Binding of Activated \( \alpha_2 \)-Macroglobulin to Its Cell Surface Receptor GRP78 in 1-LN Prostate Cancer Cells Regulates PAK-2-dependent Activation of LIMK*

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Two characteristics of highly malignant cells are their increased motility and secretion of proteinases allowing these cells to penetrate surrounding basement membranes and metastasize. Activation of 21-kDa activated kinases (PAKs) is an important mechanism for increasing cell motility. Recently, we reported that binding of receptor-recognized forms of the proteinase inhibitor \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)-M*) to GRP78 on the cell surface of 1-LN human prostate cancer cells induces mitogenic signaling and cellular proliferation. In the current study, we have examined the ability of \( \alpha_2 \)-M* to activate PAK-1 and PAK-2. Exposure of 1-LN cells to \( \alpha_2 \)-M* caused a 2- to 3-fold increase in phosphorylated PAK-2 and a similar increase in its kinase activity toward myelin basic protein. By contrast, the phosphorylation of PAK-1 was only negligibly affected. Silencing the expression of the GRP78 gene, using either of two different mRNA sequences, greatly attenuated the appearance of phosphorylated PAK-2 in \( \alpha_2 \)-M*-stimulated cells. Treatment of 1-LN cells with \( \alpha_2 \)-M* caused translocation of PAK-2 in association with NCK to the cell surface as evidenced by the communoprecipitation of PAK-2 and NCK in the GRP78 immunoprecipitate from plasma membranes. \( \alpha_2 \)-M*-induced activation of PAK-2 was inhibited by prior incubation of the cells with specific inhibitors of tyrosine kinases and phosphatidylinositol 3-kinase. PAK-2 activation was accompanied by significant increases in the levels of phosphorylated LIMK and phosphorylated cofilin. Silencing the expression of the PAK-2 gene greatly attenuated the phosphorylation of LIMK. In conclusion, we show for the first time the activation of PAK-2 in 1-LN prostate cancer cells by a proteinase inhibitor, \( \alpha_2 \)-macroglobulin. These studies suggest a mechanism by which \( \alpha_2 \)-M* enhances the metastatic potential of these cells.

Cancer of the prostate is the most commonly diagnosed malignancy of men (1). In the development of prostate cancer, deregulation of cell growth control often is accompanied by acquisition of androgen independence, a poor prognostic indicator (2, 3). Growth factors, including epidermal growth factor, insulin-like growth factor, and fibroblast growth factor play a role in the progression of androgen-independent prostate cancer (2, 3). These growth factors induce mitogenic cellular responses by activating their specific receptors. Ligand binding to these receptors induces the autophosphorylation of the receptor on specific tyrosine residues resulting in the assembly of multiprotein complexes, which activate the Ras/MAPK and PI 3-kinase signaling pathways (4). In addition to increased activation of signaling pathways that promote cellular proliferation and/or suppression of apoptosis, increased motility is often seen in malignantly transformed cells. This increase in motility, along with increased secretion of proteinases, especially matrix metalloproteinases, enables highly metastatic cancer cells to penetrate surrounding basement membranes and invade blood vessels and lymphatics. One mechanism that promotes increased motility of malignant cells is activation of members of the 21-kDa activated kinase (PAK) family.

These proteins are Ser/Thr kinases that mediate Rac and Cdc42 GTPase-dependent signaling (see reviews in Refs. 5–8 and references therein). The mammalian PAK family consists of six members, including PAK-1 and PAK-2. PAK-1 is tissue-specific in its expression, whereas PAK-2 is ubiquitously expressed. The catalytic activity of PAKs is regulated by the binding of active GTPases to the conserved p21 binding motif in the NH2-terminal domain leading to the relief of autoinhibitory interactions with the COOH-terminal catalytic domain (5–8). PAK-2 is also activated by caspase or caspase-like proteinases, which generate constitutively active p34 PAK-2, the COOH-terminal catalytic domain (9). Activated full-length PAK-2 stimulates cell survival and growth in response to various stress stimuli, whereas its proteolytic fragment, p34 protein, stimulates cell death (9, 10). Stimulation of cell survival by activated full-length PAK-2 is partly mediated by phosphorylation and inhibition of pro-apoptotic Bad (8–10). The activation of PAK-2 in response to irradiation or cytokine \( \beta \)-d-arabinoside is dependent on protein-tyrosine kinase and PI 3-kinase activity (11). PAK-1 mediates signals from the Ras/MAPK and PI 3-kinase signaling pathways to promote cell transformation. PAKs also play important roles in modulating the ability of cancer cells to move and metastasize (5–8). A number of highly metastatic human breast cancer lines exhibit constitutively elevated PAK-1 or PAK-2 activity (12).

\( \alpha_2 \)-Macroglobulin (\( \alpha_2 \)-M) is a broad specificity proteinase inhibitor that binds to cell surface receptors when activated by...
The circulating concentration of α2M in 1–5 μM, and α2M* comprises about 200–500 nM of this pool (32). It has been estimated that about 1 g of αM turns over daily (32). Prostate cancer cells also produce prostate cancer-specific antigen, a proteinase that binds readily to αM (33, 34). Thus highly aggressive prostate cancer may secrete prostate cancer-specific antigen, which by binding to αM generates α2M* further increasing the concentration of α2M* in the tumor microenvironment. Furthermore, tumors may be viewed as existing under ER stress and tumors protect themselves from ER stress by expressing unfolded protein response, of which enhanced GRP78 synthesis is a biomarker (13–18). A small pool of newly synthesized GRP78 translocates to cell surface from the ER in association with MTJ-1 (35). Therefore, it could be envisaged that, under these conditions, a substantial amount of α2M* would be available to bind to cell surface GRP78 thus triggering the activation of mitogenic signaling-dependent cell proliferation. Because it is known that PAKs can be activated via PI 3-kinase signaling (5–8, 11) and membrane localization (5–8, 36), we suggest that activated PAKs may mediate α2M*-induced effects on 1-LN prostate cancer cells. Here we demonstrated that α2M* mediates PAK2 activation in 1-LN cells, but PAK-1 is only negligibly affected. We then examined the effect of treating 1-LN cells with α2M* on the mechanism of activation of PAK-2, Rac-1, LIMK, and coflin. We report that exposure of 1-LN cells to α2M* induces autophosphorylation of PAK-2, activation of the kinase activity of PAK-2 toward myelin basic protein in a tyrosine kinase and PI 3-kinase-dependent manner, and recruitment of PAK-2 to plasma membrane via the adaptor protein NCK. Rac-1 is also activated. We further demonstrate activation of LIMK and coflin, which are essential for regulating cytoskeletal organization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture media were purchased from Invitrogen. Antibodies against PKA-1, PKA-2, and Bad, as well as the phosphorylated forms of PKA-1, PKA-2, LIMK, coflin, and Bad (Ser112 or Ser145, 4×106 cells/well) were washed twice with HBSS, with a volume of the HBSS added to the monolayers. One set of cells was stimulated with different concentrations of α2M*, and cells were incubated as above for different time periods. The other set of cells was stimulated with different concentrations of α2M* for 10 min. At the end of incubation, medium was aspirated, and the cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.6), 0.1 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM sodium orthophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 0.5% Nonidet P-40 for 10 min on ice. The DNA strands were broken by passing the lysates through a 27-gauge needle and syringe several times. The lysates were centrifuged at 800 × g for 5 min at 4°C to remove cell debris. The supernatants were transferred to clean tubes, and their protein contents were determined (37). Equal amounts of lysate proteins were electrophoresed according to Laemmli (38). Proteins from 100 μg of tissue were transferred to a Hybond P® membrane and immunoblotted with antibodies against phosphorylated and unphosphorylated PAK-2 and PKA-1, respectively, according to the manufacturer's instructions. Protein bands on the membrane were visualized by ECF (Amersham Biosciences) and quantified using a Storm 860 PhosphorImager® (Amersham Biosciences). The respective membranes were stripped and reprobed for actin according to the manufacturer's instructions.
αMβ2-induced Autophosphorylation of PAK-1 and PAK-2 in 1-LN Prostate Cancer Cells—Autophosphorylation of PAK-1 and PAK-2 in αMβ2-stimulated cells was measured essentially as described (39). 1-LN cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 12.5 units of penicillin/mL, 10 μg/mL streptomycin in 6-well plates at 37 °C in a humidified CO2 incubator till 80–90% confluent (4 × 106 cells/well). The medium was aspirated, the monolayers were washed with chilled HBSS buffer (pH 7.4), and a volume of HBSS was added to the monolayer. The cells were lysed in a volume of lysis buffer containing 40 mM HEPES (pH 7.4), 1% Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin over ice for 15 min. The lysates were pipetted into Eppendorf tubes. DNA strand breaks were prevented by passing the lysate through a 27-gauge needle several times, and the lysates were centrifuged at 1000 rpm for 5 min at 4 °C to remove cell debris. The supernatants were transferred to new tubes, and their protein contents were determined (37). To equal amounts of lysate proteins in respective tubes, 40 μl of PAK-PBD agarose was added, and the tubes incubated for 1 h at 4 °C with gentle rotation. The tubes were centrifuged at 3500 rpm for 10 min at 4 °C. The agarose pellet was washed twice with HBSS buffer as described above. In the agarose pellets 40 μl of reducing sample buffer was added, the tubes were heated at 90 °C for 5 min, then centrifuged briefly, and the supernatant was processed for protein fractionation on a 10% gel according to Laemmli (38). The protein bands on the gel were transferred to Hybond P® membranes and immunoblotted with antibodies against Rac-1 (Santa Cruz Biotechnology, Santa Cruz, CA). The bands on the membranes were visualized by ECF and quantitated using the PhosphorImager®. An aliquot of lysate was similarly processed for total Rac quantification.

Translocation of PAK-2 to Plasma Membrane in 1-LN Cells Stimulated with αMβ2—Confluent monolayers of 1-LN cells grown as above in RPMI medium in 4-well plates (12 × 105 cells/well) were washed with HBSS twice, and a volume of HBSS was added to the monolayers. The medium was aspirated and replaced with αMβ2 (25 nM) and incubated for 10 min at 37 °C. At the end of incubation, the medium was aspirated, and to the cells was added a volume of chilled HBSS buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μM benzamidine, and 10 μM leupeptin. The cell membranes were then disrupted by a few up and down strokes with a Teflon pestle in a glass homogenizing tube, and the membrane fractions highly enriched in plasma membrane were isolated as described previously. Briefly, the samples were homogenized with glass homogenizing tubes for 1 min at 4 °C. The homogenate was centrifuged at 600 × g for 5 min at 4 °C and the pellet discarded. The supernatant was layered onto a sucrose gradient (50% (3 ml each) and centrifuged at 200,000 × g for 75 min in a Beckman Coulter Ultracentrifuge (Optima LE80) at 4 °C. The membrane fraction at the interface between the sucrose layers was removed and suspended in a volume of incubation buffer (αMβ2 in a volume of 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 3 mM MgCl2, 2 μM EGTA, 2 μM leupeptin, and 1 μM Ca2+). The suspension was centrifuged at 400,000 × g for 90 min at 4 °C. The pellet was suspended in a volume of incubation buffer. The enrichment of membrane preparation for plasma membrane was assessed as described previously, including by electron microscopy (41). These analyses showed this membrane fraction was highly enriched in plasma membranes (92–95%), and hence we refer to this fraction as the “plasma membrane fraction.” The membrane pellet was lysed in lysis buffer, and the lysate was immunoprecipitated with antibodies against GRP78 (1:100) and GRP78 in the membranes quantified as above. The membranes on which these preparations were transferred after electrophoresis were rephotographed and quantified for phosphorylated PAK-2 and NCK according to the manufacturer’s instructions.

Chemical Synthesis of dsRNA Homologous in Sequence to the Target GRP78 Gene—The chemical synthesis of dsRNA homologous in sequence to the target GRP78 were as follows: 1) 5′-TGGKQQLVK376, mRNA sequence 5′-ATA AAA CAT CAA TTA GTA AAG-3′ and 2) 5′-GKKNTTTG, mRNA sequence 5′-AAG AAT AAA ATA ACA ACA ACA-3′. The oligonucleotides were annealed according to the manufacture’s instructions. Throughout the entire period of experimentation, handling of reagents was performed in a RNase-free environment. Briefly, equal amounts of sense and antisense oligonucleotides were mixed in annealing buffer and heated at 90 °C for 1 min then chilled 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 GRP78—Confluent 1-LN cells grown in 10-cm dishes (1.5 × 106 cells in 6-well plates) were incubated as described above were washed twice with HBSS and 2 mL of DMEM containing 10% of FBS and the above mentioned antibiotics added, and cells were incubated as above for 16 h. Just before each transfection, 25 μg of both GRP78 dsRNA was diluted to 100 μM of serum- and antibiotic-free DMEM in a tube. In another tube, 10 μl of Lipofectamine was diluted into 100 μM of serum- and antibiotic-free medium. The two solutions were mixed in a ratio of 2:1 and incubated for 5 min at room temperature before use. The dsRNA mixtures were added to 1-LN cells for 24 h.
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 in separate experiments. The monolayers were washed twice with serum-free DMEM, layered in each well with 1 ml of Lipofectamine-DMEM or lipid dsRNA mixtures containing 25 μg of dsRNA of each 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 incubation, 1 ml of antibiotic-free DMEM containing 10% FBS was added to each well, and cells incubated for 16 h as above. Microscopic observations of the monolayers did not show evidence of toxicity consistent with previous studies. 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 incubation, medium was aspirated and 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 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™ negative control, catalog number 4610, Ambion) under identical conditions as described above for transfection with GRP78 dsRNA. At the end of transfection period (48 h), the medium was aspirated and a volume of DMEM was added, the cells were either stimulated with buffer or αM* (50 pm) for 10 min. The reaction was stopped by aspirating the medium and adding a volume of lysis buffer as described above. Equal amounts of lysate proteins (37) were electrophoresed according to Laemmli (38). Proteins from gel (10%) were transferred to a Hybond P membrane and immunoblotted with antibodies against GRP78, unphosphorylated PAK-2, and NCK, respectively, according to the manufacturer’s instructions. Protein bands on the membrane were visualized by ECF (Amer sham Biosciences) and quantified using the PhosphorImager®. The respective membranes were stripped and reprobed for actin according to the manufacturer’s instructions. 

Chemical Synthesis of dsRNA Homologous in Sequence to the Target PAK-2 Gene—The chemical synthesis of dsRNA homologous in sequence to the target PAK-2 (5'-GGKLDTPGGEKGGG-3' and 5'-AAA TTA ACA GAT TTT GGA TTT-3') peptide; Swiss-Prot primary accession number Q8CIN4) was performed by Ambion. For making dsRNA, the sense (5'-AUU AAC AGA AGA UGG AUU UTU-3') and antisense (5'-AAA UCC AAA AUC UGU UAA UTU-3') oligonucleotides were annealed according to the manufacturer’s instructions as described above for GRP78 above. 

Transfection of 1-LN Cells with dsRNA Homologous in Sequence to PAK-2 Gene and Effect of αM* on Phosphorylated LIMK—Confluent 1-LN cells monolayers (1.5 × 10⁵/well in 6-well plates) incubated as described above were washed twice with HBSS and 2 ml of DMEM containing 10% FBS, the above mentioned antibiotics were added, and the cells were incubated as above for 16 h. For each transfection, 25 μg of dsRNA was used and cells were transfected as described above for dsRNA with Lipofectamine with or without dsPAK-2. 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. Microscopic observation of the transfected monolayers did not show evidence of toxicity expect that 1-LN cells transfected with dsPAK-2 RNA showed impairments in the uniformity of spreading of monolayers. At the end of incubation, medium was aspirated, monolayers were washed with the above medium once, a volume of same medium was added, the cells were stimulated with αM* then lysed, and lysates were processed for phosphorylated LIMK, PK-2, and LIMK protein assay by Western blotting as described above. Protein bands on the membrane were visualized by ECF (Amer sham Biosciences) and quantified using the PhosphorImager®. To demonstrate that the transfection of 1-LN prostate cancer cells with dsRNA homologous in sequence to target PAK-2 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, under identical conditions as described above. At the end of transfection (48 h), the medium was aspirated, a volume of DMEM was added, and the cells were stimulated with either buffer or αM* and processed as above for the quantification of phosphorylated LIMK, PK-2, and LIMK protein by Western blotting.

RESULTS

Binding of αM* to Cell Surface-associated GPR78 Enhances Phosphorylation of PK-2 in 1-LN Cells—PAK activity is stimulated in response to a variety of extracellular stimuli, including chemoattractants acting on G protein-coupled receptors, growth factors interacting with receptor tyrosine kinases, cytokines, and extracellular matrix molecules binding to integrins (5–8). We have previously shown that αM* promotes cellular growth of 1-LN prostate cancer cells (17, 18). To understand the possible involvement of PAK-1 and PAK-2 in αM*-stimulated cellular growth, we first examined the levels of phosphorylated PAK-1 and PAK-2 in 1-LN cells treated with αM* for different periods of time and with varying concentrations of αM* (Fig. 1 and Table I). In the Western blot, phosphorylated PAK-2 was seen as a doublet of 58–60 kDa (Fig. 1). This appears to represent differential phosphorylation of PAK-2 as previously reported. The maximal increase in phosphorylated PAK-2 occurred at about 10 min of incubation and declined slowly thereafter, whereas the maximal increase in levels of phosphorylated-PAK-2 occurred at about 50–100 μM αM*. Surprisingly, incubation of 1-LN cells with αM* under these conditions only showed a negligible effect on phosphorylation of PAK-1. Similar to the results described above, stimulation of 1-LN cells with αM* predominantly caused autophosphorylation of PAK-2, but negligible autophosphorylation of PAK-1 (Fig. 2A).

αM* Stimulates the Kinase Activity of PAK-2 in 1-LN Prostate Cancer Cells—In the next series of experiments we studied the activation of only PAK-2 by measuring its kinase activity toward MBP (Fig. 2B). Treatment of 1-LN prostate cancer cells with αM* increased PAK-2-dependent phosphorylation of MBP by 2- to 3-fold (Fig. 2B). αM* Activates Rac-1 in 1-LN Cells—p21-activated protein kinases are phosphorylated by small G proteins such as Rac-1 and Cdc42 in the presence of GTP, which binds to the G protein binding site in the NH2-terminal regulatory domain. In the preceding section we showed that stimulation of 1-LN cells with αM* induces phosphorylation and the kinase activity of PAK-2 (Fig. 2, A and B). We next determined the activation of Rac-1, by quantifying the levels of Rac-1-GTP by Western blotting (Fig. 2C). Indeed αM* treatment of 1-LN cells elevated the levels of Rac-1-GTP by about 2-fold compared with unstimulated cells (Fig. 2C). The results show the importance of
Small G proteins in \( \alpha_2 M^* \)-induced activation of PAK-2 in 1-LN cells.

Recruitment of PAK-2 to Plasma Membranes via Interaction with the Adaptor Protein NCK in \( \alpha_2 M^* \)-stimulated 1-LN Prostate Cancer Cells—In response to external stimuli, PAKs, which contain variable SH3 binding motifs/sites, can interact with the SH3-containing adaptor protein NCK (5–8). This protein is involved in the recruitment of PAKs to activated tyrosine kinase receptors in plasma membranes. NCK is either constitutively associated with PAK-2 or its association is induced (5–8, 36, 42). Interaction of the adaptor protein NCK and PAKs has been implicated in its translocation and stimulation of PAK activity by growth factors (see Refs. 5–8, 36, and 42).

We have evaluated the involvement of the adaptor protein NCK in the translocation of PAK-2 to plasma membrane GRP78 in 1-LN cells upon stimulation of cells with \( \alpha_2 M^* \) (Fig. 3, A–C). GRP78 immunoprecipitate of plasma membrane lysates showed that GRP78, NCK, and PAK-2 are communoprecipitated in 1-LN cells stimulated with \( \alpha_2 M^* \) (Fig. 4, A–C). These results demonstrate that, in plasma membranes isolated from 1-LN cells stimulated with \( \alpha_2 M^* \), PAK-2 exists in complex with the adaptor protein NCK and the receptor GRP78.

Silencing of GRP78 Gene Expression with RNA Interference Attenuates the Association of PAK-2 and NCK in \( \alpha_2 M^* \)-stimulated Cells—In the preceding section, we demonstrated the presence of PAK-2 in plasma membranes in association with the adaptor protein NCK and GRP78 after \( \alpha_2 M^* \) stimulation (Fig. 3, A–C). Ligand-induced tyrosine phosphorylation of the receptor causes the recruitment of SH3- and SH2-containing adaptor proteins, and forms a multiprotein complex responsible for generating and propagating intracellular signaling responsible for cellular responses. To assess the role of ligand-activated GRP78 in the recruitment of NCK-PAK-2 complex to the plasma membrane, we silenced the expression of GRP78 gene with dsRNA homologous in sequence to the target gene mRNA sequence (5’-AAA ATA CAG CAA TTA GTA AGG-3’) and assayed the association of PAK-2 and NCK in cell lysates in \( \alpha_2 M^* \)-stimulated 1-LN cells (Fig. 3) under identical conditions. Scrambled dsRNA showed none or negligible effects on GRP78, PAK-2, or NCK (Fig. 4, D, E, G, I, J, and K).

**Table 1**

| Time (min) | Phosphorylated PAK-2 | Phosphorylated PAK-1 |
|-----------|----------------------|----------------------|
| 0         | 1769 ± 17            | 534 ± 66             |
| 5         | 2243 ± 213           | 532 ± 73             |
| 10        | 4009 ± 100           | 540 ± 19             |
| 20        | 3169 ± 686           | 597 ± 43             |
| 40        | 2127 ± 60            | 628 ± 39             |
| 60        | 1358 ± 111           | 656 ± 35             |

* A.U., arbitrary units.

**Fig. 2.** Autophosphorylation and kinase activity of PAKs in 1-LN cells stimulated with \( \alpha_2 M^* \). See “Experimental Procedures” for details. A, autoradiograph showing autophosphorylation of PAK-1 and PAK-2, in the respective immunoprecipitates. The lanes are: 1, buffer control; 2, PAK-1 immunoprecipitate; and 3, PAK-2 immunoprecipitate. B, autoradiograph showing PAK-2 kinase activity toward MBP. The lanes are: 1, buffer control; 2, PAK-2 immunoprecipitate. C, Rac-1-GTP levels in 1-LN-cells treated with buffer (1) and \( \alpha_2 M^* \) (50 pM/10 min) (2); D, levels of total Rac-1 protein in 1-LN cells treated with buffer (1) and \( \alpha_2 M^* \) (50 pM/10 min) (2). Autoradiographs shown are representative of three independent experiments. The immunoblots shown are representative of two individual experiments performed in duplicate.

**Fig. 3.** Plasma membrane association of GRP78, NCK, and PAK-2 in 1-LN cells stimulated with \( \alpha_2 M^* \). See “Experimental Procedures” for details. A, GRP78 in the GRP78 immunoprecipitate from plasma membrane. B, PAK-2 in the GRP78 immunoprecipitate from plasma membrane. C, NCK in GRP78 immunoprecipitate from plasma membrane. The lanes are: 1, buffer; 2, \( \alpha_2 M^* \) (50 pM/10 min). D and J, effect of silencing the expression of the GRP78 gene on association of PAK-2. E and K, effect of silencing the expression of the GRP78 gene on the association of NCK. F, protein loading control, actin. G and I, effect of silencing the expression of the GRP78 gene on GRP78 protein levels. H, protein loading control glyceraldehyde-3-phosphate dehydrogenase. The lanes in D–G are: 1, buffer; 2, \( \alpha_2 M^* \); 3, dsRNA GRP78 then \( \alpha_2 M^* \); and 4, scrambled dsRNA then \( \alpha_2 M^* \). The lanes in I–K are: 1, buffer; 2, \( \alpha_2 M^* \); 3, dsRNA GRP78; and 4, dsRNA GRP78 then \( \alpha_2 M^* \). The immunoblots shown are representative of three to four independent experiments.
could regulate the assembly of actin (49). LIMK mRNA is up-regulated in prostate adenocarcinomas and is correlated with the aggressiveness of these cells (48). Active PAKs greatly enhanced phosphorylation of both LIMK and coflin in vitro (20–23, 48, 49). In the proceeding sections we demonstrated that treatment of 1-LN prostate cancer cells with α2M* activates PAK-2. 1-LN cells are highly malignant, motile, and invasive; therefore, to understand the involvement of LIMK in cytoskeleton reorganization in 1-LN cells upon treatment of α2M*, we have determined the levels of phosphorylated LIMK (Fig. 4A and Table III) and phosphorylated coflin (Fig. 4D and Table III) by Western blotting. A 2- to 3-fold increase in the levels of phosphorylated LIMK and phosphorylated coflin was observed in 1-LN cells treated with α2M*. The results indicate the involvement of phosphorylated-PAK-2 in cytoskeleton reorganization and possible prosurversive behavior of 1-LN prostate cancer cells treated with α2M* via phosphorylation of LIMK and coflin. The results indicate that, upon treatment of 1-LN cells with α2M*, the phosphorylation of LIMK and coflin is markedly increased and suggests that these effects are possibly mediated by LIMK under our experimental conditions. Because LIMK is also activated by Rho kinases, to further understand the role of PAK-2 in LIMK activation, we silenced the expression of the PAK-2 gene by RNA interference (Fig. 4). Silencing of PAK-2 gene expression (Fig. 4G) attenuated the levels of phosphorylated LIMK (Fig. 4H) without affecting the levels of LIMK protein (Fig. 4F) compared with cells treated with α2M* alone (Fig. 4, G–I) or cells transfected with scramble dsRNA and treated with α2M* (Fig. 4, G–I). The reductions in the protein levels of PAK-2 and phosphorylated LIMK were nearly comparable, which suggest that, in 1-LN prostate cancer cells, α2M*-induced PAK-2 is involved in reorganization of the cytoskeleton via LIMK and coflin. However, the role of Rho kinase in phosphorylation of LIMK in 1-LN cells is not ruled out.

**α2M* Treatment of 1-LN Prostate Cancer Cells Up-regulate the Increased Expression of Phosphorylated Bad (Ser112 and Ser136)—** Different PAK family members regulate the balance between pro-survival and pro-apoptotic proteins of the Bcl-2 family (8). As noted above, caspase-induced cleavage of PAK-2 releases the constitutively active 34-kDa catalytic COOH-terminal fragment in response to multiple stimuli that induce apoptosis; however, full-length activated PAK-2 is anti-apoptotic (5–8, 11). Members of the Bcl-2 family are intracellular proteins that either promote survival (Bcl-2 and Bcl-xL) or augment cell death (Bad and Bax) (50–53). Bad binds to Bcl-2 and neutralizes the anti-apoptotic effects of Bcl-2 and promotes cell death. Phosphorylation of Bad causes its interaction with 14-3-3 protein and prevents its binding to Bcl-2, which then interacts with Bax to inhibit apoptosis. Bad is phosphorylated on Ser112, Ser136, and Ser155—different PAK family members regulate the balance between pro-survival and pro-apoptotic proteins of the Bcl-2 family (8). As noted above, caspase-induced cleavage of PAK-2 releases the constitutively active 34-kDa catalytic COOH-terminal fragment in response to multiple stimuli that induce apoptosis; however, full-length activated PAK-2 is anti-apoptotic (5–8, 11). Members of the Bcl-2 family are intracellular proteins that either promote survival (Bcl-2 and Bcl-xL) or augment cell death (Bad and Bax) (50–53). Bad binds to Bcl-2 and neutralizes the anti-apoptotic effects of Bcl-2 and promotes cell death. Phosphorylation of Bad causes its interaction with 14-3-3 protein and prevents its binding to Bcl-2, which then interacts with Bax to inhibit apoptosis. Bad is phosphorylated on Ser112, Ser136, and Ser155 by protein kinases, including MAPKs and Akt (54, 55), the latter of which is the effector of PI 3-kinase, and PAK-1 phosphatases Bad at Ser136 either directly or indirectly through PAK-2. Treatment of 1-LN prostate cancer cells with α2M* profoundly elevated the levels of Bad phosphorylated at Ser112, Ser136, and Ser155 (Fig. 5, A and B, and Table III) with kinetics similar to that of PAK-2 activation (Fig. 1). These studies suggest that PAK-2 is involved in the cellular proliferative effects of α2M* observed in 1-LN prostate cancer cells by up-regulating the levels of anti-apoptotic proteins.

**α2M*-induced Increased Activation of PAK-2 in 1-LN Cells Is Inhibited by Tyrosine Kinase and PI 3-Kinase Inhibitors—** Externally stimuli activate members of PAK family by multiple mechanisms, including Ras/MAPK- and PI 3-kinase-dependent signal transduction (5–8). In our earlier studies, we demonstrated that binding of α2M* to cells also causes activation of...


**TABLE II**

| Additions | GRP78 | | | | |
|-----------|-------|-------|-------|-------|-------|
|           | A      | B     | A      | B     |
| Lipofectamine | 276 ± 47 | 448 ± 55 | 612 ± 60 | 2227 ± 73 |
| Lipofectamine plus αM* (50 μM/10 min) | 459 ± 28 | 834 ± 18 | 1163 ± 60 | 3795 ± 138 |
| dsRNA/GFP78 (25 μg) | 192 ± 16 | 482 ± 43 | ND³ | 2253 ± 108 |
| dsRNA/GFP78 (25 μg) plus αM (50 μM/10 min) | 188 ± 23 | 500 ± 33 | 682 ± 183 | 1112 ± 72 |
| Scrambled dsRNA (25 μg) plus αM | ND | 902 ± 87 | 1175 ± 218 | ND |

⁠* A.U., arbitrary units.

⁠¹ ND = not done.

**TABLE III**

| Time (min) | Phosphorylated LIMK | | Phosphorylated cofilin | | Bad at Ser⁵³² | Bad at Ser⁵⁷⁶ |
|------------|---------------------|--------|-----------------------|--------|----------------|----------------|
|            | A.U. × 10⁴           |        | A.U. × 10⁴            |        |
| 0          | 897 ± 69             | 130 ± 9 | 63 ± 15               | 167 ± 7 |
| 5          | 1275 ± 75            | 157 ± 20 | 98 ± 4                | 240 ± 14 |
| 10         | 1772 ± 28            | 127 ± 10 | 83 ± 8                | 224 ± 11 |
| 20         | 3481 ± 223           | 288 ± 14 | 120 ± 10              | 231 ± 2 |
| 40         | 2903 ± 48            | 295 ± 13 | 121 ± 10              | 414 ± 19 |
| 60         | 2545 ± 48            | 248 ± 18 | 106 ± 7               | 299 ± 14 |

⁠* A.U., arbitrary units.

**FIG. 5.** Phosphorylation of Bad in αM*-stimulated 1-LN cells. A, effect of time of incubation of 1-LN cells with αM* (50 μM) on phosphorylation of BAD at Ser¹³⁶ and B, effect of time of incubation of 1-LN cells with αM* (50 μM) on phosphorylation of BAD at Ser⁵³²; C, Bad protein. The immunoblots are representative of three to four independent experiments.

**FIG. 6.** Modulation of PAK-2 activity by tyrosine kinase and PI 3-kinase in 1-LN cells stimulated with αM*. A, autoradiograph showing MBP phosphorylation by PAK-2. The lanes are: 1, buffer; 2, PAK-2 immunoprecipitate; 3, PAK-2 immunoprecipitate from 1-LN cells treated with genistein (20 μM/16 h) before αM* (50 μM/10 min) stimulation; and 4, PAK-2 immunoprecipitate from 1-LN cells treated with LY294002 (20 μM/20 min) before αM* stimulation. B, immunoblot showing the effect of tyrosine kinase and PI 3-kinase inhibitors on levels of phosphorylated PAK-2. The lanes are: 1, buffer; 2, αM* (50 μM/10 min); 3, genistein (20 μM/16 h), then αM* (50 μM/10 min); 4, LY294002 (20 μM/20 min) then αM*; 5, wortmannin (30 nM/30 min) then αM*; and 6, LY353511 (20 μM/20 min) then αM*. In C, the protein loading controls immunoblots of actin are shown below the immunoblot which is representative of three independent experiments.

these pathways (16–20). Activation of Ras and PI 3-kinase in macrophages stimulated with αM* induced increase milogenic signaling culminating in an increased DNA synthesis and cellular proliferation (17, 19). In view of the functional dependence of PAK-2 activation on receptor tyrosine phosphorylation, and PI 3-kinase activation, we examined the effect of inhibiting tyrosine phosphorylation of GPR78 and PI 3-kinase activation consequent to αM* binding to 1-LN cells on the activation of PAK-2 by measuring its kinase activity toward MBP (Fig. 6A and Table IV) and then expression of phosphorylated PAK-2 (Fig. 6B and Tables IV and V). Pretreatment of cells with genistein, a specific inhibitor of tyrosine kinases greatly attenuated the αM*-induced MBP phosphorylating activity of PAK-2 (Fig. 6A and Table IV) as well as the levels of activated PAK-2 by Western blotting. Likewise, inhibition of PI 3-kinase with its specific inhibitor LY294004 profoundly inhibited the MBP-phosphorylating activity of PAK-2 (Fig. 6A and Table IV) as well as the elevated levels of phosphorylated PAK-2 (Fig. 6B and Tables IV and V) in 1-LN cells. The data presented show that αM*-induced increased activation of PAK-2 in 1-LN cells requires tyrosine phosphorylation of the receptor and activation of downstream PI 3-kinase signaling. Prior treatment of cells with genistein drastically attenuated kinase activity of PAK-2. Likewise, LY294002 also inhibited kinase activity of PAK-2, but the magnitude of inhibition was smaller than that of tyrosine kinase inhibition, which suggests that PI 3-kinase-independent mechanism may be involved in PAK-2 activation.

**DISCUSSION**

The development, progression, and metastasis of prostate cancer is a multistage phenomenon where the role of cellular metabolism, environment, and intracellular signaling play crucial roles. In this study, we have examined PAK activation in 1-LN cells treated with αM* and various PAK-induced downstream signaling events. The salient observations of this study are that αM* binding to 1-LN cells: 1) up-regulates activation of PAK-2; 2) induces autophosphorylation of PAK-2, and the tyrosine kinase- and PI 3-kinase-dependent kinase activity of PAK-2; 3) induces activation of Rac-1; 4) increases phosphorylation of LIMK and cofilin, which are greatly reduced upon silencing of PAK-2 gene expression; 5) promotes NCK-mediated...
PhosphorImager. A representative immunoblot is shown in Fig. 4, which GRP78 associates, greatly attenuated GRP78 antibodies against GRP78, silencing of the expression of the cell lines, are exposed to picomolar concentrations of important respect; namely when 1-LN, cells but not the other differ from PC-3, LnCap, and DU145 cancer cells in one very mice migrate and metastasize. 1-LN prostate cancer cells also nant and noninvasive PC-3 cells at this University a number of observations suggest a functionally close relationship between proliferative secondary to inositol 1,3,4-trisphosphate/Ca²⁺

Finally, silencing expression of the gene nearly abolishes the association of PAK-2 and NCK in the plasma membranes of α₂M*-stimulated 1-LN cells. The data presented suggest that PAK-2 is involved in α₂M*-induced cellular growth, migration, and the survival of 1-LN prostate cancer cells. The mechanism by which α₂M* activates PAK-2 appears to be dependent on receptor tyrosine phosphorylation and PI 3-kinase activation, because pretreatment of cells with genistein, LY294002, and wortmannin greatly attenuated the activation of PAK-2. Because silencing of the expression of the GRP78 gene also greatly attenuated the activation of PAK-2 in α₂M*-treated 1-LN cells, ligand-induced activation of GRP78 is a prerequisite for PAK-2 activation in these cells. That the activation of PAK-2 in 1-LN cells under these conditions was not completely abolished also suggests the involvement of other mechanisms in PAK-2 activation.

Highly malignant and invasive androgen-independent human 1-LN prostate cancer cells were derived from less malignant and noninvasive PC-3 cells at this University a number of years ago. Unlike PC-3 prostate cancer cells, 1-LN cells in nude mice migrate and metastasize. 1-LN prostate cancer cells also differ from PC-3, LnCap, and DU145 cancer cells in one very important respect; namely when 1-LN, cells but not the other cell lines, are exposed to picomolar concentrations of α₂M*, cell proliferation increases substantially (16, 18, 19). The expression of GRP78 on the surface of PC3, DU145, and LnCap cells was either absent or minimal (17, 18). By contrast, the highest levels that we have detected are on the surface of 1-LN cells, which were employed to purify α₂M* signaling receptor and identify it as GRP78 (20). Preincubation of 1-LN cells with antibodies against GRP78, silencing of the expression of the GRP78 gene and silencing of the expression of MTJ-1, with which GRP78 associates, greatly attenuated α₂M*-induced mitogenic signaling and cellular responses (16, 17, 19, 35). These observations suggest a functionally close relationship between cell surface-associated GRP78 receptor activation and growth, metastasizing, and the invasive potential of 1-LN prostate cancer cells. Ligation of this cell surface-associated GRP78 on 1-LN cells with α₂M* triggers the activation of mitogenesis and cell proliferative secondary to inositol 1,3,4-trisphosphate/Ca²⁺

**TABLE IV**

| Additions            | PKA Phosphorylation | LIMK Phosphorylation |
|----------------------|---------------------|----------------------|
| Lipofectamine        | 745 ± 70            | 530 ± 59             |
| Lipofectamine + α₂M* | 1501 ± 42           | 1730 ± 200           |
| PAK-2-dsRNA (25 μg)  | 776 ± 83            | 520 ± 120            |
| PAK-2-dsRNA + α₂M*  | 947 ± 25            | 770 ± 80             |
| Scrambled dsRNA (25 μg) + α₂M* | 1350 ± 169 | 2320 ± 210 |
|                      |                     | 435 ± 17             |

**TABLE V**

| Additions            | Kinase activity | Western blotting |
|----------------------|-----------------|------------------|
| None                 | 105 ± 2         | 1080 ± 101       |
| α₂M* (50 μg/10 min)  | 302 ± 7         | 4217 ± 782       |
| Genestin (20 μM/16 min) then α₂M* | 154 ± 4 | 2459 ± 278 |
| LY294002 (20 μM/20 min) then α₂M* | 154 ± 7 | 2659 ± 356 |
| Wortmannin (30 μM/30 min) then α₂M* | NDb       | 2212 ± 380       |
| LY93511 (20 μM/20 min) then α₂M* | ND      | 3423 ± 103       |

α₂M, arbitrary units.

ND, not done.

**FIG. 7.** A schematic representation of the mechanisms of PAK-2 activation in 1-LN prostate cancer stimulated with α₂M*.

signalizing, Ras/MAPK signaling, and PI 3-kinase signaling (17, 18). In our previous reports, we have shown that cellular responses elicited post α₂M* binding to cell surface-associated GRP78 are analogous to various growth factors and its receptor behaves like a growth factor receptor. In androgen-independent 1-LN prostate cancer cells, α₂M* could function as a growth factor (16–20). This is evidenced by increased DNA synthesis and cellular proliferation observed in 1-LN cells treated with α₂M* (16). Both these events are dependent upon tyrosine phosphorylation of GRP78, and receptor downstream Ras/MAPK and PI 3-kinase signaling. Based on positive correlation between circulating GRP78 antibodies and prostate cancer, GRP78 has been suggested as a diagnostic biomarker of prostate cancer (30, 31). Prostate cancer cells also produce prostate cancer-specific antigen and matrix metalloproteinases (33, 34), which bind readily to α₂M whose daily turnover has been estimated to be about 1 g (32). Thus highly aggressive prostate cancer may produce prostate cancer-specific antigen.
converting α₂M to α₂M*, which would then be available to bind to cell surface-associated GRP78 on prostate cancer cells promoting their growth, metastasizing, and invasive potential mediated by mitogenic signaling elicited consequent to α₂M* binding to GRP78. Taken together with the clinical data cited above, it could be argued that up-regulation of α₂M* binding receptor α₂M* signaling receptor (GRP78) is part of the aggressive phenotype in prostate cancer.

PAK-2 is ubiquitously expressed, whereas the expression of PAK-1 is tissue-specific (5–8). In dividing cells PAK-2 is inactive but is transiently activated when cells are subjected to moderate stress conditions such as hyperosmolality, ionizing radiations, and DNA-damaging drugs (5–8). Under these conditions, PAK-2 activation requires upstream tyrosine kinase and PI 3-kinase activities. GRP78 is an ER-resident protein that is involved in many cellular processes, including antigen presentation, translocation of newly synthesized polypeptides across the ER membrane, and their subsequent folding, maturation, transport, or retrotranslocation (21–26). The kinase activity of PAKs has been implicated in proliferative signaling by growth factor receptors that in turn regulate cell survival, programmed cell death, and cell cycle regulation, transport, or retrotranslocation (21–26). An increased concentration, transport, or retrotranslocation of newly synthesized polypeptides within the ER, including glucose deprivation, acidosis, and hypoxia, conditions that are generally present in poorly vascularized solid tumors (21–26). The kinase activity of PAKs has been implicated in proliferative signaling by growth factor receptor kinases that in turn regulate cell survival, programmed cell death, and malignant transformation (5–8). The dominant role of PI 3-kinase signaling in cell survival and proliferation is well documented (53). Akt, a downstream effector of PI 3-kinase, attenuates the apoptotic events by phosphorylating apoptotic proteases, apoptotic protein Bad. The results of this study are summarized in Fig. 7.

In conclusion we show here for the first time that binding of α₂M* to cell surface-associated GRP78 activates PAK-2 in highly metastatic and invasive prostate cancer cells. α₂M*-induced activation of PAK-2 requires tyrosine phosphorylation of GRP78, activation of Ras/MAPK and PI 3-kinase downstream signaling, and recruitment of PAK-2 via the adaptor protein NCK to the plasma membrane. The increased expression of phosphorylated LIMK and cofilin confers motility, necessity for metastasis, in 1-LN cells. α₂M* treatment of 1-LN cells also protects these cells from cell death by inhibiting the pro-apoptotic protein Bad.
Binding of Activated $\alpha_2$-Macroglobulin to Its Cell Surface Receptor GRP78 in 1-LN Prostate Cancer Cells Regulates PAK-2-dependent Activation of LIMK

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