Ligation of Cell Surface-Associated Glucose-Regulated Protein 78 by Receptor-Recognized Forms of α2-Macroglobulin: Activation of p21-ACTivated Protein Kinase-2-Dependent Signaling in Murine Peritoneal Macrophages

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Ligation of Cell Surface-Associated Glucose-Regulated Protein 78 by Receptor-Recognized Forms of α2-Macroglobulin: Activation of p21-Activated Protein Kinase-2-Dependent Signaling in Murine Peritoneal Macrophages

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Previous studies of the plasma proteinase inhibitor α2-macroglobulin (α2M) demonstrated that α2M-proteinase complexes (α2M*) modulate immune responses and promotes macrophage locomotion and chemotaxis. α2M* binds to cell surface-associated glucose-regulated protein 78 (GRP78), which activates downstream signaling events. The role of p21-activated protein kinase-1 and -2 (PAK-1 and -2) in promoting cellular motility is well documented. In the current study, we examined the ability of α2M* to activate PAK-1 and PAK-2. Upon macrophage stimulation with α2M*, PAK-2 is autophosphorylated, resulting in increased kinase activity; however, PAK-1 is negligibly affected. α2M*-stimulated macrophages showed a marked elevation in the levels of Rac-GTP. Receptor tyrosine phosphorylation upon binding of α2M* to GRP78, recruits PAK-2 to the plasma membrane via the adaptor protein NCK. Consistent with this hypothesis, silencing of GRP78 gene expression greatly attenuated the levels of membrane-associated PAK-2 and NCK. PAK-2 activity was markedly decreased by inhibition of tyrosine kinases and PI3K before α2M* stimulation. We further demonstrate that phosphorylation of Lin-11, Isl-1, Mec-3 (LIM) kinase and cofilin is promoted by treating macrophages with α2M*. Thus, α2M* regulates activation of the PAK-2-dependent motility mechanism in these cells. The Journal of Immunology, 2005, 175: 2525–2533.

Macrophages play a central role in immune responses and represent a major defense mechanism against a variety of infectious agents. Locomotion and chemotaxis are essential for these cells to carry out these functions. Macrophages demonstrate an abundance of actin filaments and actin-associated proteins in the cortical cytoplasm. Cortical actin polymerization and the subsequent extension of pseudopodia are important components of the host-defense mechanisms of these cells (1). Rac and Cdc42 are members of the p family of small GTPases (2, 3). A major function of p family GTPases is to regulate the organization of actin, which is essential in the events required for macrophage locomotion and cell adhesion (4–7). The effects of Rac and Cdc42 on the actin cytoskeleton are mediated by p21-activated protein kinases (PAKs) (8). The mammalian PAK family consists of six members, including PAK-1 and PAK-2. The former is tissue specific, while the latter is ubiquitous in its distribution (see Refs. 8–13 and references therein).

All PAKs contain a conserved serine/threonine kinase domain in the C-terminal half, a N-terminally located p21 binding domain, also called the Cdc42/Rac-1 interactive binding domain, and an autoinhibitory domain. In addition to a binding site for activated Rac/Cdc42, PAK-2 contains binding sites for βy subunits of heterotrimeric G proteins, the adaptor protein NCK that targets PAKs to the plasma membranes and mediates interactions with transmembrane growth factors, and PAK-interacting guanine nucleotide exchange factors (see Refs. 8–13 and references therein). The key mechanism for regulation of PAK catalytic activity is the interaction between the C-terminal domain and N-terminal domain, which maintains the catalytic activity of PAK in an inactive/low affinity state (8). Binding of GTP-bound Rac/Cdc42 to inactive PAK-2 brings about a conformational change that promotes autophosphorylation of PAK-2 at Thr402 in the activation loop, and this results in a marked increase in its activity (8–13). Akt is required for both Ras and PI3K-dependent activation of PAKs; however, 3-phosphoinositide-dependent kinase-1 can also phosphorylate PAKs (14). Membrane localization of PAK-1 via recruitment through the adaptor protein NCK in response to receptor tyrosine phosphorylation is also sufficient for activation of PAK-1 (15). The mechanism(s) of activation of membrane localized PAK-1 may depend on an increased accessibility of PAK-1 to GTP-bound Cdc42 and Rac1, phosphorylation of PAK-1 by an unidentified kinase, and/or autophosphorylation of PAK-1 at the membrane (15).

Lin-11, Isl-1, Mec-3 (LIM) kinases are dual specificity (Ser/Thr and Tyr) kinases that contain two N-terminal LIM domains, which are commonly associated with actin in the cytoskeleton (16–18). Members of the LIM kinase family can be directly phosphorylated at Thr508 by activated PAKs and p-associated kinases. Activated LIM kinases specificity phosphorylates cofilin, an actin binding protein, inhibiting its depolymerization and hence provides a mechanism by which LIM kinase can regulate the assembly of actin (16–18). α2-Macroglobulin (α2M) is a broad specificity proteinase inhibitor that binds to cell surface receptors when activated by proteinases (19). The activated form of α2M (α2M*) is also produced by...
direct reaction of internal thiol esters present in each of its four identical subunits with small amines or ammonia (19). The low-density lipoprotein receptor-related protein-1 has been identified as a cellular receptor for αM* (19). αM* binding to cells triggers the activation of a number of signaling cascades in a manner analogous to growth factors (20–28). Thus, we hypothesized that αM* functions like a growth factor and its receptor as a growth factor-like receptor. Subsequent studies suggested that a receptor distinct from low-density lipoprotein receptor-related protein-1 must account for αM*-dependent signal transduction (21, 22). These events require the presence of a small number of sites (~1500/cell) demonstrating very high ligand affinity (Kd ~50–100 pM) for cellular binding of either αM* or its receptor binding domain. This second receptor, initially termed the αM* signaling receptor, was later isolated from macrophages and 1-LN human prostate cancer cells and identified as cell surface-associated glucose-regulated protein 78 (GRP78) (28). Many studies have shown a role for αM* in regulating a diversity of immune functions, as well as macrophage locomotion and chemotaxis (29, 30). We hypothesize in the present study that like other growth factors, αM* may trigger activation of PAKs, and activated PAKs might contribute in some of the cellular responses elicited in αM*-treated cells. Therefore, we studied the effects of αM* on activation of PAKs, the role of receptor activation, and the adaptor protein NCK in PAK membrane localization and activation. We also studied the role of PI3K in PAK activation and the effect of activated PAKs on downstream effectors. We report in the present study that exposure of cells to picomolar concentrations of αM* caused phosphorylation and activation of PAK-2 and its concomitant localization in conjunction with NCK in the plasma membrane fraction. Activation of PAK-2 was inhibited significantly by silencing GRP78 expression or inhibition of tyrosine kinases and PI3K. PAK-1 was negligibly affected in these studies.

Materials and Methods

Materials

Culture media were obtained from Invitrogen LifeTechnologies. Abs against PAK-1, PAK-2, phosphorylated PAK-1 (Ser199)/Ser204, phosphorylated PAK-2 (Ser216), phosphorylated LIM kinase (Thr220), phosphorylated cofilin (Ser3), and phosphorylated tyrosine residues were procured from Cell Signaling Technology. Myelin basic protein (MBP) and Abs against actin, used as a protein loading control, were purchased from Sigma-Aldrich. Abs against GADPH, also used as a protein loading control, were purchased from Ambion. Abs against Ras-GTPase-activating protein (GAP) were purchased from Upstate Biotechnology. Abs against NCK were purchased from Santa Cruz Biotechnology. Anti-GRP78 Abs were purchased from StressGen Biotechnologies. Adenosine-5′-triphosphate (γ32P)-specific activity (3000 Ci/mmol) was purchased from PerkinElmer. The sources for the inhibitors used have been described previously (21, 22). Other reagents used in the study were of the highest quality available and were procured locally.

Effect of αM* stimulation on cellular activation of PAK-1 and PAK-2 by Western blotting

Use of mice for these studies was approved by the Institutional Animal Use Committee in accordance with relevant federal regulations. Thioglycollate-elicited peritoneal macrophages were obtained from pathogen-free 6-week-old C57BL/6 mice (National Cancer Institute) in HBBS containing 10 mM HEPES (pH 7.4) and 3.5 mM NaHCO3 (HBBS). The cells were washed with HBSS and suspended in RPMI 1640 medium containing 2 mM glutamine, 12.5 U/ml penicillin, 6 μg/ml streptomycin, and 5% FBS. They were then adhered in 6-well plates (3 × 106 cells/well) and incubated for 2 h at 37°C in a humidified CO2 (5%) incubator. The monolayers were washed with HBBS three times to remove nonadherent cell and then incubated overnight at 37°C in the above RPMI 1640 medium before the study. Macrophages incubated overnight (3 × 106 cells/well) were washed twice with HBBS, and a volume of RPMI 1640 was medium added. One set of cells was stimulated with different concentrations of αM*, and cells were incubated for 10 min. A second set of cells was stimulated with 50 pM αM*, and cells were incubated for different time periods. At the end of the incubation, the medium was aspirated, and the cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.6), 0.1 M NaCl, 1 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSE, 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 determined (31). Equal amounts of lysates proteins were electrophoresed on gels according to Laemmli (32). Proteins from gels (10%) were transferred to Hybond-P. Phosphorylated PAK-2 and PAK-1 bands on the gel were detected with Abs against phosphorylated PAK-2 or PAK-1. PAK-2 protein bands were detected with Abs against PAK-2 protein according to the manufacturer’s instructions. Protein bands on the membranes were visualized by enhanced chemifluorescence (Amersham Biosciences) and quantified using a Storm 860 PhosphorImager (Molecular Dynamics). The respective membranes were stripped and reprobed for actin according to the manufacturer’s instructions.

Measurement of the levels of Ras-GAP by Western blotting

Experimental details of electrophoresis and quantification of protein bands were as described above, except that the respective membranes were immunoblotted with Abs against Ras-GAP, respectively.

Measurement of the levels of phosphorylated LIM kinase and phosphorylated cofilin by Western blotting

Experimental details for quantifying the levels of these components, which regulate cellular shape, were as described above, except that the respective membranes were immunoblotted with Abs against phosphorylated LIM kinase or phosphorylated cofilin, respectively. Membranes were reprobed for LIM kinase protein, cofilin protein, actin, and GAPDH, respectively, as protein loading controls as described above.

Measurement of αM*-induced autophosphorylation of PAK-1 and PAK-2

Autophosphorylation of PAK-1 and PAK-2 in αM*-stimulated cells was measured essentially as described previously (33). Briefly, RPMI 1640 medium was aspirated from cells incubated overnight (3 × 105/ml/well/6-well plates), monolayers were washed twice with HBBS, and a volume of HBBS was added to the monolayers. To the respective wells, αM* (50 pM) or buffer was added, and cells were incubated for 10 min at 37°C. The reaction was terminated by aspirating the medium. 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 pelleted into Eppendorf tubes, DNA strands were broken by passing the lysate through 27-gauge needle several times, and 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. To equal amounts of lysates proteins in respective tubes, Abs against PAK-1 (1:50) and PAK-2 (1:50) were added followed by the addition of protein A-agarose, and tubes were incubated overnight at 4°C in a rotary shaker. The immunoprecipitates were recovered by centrifugation (2500 rpm/5 min) at 4°C and washed twice with lysis buffer and three times with kinase buffer containing 50 mM HEPES (pH 7.5) 10 μM MgCl2, 2 mM MnCl2, and 0.2 mM DTT. Autophosphorylation was measured in 50 μl of kinase buffer containing 10 μCi of [γ32P]ATP for 20 min at 30°C. The reaction was stopped by adding a volume of 4X sample buffer and heating the tubes for 3 min at 90°C. The tubes were centrifuged, and protein in the supernatants was transferred to membranes, which were then dried and 3P-labeled PAK-1 and PAK-2 bands detected and quantified by autoradiography in a Storm 860 PhosphorImager. The membranes from the autophosphorylation study were reprobed to confirm the identification of phosphorylated PAK-1 and phosphorylated PAK-2 by Western blotting (data not shown).
buffer and then three times with 2× kinase buffer as above. To the respective immunoprecipitates in 50 μl of kinase buffer was added 5 μg of MBP, and the tubes were incubated for 5 min over ice. The kinase reaction of the immunoprecipitates was initiated by the addition of 10 μl of [γ-32P]ATP (specific activity 3000Ci/mM) followed by the addition of ATP (20 μM final concentration). The samples were incubated for 10 min at 25°C. The reaction was terminated by the addition of 1 volume of 4× sample buffer. The samples were heated at 3 min at 90°C, electrophoresed on 12.5% gels, transferred to membranes, and 32P-labeled MBP visualized and quantified by autoradiography in a Storm 860 PhosphorImager.

**Assay for Rac-GTP in αM-stimulated macrophages**

Active GTP-bound Rac was precipitated using a PAK-p21 binding domain-based commercial assay kit (Upstate Cell Signaling). Macrophages were incubated overnight (4 × 107 cells) in 6-well plates in RPMI 1640 medium containing 10% FBS, 12.5 U/ml penicillin, 6.5 μg/ml streptomycin, and 2 mM glutamine. The cells were stimulated with αM* (50 μM/10 min) at 37°C in a humidified CO2 incubator. At the end of the incubation, the medium was aspirated and to the cells was added a 40 μl aliquot of lysate was similarly processed for total Rac-1 quantification. (32). The protein bands on the gel were transferred to Hybond-P membranes and lysate proteins were electrophoresed in gels according to Laemmli (32). These membranes on which these preparations were transferred after electrophoresis were reprobed and quantified for phosphorylated PAK-2 and NCK according to the manufacturer’s instructions.

**Modulation of PAK-2 kinase activity by inhibitors of tyrosine kinases and PI3K**

Two types of studies were performed. In the first set of studies, the effects of kinase inhibitors on the ability of PAK-2 to phosphorylate MBP were studied. In these experiments, cells were treated with the Tyr kinase inhibitor genistein (20 μM/16 h) and the P38 kinase inhibitor LY294002 (20 μM/20 min) before stimulation with αM* (50 μM/10 min). Other details of immunoprecipitation with PAK-2 Abs, kinase assay, and quantification of MBP phosphorylation were identical to those described in the preceding section. In the second set of experiments, the specific inhibitors of these Tyr kinases and PI3K were added to the respective wells containing cells (3 × 107/well) as described above. Cells were incubated for the specified time period before adding αM* (50 μM) and incubating the cells for an additional 10 min. The reactions were stopped by aspirating the medium and lysing the cells in lysis buffer as described above. Equal amounts of lysate proteins were electrophoresed in gels according to Laemmli (32). Proteins from gels (10%) were transferred to Hybond-P membranes and immunoblotted with Abs against phosphorylated and unphosphorylated PAK-2, respectively, according to the manufacturer’s instructions. Protein bands on the membrane were visualized by ECF and quantified using a Storm 860 PhosphorImager.

**Immunoprecipitation and Western blotting of GRP78, phosphorylated PAK-2, and NCK in the plasma membrane fraction**

Macrophages (30 × 106 cells/4-well plates) incubated overnight as above were washed twice with HBBS, and a volume of the medium was added. The cells were treated with αM* (50 μM) and incubated for 10 min. At the end of incubation, the medium was aspirated and to the cells was added a volume of chilled HBBS buffer containing 10 mM Tris/HCl (pH 7.5), 10 mM NaCl, 1 mM PMSF, 10 μM benzamidine, and 10 μM leupeptin. The cells were scrapped into chilled glass homogenizing tubes, and the plasma membrane fraction was isolated as described previously (26, 34, 35). Briefly, the cells were homogenized by 30 up-down strokes with a Teflon pestle 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 step gradient of 50 and 30% (3 ml each) and centrifuged at 200,000 × g for 75 min in a Beckman Coulter Ultracentrifuge (Model 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 containing 25 mM HEPES (pH 7.4), 10 mM KCl, 3 mM NaCl, 5 mM MgCl2, 2 μM leupeptin, 1 mM PMSF, and 1 μM Ca2+. The suspension was centrifuged at 400,000 × g for 90 min as above. The pellet was suspended in a volume of incubation buffer. The purity of plasma membrane preparations was assessed as previously described, including by electron microscopy (26, 34, 35). These analyses showed that this membrane fraction was highly enriched in plasma membranes (92–95%), and hence, we designated this preparation as the plasma membrane fraction. The membrane pellet was lysed in lysis buffer, the lystate immunoprecipitated with Abs against GRP78 (1:100), and GRP78 in the membranes quantified as described above. The membranes on which these preparations were transferred after electrophoresis were reprobed and quantified for phosphorylated PAK-2 and NCK.

**Chemical synthesis of dsRNA homologous in sequence to the target GRP78 gene**

The chemical synthesis of dsRNA homologous in sequence to the target GRP78 K139QQLVYK276 mRNA sequence 5′-AAATACAGCAATTAGTAAAG-3′ peptide (Swiss-Prot, GRP78 primary sequence accession number P11021), was performed by Ambion. For making dsRNA, the sense 5′- AAUCACGCAAUAAGAAGG-3′ and the antisense (5′-UCUUACAUUGCUAUAUun3′) oligonucleotides were annealed according to the manufacturer’s instructions. Through the entire period of experimentation, handling of reagents was performed in an 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 maintained for 1 h at 37°C in an incubator. The dsRNA preparation was stored at −20°C before use.

**Transfection of peritoneal macrophages with dsRNA homologous in sequence to the target GRP78 gene and the effect of αM* on PAK-2 and NCK**

Peritoneal macrophages adhered for 2 h in the above described RPMI 1640 medium were aspirated to remove nonadherent cells and washed twice with HBBS. A volume of 2 ml of DMEM containing 10% FBS and the above-mentioned antibiotics was added, and the cells were incubated as above for 16 h. Just before each transfection, 25 μg of GRP78 dsRNA were diluted to 100 μl of serum- and antibiotic-free DMEM in a tube. In another tube, 10 μl of LipofectAMINE were diluted to 100 μl of serum- and antibiotic-free medium. The two solutions were combined, mixed gently, and incubated for 45 min at room temperature followed by the addition of 800 μl of serum- and antibiotic-free medium to each tube. The monolayers were washed twice with serum- and antibiotic-free DMEM, layered in each tube with 1 ml of LipofectAMINE-DMEM or lipid dsRNA mixtures containing 25 μg of dsRNA, and gently mixed and incubated for 5 h at 37°C in a humidified CO2 incubator. At the end of the incubation, 1 ml of antibiotic-free DMEM containing 10% FBS was added to each well, and cells were incubated for 16 h as above. Microscopic observation and Trypan blue uptake by 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 an additional 24 h as above. At the end of incubation, medium was aspirated and monolayers washed with the above medium once and a volume of the same medium added, and the cells were used for the experiment outlined below. To demonstrate that the transfection of macrophages with dsRNA homologous in sequence to target GRP78 gene does not produce any nonspecific effects on target gene expression, the macrophages were transfected with equimolar concentrations of scrambled siRNA (Silencer negative control, catalog no. 4610; Ambion) under identical conditions as described above for transfection with GRP78 dsRNA. At the end of the transfection period (48 h), the medium was aspirated, and a volume of DMEM was added and cells either treated with buffer (αM* (50 μM) for 10 min). The reaction was stopped by aspirating the medium and adding a volume of lysis buffer as described above. Equal amounts of membrane proteins were then electrophoresed according to Laemmli (32). Proteins from gels (10%) were transferred to Hybond-P membranes and immunoblotted with Abs against phosphorylated and unphosphorylated PAK-2, respectively, according to the manufacturer’s instructions. Protein bands on the membrane were visualized by ECF and quantified using a Storm 860 PhosphorImager. The respective membranes were stripped and reprobed for actin according to the manufacturer’s instructions.
The data were statistically analyzed using the “kaleidagraph” program. The significance of differences between groups was calculated using Student’s t test.

Results
Tyrosine phosphorylation of GRP78 upon αM* binding
Binding of growth factors to their cognate cell surface receptors causes tyrosine phosphorylation of the receptors, and this induces the recruitment of Src homology 2 and Src homology domain-containing effector proteins and the onset of receptor-mediated downstream signaling. We first examined αM*-induced tyrosine phosphorylation of GRP78 in the GRP78 immunoprecipitate in murine peritoneal macrophages (Fig. 1A). αM* binding caused a severalfold increase in tyrosine phosphorylation of GRP78 as compared with unstimulated cells (Fig. 1A). This demonstrates that like various growth factors, αM* binding to GRP78 causes its tyrosine phosphorylation, a characteristic of protein tyrosine kinase receptors.

αM* activates PAK-2 in a concentration- and time-dependent manner
PAK activity can be stimulated by a variety of external stimuli that activate cell surface receptors. These include chemoattractants acting on G protein-coupled receptors, growth factors interacting with receptor tyrosine kinases, cytokines, and extracellular matrix molecules binding to integrins (8–13). Exposure of cells to varying concentrations of αM*-triggered phosphorylation of PAK-2 by ~2- and 3-fold compared with unstimulated cells (Fig. 1B). The levels of PAK-2 protein were not affected in cells stimulated with αM* (Fig. 1B). The maximal increase in phosphorylated-PAK-2 levels occurred at 25–50 pM αM* and ~10 min of incubation, after which the levels remained elevated for several hours. Incubation of cells with αM* (50 pM) for varying periods of time showed maximal elevation in the protein levels of phosphorylated PAK-2 occurred at 10–20 min of the incubation, which declined at longer periods of incubation (Fig. 1C). Incubation of cells with higher concentrations of αM* caused a less sustained increase in PAK-2 phosphorylation. Incubation of cells with αM* had much less of an effect on the activation of PAK-1 (Fig. 1C).

FIGURE 1. Effect of stimulation of macrophages with αM* on tyrosine phosphorylation of GRP78 and activation of PAK-2. A. Immunoblot of tyrosine phosphorylated GRP78 in GRP78 immunoprecipitated from cells stimulated with buffer (lane 1) and αM* (lane 2) (50 pM/10 min). B. Effect of αM* concentration on levels of phosphorylated PAK-1 and PAK-2. C. Effect of time of incubation of αM* (50 pM/10 min) on levels of phosphorylated PAK-2 and PAK-1. Representative immunoblots of phosphorylated PAK-2 are shown below the respective graphs. Also shown are immunoblots of unphosphorylated PAK-2, phosphorylated PAK-1, and the protein loading control actin. The data are shown in arbitrary units (×10^5) and are the mean ± SE from three to four individual experiments.
If ligation of GRP78 receptors results in the membrane recruitment of the adaptor protein NCK and phosphorylated PAK-2, one would expect the presence of NCK and activated PAK-2 in GRP78 immunoprecipitates obtained from plasma membranes, and this indeed is observed (Fig. 3).

Silencing of GRP78 gene expression by RNA interference attenuates phosphorylation of PAK-2 and NCK recruitment in αM*–stimulated cells

In the next series of experiments, we further evaluated the functional relationship between GRP78 and PAK-2 by using RNA interference. We have shown previously that silencing of GRP78 gene expression decreases GRP78 mRNA and GRP78 protein levels by ~60% in macrophages (28). Silencing the GRP78 gene expression greatly attenuated the activation of PAK-2, as well as the increase in NCK protein in the membranes of αM*-stimulated cells (Fig. 4). Under these conditions, GRP78 expression decreases >60% (Ref. 28 and Fig. 4). Protein levels of PAK-2 and NCK in cells treated with LipofectAMINE alone were comparable to those treated with buffer (data not shown). Transfection of cells with scrambled RNA had very little effect on phosphorylated PAK-2 or NCK levels (Fig. 4). Taken together, these results show that upon binding of αM* to GRP78, the receptor is tyrosine phosphorylated, which recruits NCK/PAK-2 complexes to the plasma membrane. The mechanism by which PAK-2 is activated at the membrane is not clearly understood. However, it has been reported that mere membrane localization of PAK-2 is sufficient for its activation.

αM* up-regulates the protein levels of RasGAP

Ras family proteins cycle between GTP-bound and GDP-bound forms. The kinetics of GTP hydrolysis and GDP dissociation are catalyzed by GAPs and GTP exchange factors, respectively (see Ref. 8 and references therein). Activation of GAPs results in termination of its downstream signaling. Exposure of cells to αM* caused a 2- to 3-fold increase in the protein levels of RasGAP by 15–30 min of incubation and nearly plateaued off at longer periods of incubation, suggesting a switching of activated Rac1/Cdc42 downstream signaling (Fig. 5A and Table I).

αM* up-regulates the protein levels of phosphorylated LIMK and cofilin

LIM kinases are a family of serine kinases with two N-terminal LIM domains, which are commonly associated with the actin cytoskeleton. The members of the LIM kinase family can be phosphorylated and activated by PAKs and p–associated kinases. Activated LIM kinases specifically phosphorylates cofilin, an actin binding protein that promotes the disassembly of actin filaments. Phosphorylation of cofilin inhibits its actin depolymerizing action and hence provides a mechanism by which LIMK activity could regulate the assembly of actin important in cell locomotion, cell

![Image](http://www.jimmunol.org/fig/2529fig2.jpg)

**FIGURE 2.** αM*-induced autophosphorylation and activation of kinase activity of PAK-2. See Materials and Methods for experimental details. A, Autoradiograph of autophosphorylated PAK-2 and PAK-1 in macrophages. Lane 1, Immunoprecipitate of buffer stimulated; lane 2, αM*-stimulated cells. B, Kinase activity of PAK-2 as determined by phosphorylation of MBP in their immunoprecipitates from αM*-stimulated cells. The bars are as follows: 1) buffer and 2) PAK-2. The corresponding autoradiograph of PAK-2 is shown below. C, Rac-1/GTP levels in αM*-stimulated macrophages. The lanes are as follows: buffer (lane 1) and αM* (lane 2) (50 pM/10 min). Rac-1/GTP and total Rac-1 protein immunoblots are also shown. The data shown are representative of two to three independent experiments in each case. * Significantly different at the 5% level from unstimulated cells.

αM* activates Rac-1 in macrophages

PAKs are activated 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 N-terminal regulatory domain. In the preceding section, we showed that stimulation of macrophages with αM* induces autophosphorylation 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 using Western blotting (Fig. 2C). Indeed, αM* treatment of cells elevated the levels of Rac-1/GTP by ~2-fold compared with unstimulated cells (Fig. 2C). The studies demonstrate that αM* induces activation of Rac-1, which binds to PAK-2 and induces its activation in murine peritoneal macrophages.

**FIGURE 3.** Colloimmunoprecipitation of NCK and phosphorylated PAK-2 in plasma membrane immunoprecipitates of GRP78 in αM*-stimulated cells. Experimental details are described in the section on Materials and Methods. The immunoblots shown are representative of at least two to three individual experiments in each case. Lane 1, GRP78 in the buffer-stimulated cells; Lanes 2–4, GRP78, phosphorylated PAK-2, and NCK, respectively, in the immunoprecipitate of GRP78 in αM*-stimulated cells.

**GRP78, NCK, and activated PAK-2 are coimmunoprecipitated in the plasma membrane fraction of αM*-stimulated cells**

In response to external stimuli, including growth factors, PAKs translocate to the plasma membrane where they can interact with membrane-bound receptors and activators. Interaction of the adaptor protein NCK and PAKs has been implicated in translocation and stimulation of PAK activity by growth factors (see Refs. 37–39). However, the mechanism by which membrane localization of PAKs causes its activation is not clearly understood (see Ref. 15).

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spreading, and cell adhesion (17, 18). Overexpression of LIM kinases in fibroblasts results in increased membrane ruffling and thickened actin filaments. Activation of Rac enhanced LIM kinase-induced changes in the cytoskeleton. Active PAKs greatly enhanced the phosphorylation of both LIM kinase and cofilin in vitro (40–43). PAKs directly interact with LIM kinase through binding sites at both the N and C terminus, which facilitates the phosphorylation at Thr508 in the kinase activation loop (44). The plating of thioglycolate-elicited peritoneal macrophages causes their adherence and spreading; therefore, to understand the role of $\alpha_2 M^*$-activated PAK-2 in the cytoskeleton arrangement, we have assayed the levels of phosphorylated LIM kinase and phosphorylated cofilin by Western blotting (Fig. 5B and Table I). Stimulation of macrophages with $\alpha_2 M^*$ elevated the protein levels of both phosphorylated LIM kinase and phosphorylated cofilin at 15–30 min postexposure (Fig. 5B and Table I). These results show that $\alpha_2 M^*$ regulates cytoskeletal rearrangement via LIM kinase and cofilin, which are downstream targets of activated PAK-2.

**Modulation of $\alpha_2 M^*$-induced activation of PAK-2**

In the preceding sections, we have shown that stimulation of cells with picomolar concentrations of $\alpha_2 M^*$ caused a marked stimulation of PAK-2 activation and that this event was dependent upon GRP78 activation, which was tyrosine phosphorylated upon $\alpha_2 M^*$ binding (Fig. 1). In our earlier studies, we have shown that binding of $\alpha_2 M^*$ to cells causes activation of Ras, PI3K/Akt signaling and MAPK signaling (21–24, 45, 46). PI3K is a direct effector of Ras. PI3K stimulates Rac, which is a direct activator of PAK-2, as well as Akt. Activated Akt stimulates PAK, whereas dominant negative Akt inhibits Ras activation of PAKs. Akt activates PAKs through a GTPase-independent mechanism (47–50). In view of the functional dependence of PAK-2 activation on receptor tyrosine phosphorylation, as well as Ras and PI3K activation, we have examined the effect of tyrosine kinase inhibitor, genistin, and PI3K inhibitor, LY294002, on PAK-2 activation as determined by phosphorylation of MBP (Fig. 6). Prior treatment of cells with genistin drastically attenuated kinase activity of PAK-2 (Fig. 6). 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 PI3K-independent mechanism are also involved in PAK-2 activation (Fig. 6).

**Discussion**

In this study, we have examined the role of PAK-2 in $\alpha_2 M^*$-stimulated mitogenic signaling and cellular responses by quantifying its activation and regulation in macrophages. The principal findings are as follows: 1) binding of $\alpha_2 M^*$ to cell surface-associated GRP78 caused its tyrosine phosphorylation and recruitment...
of cytosolic PAK-2 to the plasma membrane via the adaptor protein NCK and the membrane association of PAK-2 caused its activation; 2) silencing of GRP78 gene expression-abrogated membrane association of PAK-2 and NCK; 3) α2M* binding caused autophosphorylation of PAK-2 and enhanced its kinase activity toward MBP by severalfold; 4) kinase activation was greatly inhibited by prior treatment of cells with inhibitors of Tyr kinases and PI3K; and 5) α2M* binding to GRP78 elevated the levels of Ras-GAP, phosphorylated LIM kinase, and phosphorylated cofilin. The results presented strongly support the role of PAK-2 in cell cytoskeleton arrangements in α2M*-stimulated murine peritoneal macrophages. The mechanism by which α2M* activates PAK-2 appears to be dependent on receptor tyrosine phosphorylation and PI3K activation. We have shown previously that silencing of the GRP78 gene expression nearly abolished α2M*-induced calcium signaling, increased inositol 1,4,5-triphosphate synthesis and [3H]thymidine incorporation (28). In this study, we have extended the role of GRP78 in PAK-2 activation. Ligand-activated GRP78 triggers activation of downstream Ras/MAPK and PI3K signaling. Ras activates Rac/Cdc42 via PI3K, which is downstream of Ras. Binding of Rac1/Cdc42 to inactive PAKs causes a conformational change, which promotes their autophosphorylation. Under our experimental conditions, we find that the activation of PAK-2 exceeds that of PAK-1. In macrophages, PAK-2 activation also occurs independent of small G proteins. Ligand-induced tyrosine phosphorylation of GRP78 recruits PAK-2 to the membrane via the adaptor protein NCK. The membrane association of PAK-2 in complex with GRP78 and NCK activates PAK-2. It has been reported that mere membrane localization of PAK-2 is sufficient for its activation (15). The activation of PAK-2 also appears to be involved in cytoskeleton organization in α2M*-stimulated macrophages as evidenced by an increase in the protein levels of phosphorylated LIM kinase and phosphorylated cofilin.

PAK-2 is expressed ubiquitously whereas the expression of PAK-1 is tissue specific; PAK-2 also has very different properties than PAK-1 (see Refs. 8–13). In dividing cells PAK-2 is inactive, but it is transiently activated when cells are subjected to moderate stress conditions such as hyperosmolarity, ionizing radiation, or DNA damaging drugs. Under these conditions, PAK-2 activation requires upstream tyrosine kinase and PI3K activities (8, 51). In α2M*-stimulated macrophages we also show the requirement of tyrosine kinase and PI3K for PAK-2 activation (Fig. 6). The kinase activity of PAKs has been implicated in proliferative signaling by growth factor receptor kinases, which in turn regulate cell survival, programmed cell death, and malignant transformation (see Refs. 8–13). It has been demonstrated that activated full length PAK-2 stimulates cell survival and cell growth (52). Expression of constitutively active PAK-2 also promotes cell survival and suppresses cell death of BALB 3T3 fibroblasts in response to stress stimuli such as TNF-α, growth factor withdrawal, or UV light (see Refs. 8–13).

PAK-2, by virtue of its activation both by Rac, Cdc42, and heterotrimeric G proteins and binding of the adaptor protein NCK, is capable of receiving messages from a variety of signal transduction pathways (see Refs. 8–13). PI3Ks and their products have also been implicated in the regulation of the cytoskeleton (8, 48). Cdc42 binds to and weakly activates PI3K in vitro (53). Activation of PAK-1 in a Cdc42/Rac-1-independent manner has been shown by a direct association of PI3K with the N-terminal regulatory

Table I. Effect of time of incubation on protein levels of Ras • GAP, phosphorylated LIMkinase, and phosphorylated cofilin

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Ras • GAP (AU × 10^3)</th>
<th>Phosphorylated LIM kinase (AU × 10^3)</th>
<th>Phosphorylated Cofilin (AU × 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>640 ± 17</td>
<td>418 ± 35</td>
<td>422 ± 41</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>501 ± 55</td>
<td>675 ± 85</td>
</tr>
<tr>
<td>10</td>
<td>1021 ± 50</td>
<td>641 ± 58</td>
<td>826 ± 76</td>
</tr>
<tr>
<td>20</td>
<td>1627 ± 50</td>
<td>806 ± 81</td>
<td>921 ± 85</td>
</tr>
<tr>
<td>40</td>
<td>ND</td>
<td>902 ± 89</td>
<td>861 ± 80</td>
</tr>
<tr>
<td>60</td>
<td>1350 ± 102</td>
<td>800 ± 75</td>
<td>785 ± 80</td>
</tr>
<tr>
<td>120</td>
<td>1504 ± 39</td>
<td></td>
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</tbody>
</table>

* Values are expressed as arbitrary units (AU) and are the mean ± SE from three to four individual experiments.

FIGURE 6. Modulation of PAK-2 activation by tyrosine kinase and PI3K inhibitors by phosphorylation of MBP (A) and Western blotting (B).

The Journal of Immunology
domain of PAK-1 (53). In macrophages, we find that the inhibition of PI3K greatly reduces activation of PAK-2. This may occur either by attenuated interaction of PI3K with small G proteins upstream or downstream effectors of PI3K.

For many cell types, the ability to move across a solid surface is fundamental to their biological functions. Protrusion of the plasma membrane in lamellipodia and filopodia are driven by polymerization of actin filaments (54, 55). Members of the Wiskott-Aldrich syndrome protein (WASP) family, including WASP and WASP family verprolin-homologous proteins (WAVE), regulate several aspects of the actin polymerization cycle, including nucleation of new actin filaments and elongation of the existing ones (56–58). WASP mediates these processes through interactions with Arp2/3 complex and by binding to GTP-bound Cdc42 and phosphorylaminositol, 4,5-bisphosphate (PIP2) (58–56). The adaptor protein NCK has been implicated in the recruitment of WASP and N-WASP to the site of tyrosine phosphorylation (59). NCK stimulated actin nucleation by the N-WASP/Arp2/3 complex, which was further stimulated by PIP2. αM* treatment of macrophages activated Rac/Cdc42 as evidenced by the autophosphorylation of PAK-2 and activated PAK-2 by its recruitment to GRP78 in plasma membrane via its binding to NCK. αM* also up-regulated PAK-2-induced activation of LIM kinase, which in turn phosphorylated cofilin, thus promoting actin assembly. The PIP2 pool in macrophage plasma membranes is effectively used in αM*-stimulated cells as substrate for phosphorylaminositol-specific phospholipase C (20, 21, 60) and PI3K (24), generating vital intracellular signaling cascades. We suggest that under the experimental conditions, both PIP2 and NCK may mediate actin polymerization in αM*-treated macrophages.

In conclusion, we show in the present study that binding of αM* to GRP78 on murine peritoneal macrophages causes activation of PAK-2, its binding to the adaptor protein NCK, and the recruitment of NCK/PAK-2 complex to the tyrosine phosphorylated receptor, which initiates receptor downstream signaling pathways. The pivotal role of GRP78 in plasma membrane recruitment and activation PAK-2 in complex with NCK in αM*-stimulated cells is supported by the effect of silencing the expression of the GRP78 gene. This caused significant reductions in the activation of GRP78 upon αM* binding. That these events are a prerequisite for recruitment and activation of PAK-2 is further supported by experiments where pretreatment of cells with GRP78 Abs significantly reduced the phosphorylation of GRP78 as well as autophosphorylation of PAK-2 (unpublished observation). Because inhibition of PI3K inhibited PAK-2 activation, it can be inferred that PI3K signaling cascades is also involved in PAK-2 activation under our experimental conditions. Increased intracellular calcium is a ubiquitous second messenger of downstream signaling. Heterotrimeric G proteins can activate Ras via phosphorylaminositol-specific phospholipase C and Ca2+/calmodulin complex (61–63). Inhibition of intracellular calcium channels, Ca2+/calmodulin complex, and Ca2+/calmodulin kinase blocked PAK activation in IMLP-stimulated neutrophils (62). In PC-3 prostate cancer cells, thrombin-activated Rac activation was blocked by chelation of intracellular calcium (61, 63). Furthermore, we have shown that silencing of the expression of GRP78 by RNA interference significantly reduced the mRNA and protein levels of GRP78, reduced expression of GRP78 on the cell surface, reducing binding of αM*, and reduced receptor downstream signaling (64). Thus, receptor activation appears to be critical for the recruitment of PAK-2 via NCK to the receptor and induction of downstream signaling (64). Thus, αM* appears to activate PKA-2 by multiple mechanisms, which all contribute to the locomotive and cellular proliferative effects of αM*.

Disclosures

The authors have no financial conflict of interest.

References


