP regulates DNA and protein synthesis by human prostate cancer cells in a manner comparable to that of α_2M*. Akt and mTORC1 activation and signaling are also comparably upregulated by α_2M*, RBD, or mutant K1370A. Antibodies directed against the carboxyl-terminal domain of GRP78 are antagonists that block α_2M*-mediated effects on pro-proliferative and anti-apoptotic signaling cascades and protein and DNA synthesis. The effects of RBD and its mutant were similarly blocked by these antibodies. Finally, proteolysis of α_2M at pH values from 5.7 to 7.0 causes production of free RBD and RBD-containing fragments. Thus, while α_2M* ligates only one GRP78 receptor molecule per α_2M*, it may potentially serve as a reservoir for release of up to four binding fragments per molecule.



Human α_2-macroglobulin (α_2M), consisting of four identical $M_r \sim 180000$ subunits, is a broad spectrum proteinase inhibitor.[1] It is expressed in most animal species and has been studied in frogs, horseshoe crabs, octopi, birds, and mammals.[1−5] The protein is synthesized by many cells, including hepatocytes and macrophages, and is present in plasma at a concentration of 1−5 μM.[1] Tumors and their associated stroma also produce this protein as well as various proteinases that react with α_2M.[6] Thus, prostate cancer cells produce prostate specific antigen (PSA), a chymotrypsin-like serine proteinase, metalloproteinases, and other proteinases, while prostate cancer stroma secretes large amounts of α_2M.[6] When α_2M reacts with a proteinase, the so-called "bait region" in each subunit is cleaved, and consequently, a β-cysteinyl-γ-glutanyl thiolester ruptures followed by a large conformational change in the molecule.[1] This exposes a receptor recognition site in each subunit that is present in the carboxyl-terminal domain.[7] Small nucleophiles such as methylamine or ammonia can directly rupture the thiolesters, triggering a similar conformational change exposing the receptor recognition sites.[1]

The lipoprotein receptor-related protein (LRP) was first identified as a receptor for proteinase or amine-activated α_2M (α_2M*).[1] LRP is a scavenger receptor capable of binding more than 20 ligands in addition to α_2M*, including *Pseudomonas* exotoxin A, lipoprotein lipase, apolipoprotein E, LDL, and serine proteinase inhibitor complexes with proteinase.[1] The reported K_d for LRP ranges from 1 to 10 nM, and the receptor density ranges from 50000 to 250000 per cell.[1] We subsequently identified a second extremely high affinity receptor on the surface of activated macrophages and many types of tumor cells.[7−14] The K_d for this receptor is 50−100 pM, and the copy number is relatively low, ∼1500 sites per cell.[15−18] This second receptor was identified as cell surface-associated glucose-regulated protein with an M_r of ∼78000 (GRP78).[13] The best known function of GRP78 is as an ER chaperone, critical to the unfolded protein response;[16] however, under high cell stress such as rapid growth, an increased level of synthesis of GRP78 results in a fraction of the pool migrating to the cell surface. GRP78 possesses an ATP binding site and when activated by binding of α_2M* to a region in the amino-terminal domain undergoes autophosphorylation.[14,19,20] Through coupling of GRP78 with several different classes of G proteins, α_2M* triggers pro-proliferative, anti-apoptotic, and pro-migratory signaling cascades in tumor cells.[12,21,22]

GRP78 is found on the surface of activated macrophages but is not present on the surface of naïve cells.[23] With respect to tumor cells, it is present at higher density on the more malignant tumor cells and stem cell subpopulations.[14,21,22] The best studied example of this behavior is human prostate cancer in which the highly metastatic human 1-LN cell line

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demonstrates cell surface GRP78, but not LRP, while the less aggressive PC-3 line expresses LRP but not GRP78.[6,19−21] This has allowed us to tease out signaling cascades dependent on GRP78, but not LRP.[6,16,20,21,24] We and others have shown that some LRP ligands can activate signal transduction by a distinct mechanism involving a pertussis toxin sensitive G protein while GRP78 is coupled to Gαq11, which is pertussis toxin insensitive.[11,12] GRP78-dependent signal transduction is maximal at ∼100 pM ligand; however, α_2M* triggers signaling via LRP only at concentrations of 25 to >100 nM, which may be difficult to achieve *in vivo*, especially because α_2M* has a circulatory half-life of only 2−5 min.[1,25,26] In addition to hepatic clearance, it is also taken up by reticuloendothelial cells that are present throughout the tissues.[1] We have, therefore, chosen to focus on GRP78-mediated signal transduction.

Studies by Mintz et al. demonstrated that autoantibodies to GRP78 in the serum of prostate cancer patients are indicative of a poor prognosis.[27] We subsequently characterized these autoantibodies and demonstrated that they are receptor agonists that bind to the amino-terminal domain close to or at the α_2M* binding site of GRP78.[27−29] The entire GRP78 molecule is expressed on the cell surface and is anchored to the membrane by a complex of proteins that are required to transduce a signal.[22,30] Because the carboxyl-terminal domain is present on the cytosolic face of the cell membrane, we studied the effects of antibodies directed against this region of GRP78. These studies demonstrate that ligating this domain blocks the pro-proliferative and anti-apoptotic effects of α_2M*, but more significantly, such binding in and of itself triggers pro-apoptotic signaling leading to cancer cell death.[31−33] These effects were subsequently extended to an *in vivo* murine model of melanoma.[34,35]

α_2M* ligation of LRP or GRP78 as studied by Scatchard analysis demonstrates no evidence of a cooperative binding isotherm.[15−18] Thus, despite the fact that α_2M* contains four receptor binding sites, it behaves as if only one site engages a cell surface receptor of either class. α_2M*, therefore, behaves as if it were a monomeric ligand. Structural studies, however, suggest that all four receptor recognition sites should be available for cell binding.[8−10]

Here we consider this phenomenon with respect to signal transduction because previous studies comparing a monomeric α-macroglobulin, rat α_1-inhibitor-3, and the cloned and expressed receptor binding domain (RBD), while not extensive, suggest similar signaling behavior for these monomeric forms as compared to that of α_2M*.[36,37] Both monomeric ligands and α_2M* bind with the same affinity to a comparable number of sites on cells.[36,37] Here we study two prostate cancer cell lines, namely, 1-LN cells, strong expressors of cell surface GRP78, and DU-145 cells, which have an intermediate level of cell surface GRP78.[6−14] 1-LN cells express no cell surface LRP, but DU-145 cells do demonstrate cell surface LRP.[6,14] Both cloned and expressed RBD and its K1370A mutant, which binds to GRP78 but not LRP,[15−18] were compared to α_2M* for their ability to activate signaling cascades. We studied the effects of these three ligands on protein synthesis, [^{3}H]thymidine uptake, regulation of GRP78 and PSA message and protein levels, and signal transduction. Further, we demonstrate that at pH values typically present in tumor beds proteolytic attack can release α_2M* fragments containing the GRP78 receptor binding site. Thus, α_2M, which when it binds to cells behaves as if only one of its receptor binding sites is engaged, may release four RBDs per α_2M*. This potentially is a multiplier of signal transduction because each RBD induces receptor activation comparable to that of tetrameric α_2M* as demonstrated here.

EXPERIMENTAL PROCEDURES

Materials. Culture media were purchased from Invitrogen (Carlsbad, CA). Receptor-recognized α_2M* was produced by reaction of α_2M prepared and activated with methylamine as described previously.[6] Antibodies against ERK1/2, p-ERK1/2, MEK, p-MEK, S6-kinase-1 (S6K1), p-S6K1^{T389}, p-4EBP1$^{T37/46}$, Akt1, p-Akt^{T308}, and p-Akt^{S473} were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin antibody was from Sigma (St. Louis, MO). Antibodies against the carboxyl-terminal domain of GRP78 and the IgG isotype control were purchased from Aventa Biopharmaceutical Corp. (San Diego, CA). [^{3}H]Leucine (specific activity of 115.4 Ci/mmol) [^{3}H]thymidine (specific activity of 174 Ci/mmol), and [γ-^{33}P]ATP (specific activity of 3000 Ci/mmol) were purchased from Perkin-Elmer Life Sciences. Rapamycin and LY294002 were purchased from Biomol (Plymouth, PA). All other materials used were of analytical grade and were procured locally.

Cloning and Mutagenesis of the Receptor Binding Domain (RBD) and Its Purification. RBD and its K1370A binding site mutant were prepared as described previously.[36] Briefly, the FLAG-1/rat α_1M construct containing coding sequences for residues 1313−1451 (human numbering; residues 1336−1476 in rat numbering) of the rat α_1M receptor binding domain (RBD) served as a template for polymerase chain reaction (PCR).[17] Plasmid DNA was amplified using the following oligonucleotides: 5′-GCC GGA TCC GGC GGT GGA GAA GCA CCC-3′ and 5′-GAG AGA TCT GTC GAC GAT-3′. These primers contained restriction sites for BamHI and SalI, respectively. Following amplification, the DNA was cloned into pBluescript SK- (Stratagene, La Jolla, CA). Mutagenesis was performed on the RBD using Muta-Gene Phagemid Invitro mutagenesis kit version 2 (Bio-Rad, Hercules, CA). Manipulations were conducted according to the manufacturer's instructions, and the uracil-laden single-stranded DNA was purified using the Prep-AS-Gene DNA purification kit (Bio-Rad); 24-base oligonucleotide were used to create mutations and silent restriction sites. Mutant plasmids were transformed in *Escherichia coli* strain DH5a and screened by colony PCR, restriction digestion, and DNA sequencing using Sequence version 2 (U.S. Biochemicals, Cleveland, OH). Using BamH2 and SalI restriction sites, wild-type or mutant RBD was cloned into the pGEX-4T-3 expression vector (Pharmacia Biotech, Piscataway, NJ). DH5a bacteria containing the pGEX-4T-3/RBD construct were grown to an A_{600} of 0.6−0.8, induced with isopropyl thio-β-D-galactoside at a final concentration of 1 mM, and grown for an additional 2 h at 37 °C. To the bacterial lysate were added Dnase (20 mg/L) and $MgCl_2$ (10 mM), and the lysate was passed through needles. The lysates were incubated on ice for 30 min, and cell debris was removed by centrifugation at 14000*g* for 40 min. Affinity chromatography was performed using glutathione-Sepharose 4B (Pharmacia Biotech). The RBD was cleaved from the RBD−GST fusion protein by incubating 1 mg of fusion protein with 10 units of bovine thrombin (U.S. Biochemicals) in a volume of 1 mL for 2 h at room temperature. The incubation mixture was applied to a glutathione-Sepharose 4B column and free RBD collected in the flow through. Protein samples were dialyzed against 20 mM HEPES (pH 7.3) containing 150 mM NaCl, sterile filtered, and stored in aliquots

at −80 °C. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) using BSA as a standard.

Prostate Cancer Cell Lines. The highly metastatic 1-LN cell line is derived from the PC-3 line and was a kind gift of P. Walther (Duke University Medical Center). DU-145 cells were purchased from ATCC. As needed, these cells were grown in 6-, 12-, 24-, or 48-well plates to confluence in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 12.5 units/mL penicillin, 6.5 μg/mL streptomycin, and 10 nM insulin (RPMI-S) in a humidified CO_2 (5%) incubator. At 90% confluency, the medium was aspirated, the monolayers were washed with ice-cold HHBSS, a fresh volume of medium without fetal bovine serum was added, and the cells were used for the experiments described below.

Determination of the Effects of α_2M*, RBD, or Its K1370A Mutant on Protein Synthesis in 1-LN Cells. 1-LN cells (300×10^3 cells/well) in 48-well plates were grown in RPMI-S medium in a humidified CO_2 (5%) incubator at 37 °C. At ∼90% confluence, the medium was aspirated and a volume of RPMI was added followed by the addition of either buffer or RBD (50 pM). To each well was added [^{3}H]leucine (2 μCi/mL), and cells were incubated overnight as described above. The reaction was terminated by aspirating the medium, and monolayers were washed thrice with ice-cold 5% TCA (two times), followed by three washings with ice-cold PBS. Cells were lysed in a volume of 1 NaOH (40 °C for 2 h); the amount of protein was estimated, and the lysates were counted in a liquid scintillation counter.[6] In experiments in which modulation of RBD-induced protein synthesis was studied, cells were pretreated with PI 3-kinase inhibitor LY294002 (20 μM for 20 min), mTOR inhibitor rapamycin (100 nM for 15 min), or antibodies against the carboxyl-terminal domain of GRP78 (3 μg/mL for 2 h) before RBD was added (50 pM overnight). Other details of quantifying the incorporation of [^{3}H]leucine by cellular proteins were the same as described.

Determination of the Effects of α_2M*, RBD, or Its K1370A Mutant on [^{3}H]Thymidine Uptake in 1-LN Cells. 1-LN cells (300×10^3 cells/well) in 48-well plates were grown in RPMI-S medium in a humidified CO_2 (5%) incubator at 37 °C. At ∼90% confluence, the medium was aspirated and a volume of RPMI was added followed by the addition of either buffer or RBD (50 pM). To each well was added [^{3}H]-thymidine (2 μCi/mL), and cells were incubated overnight as described above. The reaction was terminated by aspirating the medium, and monolayers were washed thrice with ice-cold 5% TCA, followed by three washings with ice-cold PBS. Cells were lysed in a volume of 1 NaOH (40 °C for 2 h); the amount of protein was estimated, and the lysates were counted in a liquid scintillation counter.[6] In experiments in which modulation of RBD-induced DNA synthesis was studied, cells were pretreated with PI 3-kinase inhibitor LY294002 (20 μM for 20 min), mTOR inhibitor rapamycin (100 nM for 15 min), or antibodies against the carboxyl-terminal domain of GRP78 (3 μg/mL for 2 h) before RBD was added (50 pM overnight). Other details of quantifying the uptake of [^{3}H]thymidine by cellular DNA were the same as described.

Measurement of Prostate Specific Antigen (PSA) mRNA Levels by Reverse Transcription. We have previously shown that α_2M* ligation of GRP78 promotes PSA production and secretion.[6] Therefore, we examined the effect of RBD ligation of GRP78 on PSA message production. Total RNA from 1-LN prostate cancer cells treated with buffer or RBD was extracted by a single method using an Rneasy Mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Total RNA was reverse-transcribed with 1 μg of RNA in a 20 μL reaction mixture, using Moloney murine leukemia virus reverse transcriptase (200 units) and oligo(dt) as a primer for 1 h at 4 °C. The resulting DNA (5 μg) was used as a template, and a 225 bp segment of the PSA cDNA was amplified using a 20mer upstream primer (5′-CCA ACA CCC GCT CTA CGA TA-3′) and a 22mer downstream primer (5′-ACC TTC TGA CGG TGA ACT TGC G-3′). A 302 bp segment of mouse β-actin (constitutive internal control) cDNA was co-amplified using a set of primers provided in an R&D Systems (Minneapolis, MN) kit. Amplification was conducted in a Techne Thermal Cycler PHC-3 for 28 cycles (one cycle at 94 °C for 45 s, 60 °C for 45 s, and 70 °C for 45 s). PCR products were analyzed on a 1.2% (w/v) agarose/ethidium bromide gel. The gels were photographed, and then the intensities of individual PSA and β-actin mRNA bands were quantified as PSA/β-actin ratios.

Measurement of GRP78 mRNA Levels by Reverse Transcription. Previous studies demonstrate that binding of α_2M* to prostate cancer cells upregulates GRP78.[38] Thus, we studied the effect of RBD on GRP78 message levels. Total RNA from 1-LN prostate cancer cells treated with buffer on RBD was extracted by a single method using an Rneasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was reverse-transcribed with 1 μg of RNA in a 20 μL reaction mixture, using Moloney murine leukemia virus reverse transcriptase (200 units) and oligo(dt) as primer for 1 h at 4 °C. The resulting DNA (5 μg) was used as a template, and a 225 bp segment of the GPR78 cDNA was amplified using a 17mer upstream primer (5′-CCA CTT GGG CTA TAG CA-3′) identical to positions corresponding to amino acids 332−338 and a 16mer downstream primer (5′-ACC GCC TGA CAC CTG A-3′) complementary to positions 253−259 of the amino acids encoded in the GRP78 mRNA. A 302 bp segment of mouse β-actin (constitutive internal control) cDNA was co-amplified using a set of primers provided in an R&D Systems kit. Amplification was conducted in a Techne Thermal Cycler PHC-3 for 28 cycles (one cycle at 94 °C for 45 s, 60 °C for 45 s, and 70 °C for 45 s). PCR products were analyzed on a 1.2% (w/v) agarose/ethidium bromide gel. The gels were photographed, and then intensities of individual PSA and β-actin mRNA bands were quantified as PSA/β-actin ratios.

Measurement of Levels of PSA, GRP78, p-ERK1/2, and p-MEK1/2 by Western Blotting in Cells Stimulated with α_2M*, RBD, or Its K1370A Mutant. The effect of treatment of 1-LN prostate cancer cells with buffer or RBD on levels of PSA, GRP78, p-ERK1/2, and p-MEK1/2 was measured by Western blotting. Cells incubated overnight in 6-well plates (3×10^6 cells/well) in RPMI-S medium were stimulated with buffer or RBD (50 pM for 25 min) and incubated at 37 °C. The reactions were terminated by aspirating the medium. A volume of lysis buffer A containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% Nonidet P-40, 25 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 well. Cells were lysed for 15 min over ice, cell lysates scraped into respective tubes and centrifuged for 5 min at 800*g* at 4 °C, and protein contents of lysates determined.[6] Equal amounts of lysate protein were used for electrophoresis. Protein bands on the gel were transferred to a PVDF membrane and the respective membranes immunoblotted with antibodies

against PSA, GRP78, p-ERK1/2, and p-MEK1/2. The membranes, after stripping, were reprobed for actin or ERK1/2 or MEK1/2. The specificities of antibodies used in this experiment and the experiments described in the following studies were determined by treating the cells with nonimmune serum and cell lysates processed as described above. Under the experimental conditions, no reactivity of antibodies was observed.

Assay of mTORC1 Kinase in Raptor Immunoprecipitates in Prostate Cancer Cells Treated with α_2M*, RBD, or Its K1370A Mutant. α_2M* (50 pM)-induced activation of mTORC1 kinase in prostate cancer cells was assessed in Raptor immunoprecipitates of cells by assaying the phosphorylation of S6K1 and 4EBP1 as described previously.[39−41] Briefly, 1-LN prostate cancer cells (3×10^6 cells/well in 6-well plates) incubated overnight in RPMI-S medium were washed twice with ice-cold HHBSS, and a volume of RPMI-S medium was added to each well. After temperature equilibration, cells in the wells were exposed to either buffer or α_2M* (50 pM for 25 min), RBD (50 pM for 25 min), or RBD mutant K1370A (50 pM for 25 min) and incubated as described above. Reactions were stopped by aspirating the medium; a volume of CHAPS lysis buffer (buffer B) containing 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.3% CHAPS, and a Roche protease inhibitor cocktail tablet (1 tablet/10 mL) was added, and cells were lysed over ice for 15 min. The lysates were transferred to separate Eppendorf tubes and centrifuged (1000 rpm for 5 min at 4 °C), and supernatants were used for protein estimation and immunoprecipitation. In separate experiments, equal amounts of lysate protein (200−250 μg) were used for immunoprecipitation with Raptor antibodies (1/50), followed by the addition of 40 μL of protein A agarose, and contents incubated with rotation overnight at 4 °C. Raptor immunoprecipitates were recovered by centrifugation (2000 rpm for 5 min at 4 °C). In experiments in which Raptor immunoprecipitates were used for S6K1 phosphorylation, the respective immunoprecipitates were first washed once with lysis buffer containing 10 mM Tris-HCl (pH 7.2), 0.5% sodium deoxycholate, 0.1% NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM DTT, 10 μg/mL leupeptin, and 5 μL of pepstatin, then with this mixture without NP-40 and with 1 M NaCl, and then with buffer containing 50 mM Tris-HCl (pH 7.2), 5 mM Tris base, and 100 mM NaCl. The respective immunoprecipitates after each wash were recovered by centrifugation (2500 rpm for 5 min at 4 °C). To the respective immunoprecipitates was added 40 μL of reaction buffer containing 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 50 μM ATP, and 3 μg of substrate peptide. The reaction was started by adding 5 μCi of [γ-^{33}P]ATP. The contents were incubated for 15 min at 30 °C in a shaking water bath. The reaction was stopped by adding a volume of 4× sample buffer; contents of tubes were boiled for 5 min, centrifuged, electrophoresed in a 12.5% gel, and transferred to membranes, and the membrane was autoradiographed. Phosphorylated S6K1 substrate peptide bands were visualized and quantified as described above. We also assessed α_2M*-induced activation of mTORC1 kinase by assaying the phosphorylation of S6K1 in Raptor immunoprecipitates by Western blotting. Experimentation details were identical to those described above except that Raptor immunoprecipitates isolated from cells treated as described above were added a volume of 4× sample buffer, contents of tubes were boiled, centrifuged, electrophoresed (12.5% gel), transferred to a membrane, and immunoblotted with a p-S6K Thr^{389} antibody, and protein bands were detected by ECF and quantified via a Storm 860 Phosphorimager.[39−41]

Determination of mTORC1 Kinase Activity in Raptor Immunoprecipitates by Phosphorylation of 4EBP1 in Cells Stimulated with α_2M*, RBD, or Its K1370A Mutant. RBD-induced activation of mTORC1 kinase in prostate cancer cells was determined in Raptor immunoprecipitates of 1-LN prostate cancer cells (3×10^6) incubated overnight in RPMI-S medium and washed twice with ice-cold HHBSS and a volume of RPMI-S medium added to each well. After temperature equilibration, cells in the wells were exposed to either buffer, RBD (50 pM for 25 min), or K1370A (50 pM for 25 min) and incubated as described above. Reactions were stopped by aspirating the medium; a volume of CHAPS lysis buffer (buffer B) was added, and cells were lysed over ice for 15 min. The lysates were transferred to separate Eppendorf tubes and centrifuged (1000 rpm for 5 min at 4 °C) and supernatants used for protein estimation and immunoprecipitation with Raptor antibodies. In separate experiments, to equal amounts of lysate protein (200−250 μg) were added 40 μL of protein G agarose and Raptor antibodies (1/50), and the contents were incubated with rotation overnight at 4 °C. Raptor immunoprecipitates were recovered by centrifugation (2000 rpm for 5 min at 40 °C), washed twice with lysis buffer B, and centrifuged (2500 rpm for 5 min at 4 °C). The respective immunoprecipitates were suspended in 40 μL of kinase buffer containing 10 mM HEPES (pH 7.4), 50 mM NaCl, 50 mM β-glycerophosphate, 0.1 mM EDTA, 1 mM DTT, 20 mM $MnCl_2$, 200 μM ATP, and 4 μg of PHAS. The reaction was initiated by adding 5 μCi of [γ-^{33}P]ATP, and tubes were incubated in a shaking water bath for 15 min at 30 °C. The reaction was stopped by adding a volume of 4× sample buffer; contents of tubes were boiled for 5 min, and supernatants were electrophoresed via 12.5% acrylamide gels. The protein bands were transferred to membranes and membranes autoradiographed. Phosphorylated PHAS peptide bands were visualized and quantified as described above. We also assessed RBD-induced activation of mTORC1 kinase by assaying the phosphorylation of 4EBP1 in Raptor immunoprecipitates by Western blotting. Experimentation details were identical to those described above except that to Raptor immunoprecipitates isolated from cells treated as described above was added a volume of 4× sample buffer, contents of tubes were boiled, centrifuged, electrophoresed (12.5% gel), transferred to membranes, and immunoblotted with an anti-p-4EBP1$^{T37/46}$ antibody, and protein bands were detected by ECF and quantified in a Storm 860 Phosphorimager.[39−41]

Determination of α_2M*-, RBD-, or K1370A-Induced Activation of Akt^{T308} and Akt^{S473} Kinases in Cancer Cells. 1-LN prostate cancer cells (3×10^6 cells/well in 6-well plates) incubated overnight in RPMI-S medium were washed twice with ice-cold HHBSS, and a volume of RPMI-S medium was added to each well. After temperature equilibration, cells in the wells were exposed to either buffer or α_2M* (50 pM for 25 min), RBD (50 pM for 25 min), or K1370A (50 pM for 25 min) and incubated as described above. Reactions were stopped by aspirating the medium; a volume of CHAPS lysis buffer (buffer B) was added, and cells were lysed over ice for 51 min. The lysates were transferred to separate Eppendorf tubes and centrifuged (1000 rpm for 5 min at 40 °C) and supernatants used for protein estimation and immunoprecipitation. In separate experiments, equal amounts of lysate protein (200−

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250 μg) were used for immunoprecipitation with Akt antibodies (1/50) followed by the addition of 40 μL of protein A agarose and contents incubated with rotation overnight at 4 °C. Akt immunoprecipitates were washed with (1) lysis buffer B supplemented with 0.5 M NaCl, (2) lysis buffer, and (3) Tris-HCl (pH 7.4) supplemented with 1 mM DTT, 1 mM PMSF, and 1 mM benzamidine by centrifugation at 2500 rpm for 5 min at 40 °C. To the respective immunoprecipitates was added 40 μL of kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 20 μg/mL leupeptin followed by the addition of 30 μM Akt^{S473} kinase substrate (NH_2-RRPHFPQFSYSA-COOH) to the respective tubes. Peptide NH_2-GGEEEEYFELVKKKK-COOH (Zak 3 peptide) served as the control. The reaction was initiated by adding 50 μM ATP and 5 μCi of [γ-^{33}P]ATP to each tube, and the tubes were incubated for 30 min at 30 °C in a shaking water bath. The reaction was stopped by the addition of 5 μL of 0.5 M EDTA to each tube. The tubes were centrifuged at 3000 rpm for 3 min; 40 μL of each supernatant was applied on p81 phosphocellulose paper (Whatman) and allowed to dry, and papers were washed four times each by being immersed in 1 L of 1-LN phosphoric acid for 5 min. The papers were rinsed with acetone, and their radioactivity was counted in a liquid scintillation counter. Like phosphorylation of Akt at Ser^{473}, phosphorylation of Akt at Thr^{308} was assessed in Akt immunoprecipitates except that Akt^{Thr308} kinase substrate peptide (NH_2-KTFCGTPEYLAPEVRR-COOH) was used in the kinase assay of p-Akt^{T308}. In preliminary experiments, the kinase activities of Akt^{S473} and Akt^{T308} kinase toward control Zak 3 peptide were always 50−60% of buffer control. Hence, control peptide activities are not shown. We also assessed RBD-induced activation of Akt^{T308} and Akt^{S473} by Western blotting. Cells incubated overnight in RPMI-S medium were treated as described above. In separate experiments, to an equal amount of CHAPS cell lysate protein was added a volume of 4× sample buffer; contents of tubes were boiled for 5 min and centrifuged, supernatants electrophoresed on a 10% gel, proteins transferred to Hybond-P membranes, and membranes immunoblotted with antibodies specific for p-Akt^{S473} and p-Akt^{Ser473} bands detected by ECF and quantified in a Storm 860 Phosphorimager.

Proteolytic Degradation of α_2M. Previous studies demonstrated that proteolytic cleavage of α_2M quantitatively releases the RBD.[42−44] However, these studies generally were conducted at low pH.[43] Here we studied the cleavage of α_2M at pH 5.7, 6.0, 6.5, and 7.0 by chymotrypsin otherwise as described previously.[43] The molar ratio of proteinase to α_2M employed was 14/1, and the time of incubation was 30 min as in the previous study.[43] Samples were treated with Halt (Pierce) at a 1/100 dilution as recommended by the manufacturer, and PMSF at a final concentration of 1 mM was also added prior to electrophoresis. Digested samples were electrophoresed on a 10% Bis/Tris gel in MES buffer under reducing conditions. Gels were stained with Coomassie Brilliant Blue or transferred to nitrocellulose where the blot was probed with a rabbit anti-RBD antibody.

Statistical Analysis. Each study reported here was repeated independently at least three times. Each sample from these separate studies was analyzed in triplicate. The statistical significance of the data was determined by a Student's *t* test.

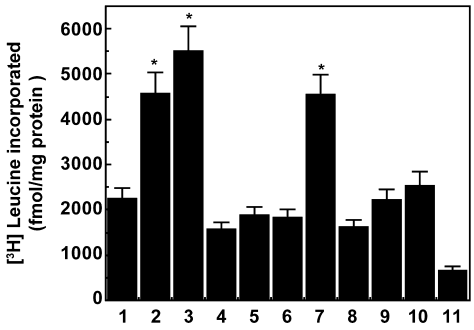

Figure 1. Effect of α_2M*, RBD, or RBD mutant K1370A on protein synthesis in 1-LN prostate cancer cells. The incorporation of [^{3}H]leucine into cellular protein is expressed as femtomoles per milligram of protein and is the mean ± standard error (SE) from three separate experiments. The bars in the diagram are (1) buffer-treated, (2) α_2M* (50 pM), (3) RBD (50 pM), (4) LY294002 (25 μM for 20 min) and then RBD (50 pM), (5) rapamycin (100 nM for 20 min) and then RBD, (6) antibody against the carboxyl-terminal domain of GRP78 (3 μg/mL for 2 h) and then RBD, (7) K1370A (50 pM), (8) LY294002 (25 μM for 20 min) and then K1370A, (9) rapamycin (100 nM for 20 min) and then K1370A, (10) antibodies against the carboxyl-terminal domain of GRP78 (3 μg/mL for 2 h) and then K1370A, and (11) isotype control IgG (2 μg/mL). The results in lanes 2, 3, and 7 are statistically significant at the 5% level compared to the appropriate controls in the figure as indicated by the asterisks.

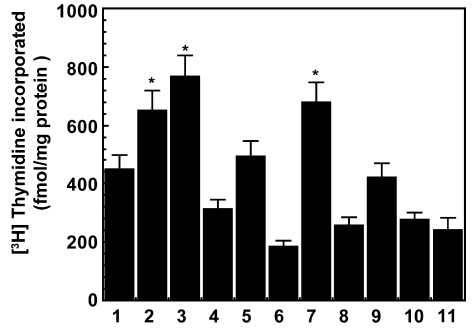

Figure 2. Effect of α_2M*, RBD, and RBD mutant K1370A on [^{3}H]thymidine uptake by 1-LN prostate cancer cells. The uptake of [^{3}H]thymidine by cells is expressed as femtomoles per milligram of cell protein and is the mean ± SE from three independent experiments. The bars in the diagram are as described in the legend of Figure 1. The statistical analysis is as described in the legend of Figure 1.

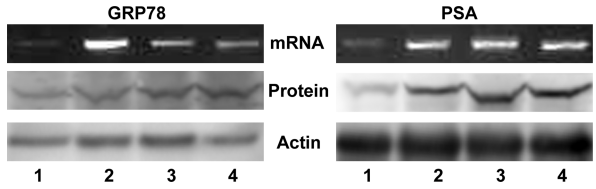

Figure 3. RBD and mutant K1370A upregulate GRP78 (left) and PSA (right) in 1-LN prostate cancer cells. The cells were treated with (1) buffer, (2) α_2M* (50 pM for 25 min), (3) RBD (50 pM for 25 min), or (4) K1370A (50 pM for 25 min). Representative RNA gels and immunoblots from three or four experiments are shown. The protein loading control actin is shown below the respective immunoblot.

RESULTS

RBD Treatment of 1-LN Prostate Cancer Cells Causes an Increased Level of Protein Synthesis. Binding of monomeric RBD or its K1370A mutant to GPR78 induces

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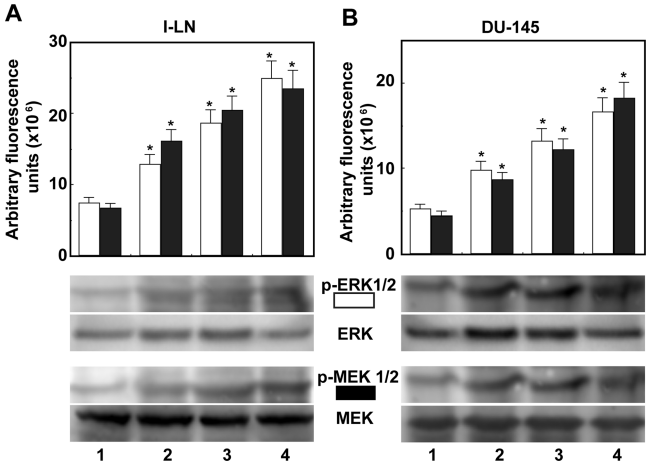

Figure 4. Upregulation of ERK1/2 and MEK1/2 phosphorylation in 1-LN (A) and DU-145 (B) cells treated with RBD (50 pM for 25 min) and its K1370A mutant (50 pM for 25 min). The bars are (1) buffer-treated, (2) α_2M* (50 pM for 25 min), (3) RBD (50 pM for 25 min), and (4) K1370A (50 pM for 25 min). The data are expressed in arbitrary units and are the mean ± SE from three independent experiments. The asterisk indicates a value statistically different from the control, lane 1 at the 5% level. A representative immunoblot of p-ERK1/2 and p-MEK1/2 along with their protein loading controls is shown.

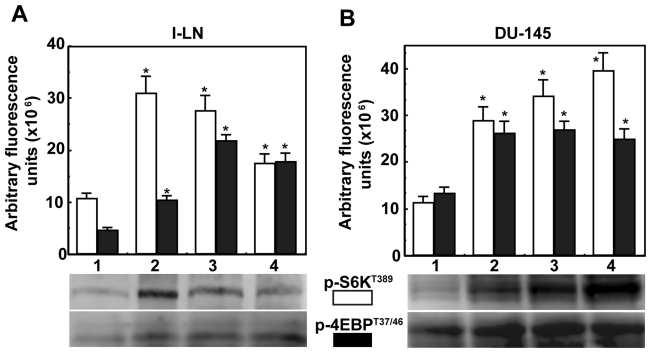

Figure 5. Stimulation of 1-LN (A) and DU-145 (B) prostate cancer cells with RBD and its K1370A mutant causes activation of mTORC1 as measured by phosphorylation of S6K at Thr^{389} and 4EBP1 in Raptor immunoprecipitates. Representative immunoblots of three or four independent experiments with p-S6-kinase^{T389} and p-4EBP1$^{T37/46}$, along with their respective protein loading controls S6K and 4EBP1, are shown. The lanes in the immunoblots are (1) buffer-treated, (2) α_2M* (50 pM for 25 min), (3) RBD (50 pM for 25 min), and (4) K1370A (50 pM for 25 min). The data are expressed in arbitrary units, and the asterisk indicates a value significantly different from the control (lane 1).

comparable signals; however, K1370A does not bind to LRP.[17,18] Treatment of 1-LN prostate cancer cells with RBD and RBD mutant K1370A caused an ∼2-fold increase in the level of protein synthesis, which was comparable to that observed in α_2M*-treated cells (Figure 1). Like that of α_2M*, this increase in the level of protein synthesis was dependent on PI 3-kinase-Akt and mTOR signaling because pretreatment of cells with the PI 3-kinase inhibitor LY294002 (20 μM for 20 min) and mTOR inhibitor rapamycin (100 nM for 20 min) drastically reduced the RBD- or K1370A-induced increase in the level of protein synthesis (Figure 1). Pretreatment of cells with antibodies against the carboxyl-terminal domain of GRP78 abrogated RBD- and K1370A-induced protein synthesis (Figure 1).

RBD Treatment of 1-LN Prostate Cancer Cells Causes an Increased Rate of [^{3}H]Thymidine Uptake. Like protein synthesis, treatment of 1-LN prostate cancer cells with RBD or RBD mutant K1370A caused an ∼1.5−2-fold increase in the rate of [^{3}H]thymidine uptake, which is comparable to that observed in α_2M*-treated cells (Figure 2). Like that with α_2M*, this increase was dependent on its binding to GRP78 because pretreatment of cells with antibodies against the carboxyl-terminal domain of GRP78 abolished the RBD- or K1370A-induced increase in the rate of [^{3}H]thymidine uptake (Figure 2). The increase in the rate of [^{3}H]thymidine uptake was regulated by PI 3-kinase-Akt and mTOR signaling because pretreatment of cells with PI 3-kinase inhibitor LY294002 and mTOR inhibitor rapamycin significantly reduced the rate of [^{3}H]thymidine uptake in RBD-, mutant K1370A-, or α_2M*-stimulated cells (Figure 2). Because pretreatment of cells with antibodies against the carboxyl-terminal domain of GRP78 nearly abrogated RBD- or K1370A-induced protein (Figure 1) and DNA synthesis (Figure 2), the requirement of cell surface GRP78 in promoting proliferation of these cells is demonstrated.

Transcriptional and Translational Upregulation of Prostate Specific Antigen and GRP78 in 1-LN Prostate Cancer Cells Stimulated with RBD. PSA is a serine proteinase that forms complexes with serum proteinase inhibitors, including α_2M*. Recently, we reported that consequent to the binding of α_2M* to GRP78 in prostate cancer cells expressing GRP78 on their cell surface, a 2−3-fold increase in the level of PSA protein synthesis and in mRNA levels occurred.[6] Similar transcriptional and translational upregulation of GRP78 also occurred under these conditions.[38] Newly synthesized PSA is secreted to the medium as an active proteinase where it binds to native α_2M.[38] The resultant α_2M−PSA complex, like the α_2M−NH_2 complex, binds to GRP78 and induces RAS/MAPK and PI 3-kinase/Akt-mTOR signaling that promote cancer proliferation and survival.[38] Transfection of cells with GRP78 dsRNA or pretreatment with antibodies against the carboxyl-terminal domain of GRP78 greatly attentuates PSA synthesis, showing the requirement of cell surface GRP78 and binding of α_2M* to GRP78 for PSA synthesis and induction of downstream signaling.[6] If the mode and kinetics of binding of tetrameric α_2M* and monomeric RBD to GRP78 are similar, then one would expect that the downstream signaling events and cellular responses consequent to the binding of RBD would also be similar to those observed in cells stimulated with α_2M*. Stimulation of 1-LN prostate cancer cells with RBD or RBD mutant K1370A upregulated mRNA and protein levels of PSA and GRP78 (Figure 3). Monomeric RBD, by binding to GRP78 to induce PSA synthesis and secretion into the medium, will promote promotion of the complex with native α_2M, causing the exposure of receptor binding sites on each of its four subunits. Activated α_2M* will bind to GRP78 and promote proliferative and survival signaling in cancer cells. Thus, just as reported for binding of α_2M* to prostate cancer cells, RBD binding activates an autocrine-like response in prostate cancer cells. The results presented show that the site, mode, and kinetics of binding of α_2M* and RBD to cell surface GRP78 and triggering of downstream signaling and cellular responses consequent to their binding are identical.

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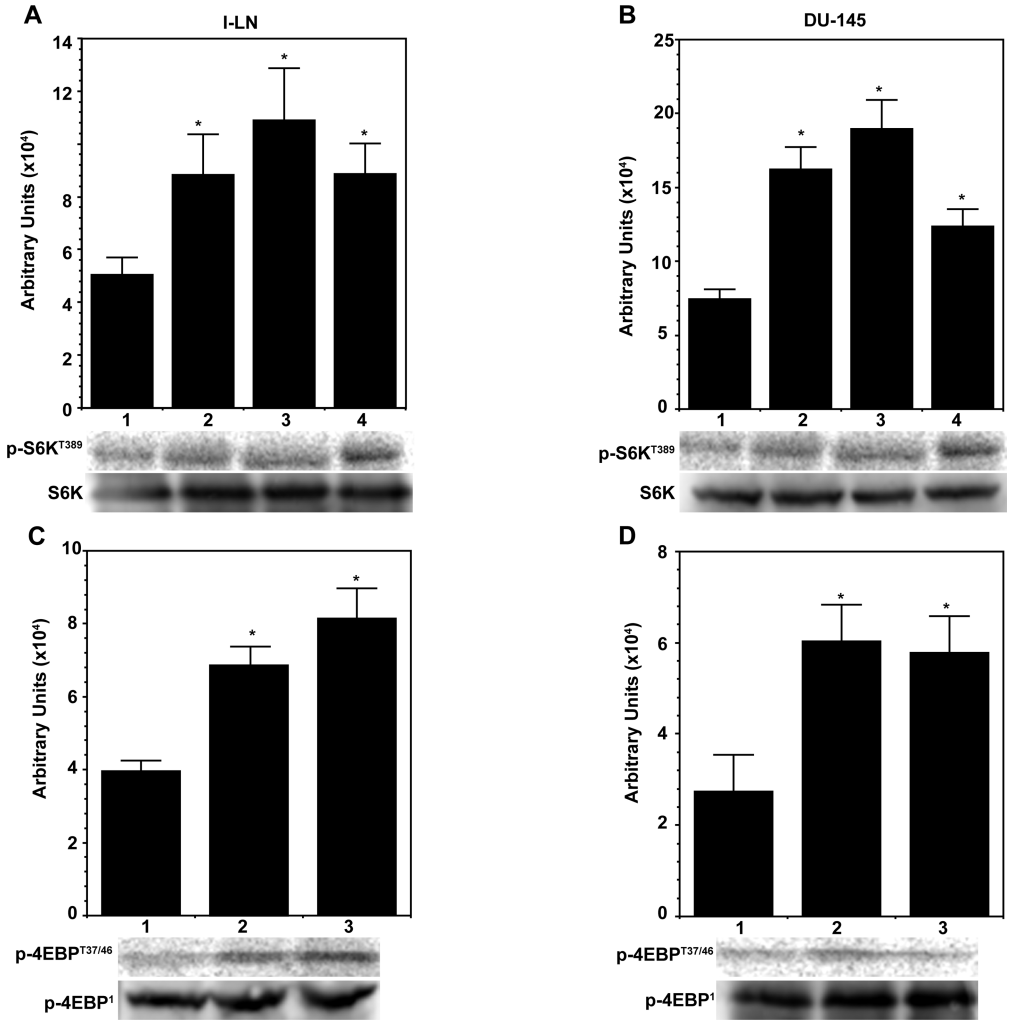

Figure 6. Assay of mTORC1 activation by phosphorylation of S6K and 4EBP1 in 1-LN (A) and DU-145 (B) prostate cancer cells stimulated with RBD and its mutant. Panel A shows the data for 1-LN cells and Panel B the data for DU-145 cells. The bars are (1) buffer-treated, (2) α_2M* (50 pM for 25 min), (3) RBD (50 pM for 25 min), and (4) K1370A (50 pM for 25 min). The bars in panels C (1-LN) and D (DU-145) are (1) buffer-treated, (2) RBD (50 pM for 25 min), and (3) K1370A (50 pM for 25 min). Representative autoradiographs of phosphorylated S6K and 4EBP1 from three independent experiments are shown below their respective bar diagrams. The phosphorylation of S6K and 4EBP1 data are expressed in arbitrary units and are the mean ± SE from three independent experiments. In all four panels, data are statistically significant at the 5% level for lanes 2−4, which are statistically equivalent to lane 1.

RBD and Mutant K1370A Activate MEK1/2 and ERK1/2 MAPK in 1-LN Cells. In mammalian cells, the Ras/MEK/ERK pathway provides a common route by which signals from different growth factor receptors converge at a major regulatory element of the promoters of the c-fos and other coregulated genes. Ras proteins act as molecular switches that relay the proliferative signals from the cell surface to the nucleus and cytoskeleton. Activation of receptor tyrosine kinase initiates a signaling cascade, including transient formation of RAS·GTP and activation of Raf kinase at the membrane, followed by the sequential activation of MEK and ERK. In an earlier report, we showed that like α_2M* treatment, RBD treatment of macrophages also induced the formation of RAS·GTP.[45] Therefore, we next measured phosphorylation of MEK1/2 and ERK1/2 in 1-LN prostate cancer cells by Western blotting (Figure 4). RBD and its mutant K1370A-stimulated cells showed an ∼1.5−2-fold increase in the level of phosphorylation of MEK1/2 and ERK1/2, which was comparable to that of α_2M*-stimulated cells (Figure 4).

Activation of mTORC1 Signaling in 1-LN Prostate Cancer Cells Stimulated with RBD. Eukaryotic cells employ Ras·MAPK and PI 3-kinase/Akt-mTOR signaling pathways for their survival and proliferation in both physiological and pathological environments. The mammalian target of rapamycin (mTOR), an evolutionarily conserved Ser/Thr kinase, is a key regulator of Akt phosphorylation. mTOR assembles into two physically and functionally distinct protein complexes designated mTORC1 and mTORC2. These two protein complexes differ in their regulation, downstream targets, and sensitivity to allosteric inhibitor rapamycin. mTORC1 is a homodimer and in addition to mTOR consists of Raptor and GβL (mLST8). It is sensitive to rapamycin. Activated mTORC1 promotes cell growth in part by directly phosphorylating translational regulators S6K and eIF4E binding protein (4EBP). Akt-induced phosphorylation of PRAS40 and Deptor causes their dissociation from Raptor and promotes recruitment of its downstream substrates S6K and 4EBP. TSC2 in complex with TSC1 acts as a GTPase activating protein for Rheb. Binding of Rheb·GTP to mTORC1 results in mTORC1

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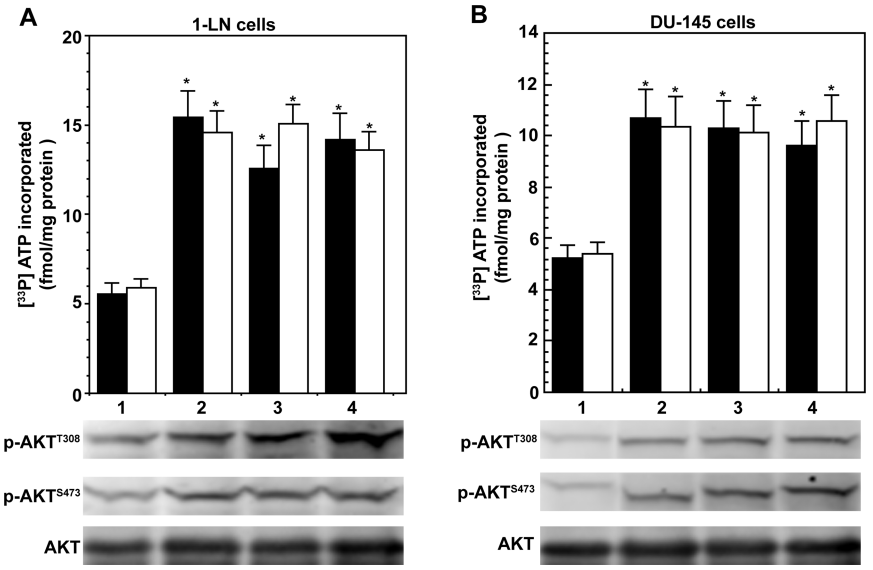

Figure 7. Upregulation of Akt phosphorylation at Thr^{308} and Ser^{473} in 1-LN and DU-145 prostate cancer cells stimulated with RBD or K1370A. Panel A shows the data from 1-LN cells and panel B the data for DU-145. Representative immunoblots of p-Akt^{T308} and p-Akt^{S473} along with their respective protein loading controls from three independent experiments are shown. Assay of Akt phosphorylation at Thr^{308} (■) and Ser^{473} (□) by a kinase assay of Akt immunoprecipitates of 1-LN and DU-145 prostate cancer cells stimulated with RBD or its K1370A mutant. The bars in the diagram are (1) buffer-treated, (2) α_2M* (50 pM for 25 min), (3) RBD (50 pM for 25 min), and (4) K1370A (50 pM for 25 min). The activation of Akt at Thr^{308} and Ser^{473} is expressed as [γ-^{33}P]ATP incorporated (femtomoles per milligram of protein) and is the mean ± SE from three or four independent experiments. In both panels, the data are statistically significant at the 5% level for lanes 2−4 compared to the control (lane 1).

activation but that of Rheb·GTP in its inhibition. Phosphorylation of TSC2 by Akt1 inhibits its GTPase activity, leading to an increased level of GTP loading on Rheb and consequent increase in mTORC1 activity. S6K and 4EBP are direct downstream targets of mTOR and upon phosphorylation of mTOR kinase regulate protein synthesis. We next measured mTORC1 activation in cells stimulated with RBD and mutant K1370A by quantitating p-$S6K^{T389}$ and p-$4EBP1^{T37/46}$ protein levels by Western blotting (Figure 5A,B). The effects were compared to that of α_2M*-induced mTOR activation analyzed as described above under identical conditions. Both RBD and its K1370A mutant caused an ∼2-fold increase in the level of mTOR activation as measured by incorporation of [γ-^{33}P]ATP into S6K1 or 4EBP1 (Figure 6A and B). The RBD-induced increase in the level of mTOR kinase activation in these cells is very comparable with that observed with α_2M*.[46]

Stimulation of Cancer Cells with RBD Promotes Phosphorylation of Akt at Thr^{308} and Ser^{473}. At the plasma membrane, Akt is phosphorylated at Thr^{308} in the catalytic domain by PDK1 and Ser^{473} in the hydrophobic motif domain by mTORC2. Phosphorylation at both these positions is required for full activation of Akt1. Akt1 is also phosphorylated cotranslationally at Thr450 in the turn motif by mTORC2. The turn motif phosphorylation of Akt is essential for newly synthesized forms by facilitating its proper folding and stability. Akt is frequently hyperactivated in human cancers via multiple mechanisms, including activating mutations or deletions in PTEN, with a high incidence in prostate cancer, amplifications, and overexpression of genes encoding p110 isoforms of PI 3-kinase.[40] Mutations of Ras that activate Akt occur in ∼30% of epithelial tumors. Akt gene amplification also occurs in a subset of human cancers. Hyperactivation of Akt is crucial for the genesis of cancer because partial ablation of Akt activity is sufficient to inhibit the development of tumors in PTEN +/− mice.[47,48] In clinical specimens, the overexpression and activation of Akt1 have been associated with high preoperative PSA levels, higher Gleason grades, and shorter disease relapses.[49,50] Immunohistochemical studies showed a greatly enhanced staining of p-Akt^{S473} in poorly differentiated prostate cancer cells.[49,50] In high-grade prostatic intraepithelial neoplasia and prostatic adenocarcinomas, elevated levels of expression of p-mTOR, Raptor, and p-S6K were observed.[51] We have reported that stimulation of activated murine peritoneal macrophages and prostate cancer cells with α_2M activates PI 3-kinase, resulting in activation of Akt1 as measured by a kinase assay of Akt phosphorylation at T^{308} and S^{473} residues and by quantitating protein levels of p-Akt^{T308} and p-Akt^{S473} by Western blotting.[39,40] The phosphorylation of Akt at Thr^{308} was sensitive to both rapamycin and LY294002, but that of Akt at Ser^{473} was sensitive to only LY294002.[40] To further support the premise that tetrameric α_2M* and monomeric RBD bind to same site on GRP78 and elicit triggering of the same signaling and same cellular responses, we assayed Akt1 activation by phosphorylation of Akt1 at T^{308} and $Akt1^{S473}$ by kinase assays (Figure 7B) by quantitating protein levels of p-Akt^{T308} and p-Akt^{S473} by Western blotting (Figure 7A). The RBD-induced increases in the level of phosphorylation of Akt at T^{308} and S^{473} and protein levels of p-Akt^{T308} and p-Akt^{S473} were identical to those observed in cells treated with α_2M* under identical conditions.

Proteolytic Degradation of α_2M. Figure 8 demonstrates that both RBD and larger proteolytic fragments of α_2M containing RBD are released at pH values ranging from 5.7 to 7.0 by chymotrypsin treatment, consistent with previous studies at pH <5.0.[43]

■ DISCUSSION

Human α_2M is a tetrameric protein that when activated by proteinases undergoes a change in its conformation, exposing receptor recognition sites at the tips of each long

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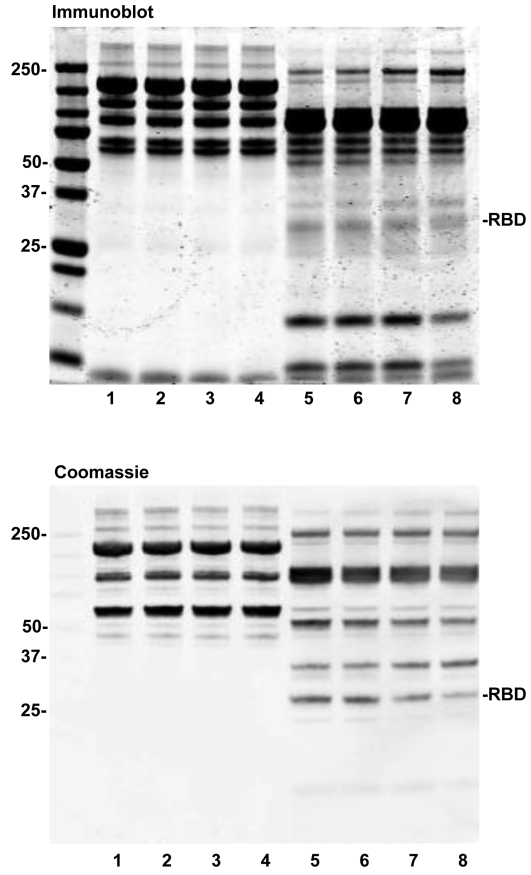

Figure 8. Effect of chymotrypsin treatment of α_2M at different pH values. The bottom panel is a Coomassie brilliant blue-stained gel indicating cleavage of α_2M at different pH values, while the top panel is an immunoblot employing the anti-RBD antibody demonstrating RBD and RBD-containing fragments. Lanes 1−4 non-digested; lanes 5−8 digested.

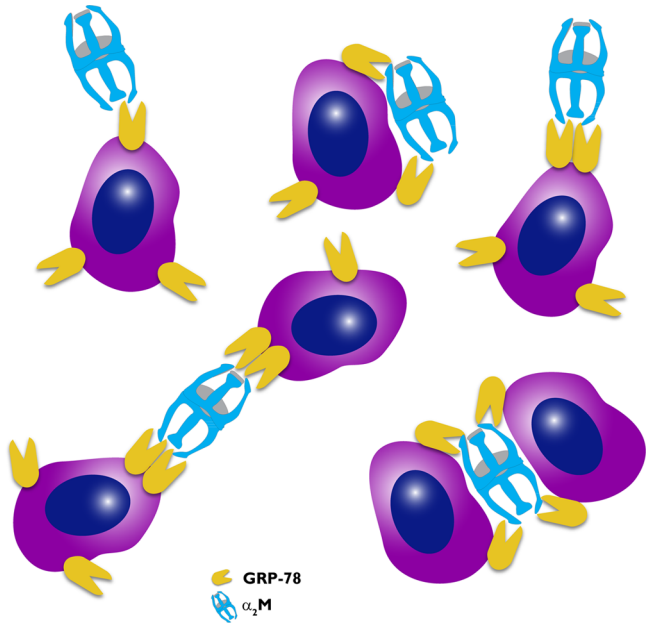

Figure 9. Model for binding of α_2M* to cells. While the model suggests two-point binding to cells, or potentially four-point binding to two different cells simultaneously, binding studies do not suggest cooperative binding as would be expected for a multivalent ligand.[52] In the closed, receptor-recognized form of α_2M, the tips of the long arms approach closely and steric hindrance may prevent simultaneous two-point binding for α_2M* molecules that dock in either orientation as shown in the second and third examples of line 1. The theoretical possibility of binding to four cells is not shown. Multicell binding may be difficult in tissue culture monolayers but cannot be ruled out *in vivo* where the tumor mass is three-dimensional.

arm.[1,7−10,13,15,17] (Figure 9). The model of α_2M and its activated form α_2M* proposed by Feldman et al.[7] are in reasonable agreement with X-ray crystallographic studies.[8−10] The protein consists of two identical "half-molecules" with three *C*2 axes of symmetry.[7] With respect to cell binding, the molecule could "dock" either on its side or like a lunar landing module (Figure 9). In either case, one might expect binding to two receptor sites on the cell surface, irrespective of whether GRP78 or LRP is the target receptor. The mathematical modeling of two-point attachment was analyzed well by Reynolds many years ago,[52] and one would have expected a curvilinear Scatchard plot. It is also conceivable that one α_2M* molecule could bridge two or more adjacent cells with either three- or four-point attachment. However, binding studies do not demonstrate evidence of cooperative binding when either GRP78 or LRP is the target receptor.[15] This behavior remains unexplained. When α_2M is activated, the pair of receptor binding sites on the same side of the molecule are in close opposition and thus steric hindrance is a possible explanation for why only one site of the pair can bind to the cell surface if the molecule binds like a lunar landing module. However, it is unclear why α_2M* might not bind on its side to two binding sites simultaneously. One possible suggestion is that the geometry of the receptor recognition sites with respect to that of the molecular binding sites makes it difficult even in this orientation to bind to two receptors simultaneously. As for binding of one molecule to two or more cells, the data do not address this question. *In vitro*, the tumor cells are grown in a monolayer, while *in vivo*, the tumor exists as a three-dimensional structure. Thus, one could conceive of an α_2M* molecule binding to two or more cells simultaneously. There are no data currently to address this question.

With respect to signal transduction studies, a few studies provide some data suggesting that these monomers trigger comparable signaling events at the same low ligand concentration.[24,37] The maximal activation of signal transduction by binding of α_2M* to GRP78 on prostate cancer cells occurs near the K_d for binding, 50−100 pM.[6,14] Here we compared the effects of RBD and its K1370A mutant on α_2M* signaling of prostate cancer cells at 50 pM. With respect to DNA and protein synthesis, upregulation of PSA and GRP78, activation of MEK1/2 and ERK1/2, Akt activation, or mTORC1 signaling, α_2M* and RBD exhibited nearly identical behavior. These studies suggest that from the standpoint of activation of signal transduction, binding of α_2M* behaves as if it were a monomeric ligand just as is the case for its cell binding behavior. One issue that should be addressed is the possibility that RBD might dimerize, thereby creating a dimeric ligand. Indeed, this has been observed in X-ray analysis of RBD.[53] However, the K_d for association is ~50 μM, some 6 orders of magnitude greater than the concentration of RBD employed in this study. Thus, we believe that this possibility can be dismissed.

Another issue to be considered is whether the signaling events in this study involve only GRP78 or could involve LRP, thus clouding the interpretation. This issue is pertinent to this

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study in view of previous studies suggesting high- and low-affinity binding of α_2M* to LRP (K_d values of 40 pM and 2 nM, respectively).[54] This study must be reinterpreted in view of subsequent studies.[11−13,15−18] In this earlier work, the investigators employed an α_2M*-agarose affinity column to purify α_2M* receptors from detergent-solubilized placenta villous tissue.[55] We have employed a similar approach to purify α_2M* receptors from macrophages and 1-LN prostate cancer cells.[13] In these studies, only one class of receptors, GRP78, a K_d of 50−100 pM is obtained from 1-LN cells, while macrophages yield both GRP78 and LRP, the latter showing a K_d of ∼2−5 nM. These K_d values are in striking agreement with those reported previously on the assumption of a "two-state" binding model (K_d values of 40 pM and 2 nM, respectively).[54] We conclude that this earlier study purified both GRP78 and LRP. For a variety of reasons, we can also rule out LRP as a mechanism for activating signaling cascades in the study presented here. First, the target 1-LN cells do not express LRP.[6,19,20] Second, RBD mutant K1370A binds to GRP78, but not LRP.[16,18,20,25] Third, antibodies directed against the carboxyl-terminal domain of GRP78 that block α_2M* signaling also block RBD- and RBD mutant K1370A-dependent signal transduction. Fourth, LRP-dependent signaling occurs only at a high concentration of α_2M* of >25 nM.[25]

Previous studies have demonstrated that α_2M can be proteolytically degraded to release RBD and fragments containing RBD.[42−44] Thomsen et al. demonstrated quantitative release of RBD by proteolysis of α_2M with chymotrypsin.[43] Unfortunately, these studies were performed at pH 4.5−5.0, not likely to occur *in vivo*, even in tumor beds. Here we demonstrate in the pH range from 5.7 to 7.0 release of RBD-containing fragments from α_2M after a digestion time of only 30 min. As noted above, within the prostate tumor bed, both α_2M[6] and a number of proteinases are secreted. These proteinases include PSA[6,56] and a variety of matrix metalloproteinases, including MMP-1, -2, -3, and -9.[56−59] Levels of PSA above 3−4 ng/mL in plasma are often employed as a screening criterion for prostate cancer,[58] but levels may excede 100 ng/mL.[60,61] This PSA circulates as free proteinase and complexes with α_2M and α_1-anti-chymotrypsin.[62] As prostate cancer progresses, levels of serum α_2M may reach levels as low as 20% of normal because of the action of PSA and presumably MMPs.[63] Thus, significant excesses in the ratio of proteinases to α_2M should occur in the prostate tumor bed. This leads us to propose that tetrameric α_2M by releasing of up to 4 mol of RBD/mol of α_2M might be viewed as a reservoir releasing RBD in tumor beds where both α_2M and proteinases of a variety of classes and at high concentrations are produced. Because α_2M* exerts no more activity than RBD, effectively such proteolysis could result in a 4-fold amplification of signals that are pro-growth, anti-apoptotic, and pro-migratory. RBD itself is expected to resist further proteolysis based on previous studies of the domain structure of α-macroglobulin subunits obtained by proteolytic digestion.[64]

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

α_2M, α_2-macroglobulin; α_2M*, activated forms of α_2M that bind to cell surface receptors; LRP, low-density lipoprotein receptor-related protein; RBD, receptor binding domain of α_2-macroglobulin; GRP78, glucose-regulated protein (M_r ∼ 78000); S6K, S6-kinase; PSA, prostate specific antigen; mTOR, mammalian target of rapamycin.

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