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

Uma Kant Misra, Tushar Sharma and Salvatore Vincent Pizzo

*J Immunol* 2005; 175:2525-2533; doi: 10.4049/jimmunol.175.4.2525

http://www.jimmunol.org/content/175/4/2525

**References**

This article *cites 63 articles*, 35 of which you can access for free at: http://www.jimmunol.org/content/175/4/2525.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
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

Uma Kant Misra, Tushar Sharma, and Salvatore Vincent Pizzo

Previous studies of the plasma protease inhibitor α2-macroglobulin (α2M) demonstrated that α2M-protease 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 βγ 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 Thr508 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 βγ-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 anal-
ogous 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 glu-
cose-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 con-
tribute 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 phosphory-
lation and activation of PAK-2 and its concomitant localization in
conjunction with NCK in the plasma membrane fraction. Activ-
tion 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 Life Technologies. Abs
against PAK-1, PAK-2, phosphorylated PAK-1 (Ser199)/Ser204, phos-
phorylated PAK-2 (Ser256), phosphorylated LIM kinase (Thr218), phos-
phorylated cofilin (Ser3), and phosphorylated tyrosine residues were procured
from Cell Signaling Technology. Myelcin 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 con-
trol, 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′-triphos-
phate (γ32P)-specific activity (3000 Ci/mmol) was purchased from
PerkinElmer. The sources for the inhibitors used have been described pre-
viously. αM-stimulated cells were prepared as described previously (21, 22). Other
agents 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-wk-
old C57BL/6 mice (National Cancer Institute) in HBSS containing 10 mM
glutamine, 12.5 U/ml penicillin, 6 μg/ml streptomycin, and 5% FBS.
The tubes were then adhered in 6-well plates (3 × 106/well/6-well plate),
monolayers were washed twice with HHBSS, and a volume of
HHBSS was added to the monolayers. To the respective wells,
106 cells/well/6-well plates), monolayers were washed twice with HHBSS, and a volume of
HHBSS was added to the monolayers. To the respective wells,

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 immu-
unoblotted 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 phosphory-
lated LIM kinase or phosphorylated cofilin, respectively. Membranes were
reprobed for LIM kinase protein, cofilin protein, actin, and GADPH, re-
spectively, 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 × 106/well/6-well plates),
monolayers were washed twice with HHBSS, and a volume of
HHBSS 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 orthovana-
date, 10 μg/ml leupeptin, and 10 μg/ml aprotonin over ice for 15 min. The
lysates were pipetted 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 super-

Measurement of PAK-1 and PAK-2 kinase activity in
immunoprecipitates

The kinase activities of PAK-1 and PAK-2 toward MBP were determined
essentially as reported previously (33). Briefly, macrophage lysates from the
respective groups were immunoprecipitated with PAK-1 or PAK-2 Abs
as above. The immunoprecipitates were washed three times with 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 ortho-

PAK-2 by Western blotting

Experimental details of electrophoresis and quantification of protein bands
were as described above, except that the respective membranes were immu-
unoblotted with Abs against Ras, respectively.
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 132P[ATP] (specific activity 3000 Ci/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 for 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 αs2-M-stimulated macrophages

Active GTP-bound Rac was precipitated using a PKA-p21 binding domain-based commercial assay kit (Upstate Cell Signaling). Macrophages were incubated overnight (4×10^6 well) in 6-well plates in RPMI 1640 medium containing 10% FBS, 12.5 U/ml penicillin, 0.65 μg/ml streptomycin, and 2 mM glutamine. The cells were stimulated with αM* (50 μM/10 min) at 37°C in a humidified CO2 (5%) incubator. The reaction was stopped by aspirating the medium and washing the cells twice with ice-cold HHBSS buffer (pH 7.4). The cells were lysed by adding a buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMFSF, 1 mM benzamidine, 10 μg/ml leupeptin, and 1% Nonidet P-40 on ice for 10 min. The lysates were transferred into Eppendorf tubes, DNA strands broken by passing the lysate through a 27-gauge needle, and the lysates 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 determined (31). To equal amounts of lysozyme in the respective tubes, 40 μl of PKA-p21 binding domain-agarose were added, and the tubes incubated at 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 three times with the above lysozyme buffer. 4×10^5 agarose pellets, 40 μl of reducing sample buffer were added, the tubes were heated at 90°C for 5 min, centrifuged briefly, and the supernatant was processed for protein fractionation on a 10% gel according to Laemmli (32). Proteins from gels (10%) were transferred to Hybond-P membranes and immunoblotted with Abs against Rac-1 (Santa Cruz Biotechnology). Protein bands on the membranes were visualized by ECF and quantified using a Storm 860 PhosphorImager (Molecular Dynamics). An aliquot of lysate was similarly processed for total Rac-1 quantification.

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 genistin (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 P38K were added to the respective wells containing cells (3×10^6 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. The respective membranes were stripped and reprobed for actin according to the manufacturer’s instructions.

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

Macrophages (3×10^6 cells/4-well plates) incubated overnight as above were washed twice with HHBSS, 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 HHBSS buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM PMFSF, 10 μM benzamidine, and 10 μM leupeptin. The cells were scraped 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 L80) 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 PMFSF, and 1 μM CaCl2. 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 lysate 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 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 K139QOLVKG376 mRNA sequence 5’−AAAATACAGCAATT AGTAAAGG−3’ peptide (Swiss-Prot, GRP78 primary sequence accession number P11021), was performed by Ambion. For making dsRNA, the sense (5’−AAUACGCAAUAGGAAAG−3’) and the antisense (5’−CU UUACAUUGCGUAGUUn−3’) 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 HHBSS. 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 into 100 μl of serum- and antibiotic-free medium. The two solutions were combined, mixed gently, and incubated for 45 min at room temperature followed by the addition of 800 μl of serum- and antibiotic-free medium to each tube. The monolayers were washed twice with serum- and antibiotic-free DMEM, layered in each well 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 the 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 or 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 and NCK, respectively, according to the manufacturer’s instructions. The membranes were incubated as above with scrambled siRNA or GRP78 homologous in sequence to the GRP78 gene and the effect of αM* on PAK-2 and NCK.
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 α2M* 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 α2M*-induced tyrosine phosphorylation of GRP78 in the GRP78 immunoprecipitate in murine peritoneal macrophages (Fig. 1A). α2M* binding caused a severalfold increase in tyrosine phosphorylation of GRP78 as compared with unstimulated cells (Fig. 1A). This demonstrates that like various growth factors, α2M* binding to GRP78 causes its tyrosine phosphorylation, a characteristic of protein tyrosine kinase receptors.

α2M* 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 chemotactic agents 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 α2M*-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 α2M* (Fig. 1B). The maximal increase in phosphorylated-PAK-2 levels occurred at 25–50 pM α2M* and ~10 min of incubation, after which the levels remained elevated for several hours. Incubation of cells with α2M* (50 pM) for varying periods of time showed maximal elevation in the protein levels of phosphorylated PAK-2 occurred at 10–20 min of incubation, which declined at longer periods of incubation (Fig. 1C). Incubation of cells with higher concentrations of α2M* caused a less sustained increase in PAK-2 phosphorylation. Incubation of cells with α2M* had much less of an effect on the activation of PAK-1 (Fig. 1C).

**FIGURE 1.** Effect of stimulation of macrophages with α2M* 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 α2M* (lane 2) (50 pM/10 min). B. Effect of α2M* concentration on levels of phosphorylated PAK-1 and PAK-2. C. Effect of time of incubation of α2M* (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 (×10^4) and are the mean ± SE from three to four individual experiments.

**Autophosphorylation of PAK-1 and PAK-2 in macrophages stimulated with α2M***

PAKs are present primarily as inactive holoenzymes (36). Binding of Cdc42-GTP and Rac-GTP to PAK causes an autophosphorylation of serine/threonine residues in the regulatory domain (see reviews Refs. 8–13). This leads to the opening of the molecule, transphosphorylation of Thr^23 in PAK-1 and Thr^402 in PAK-2, and substrate access to the kinase domain. By Western blotting techniques, we observed that treatment of macrophages with α2M* caused a 2- to 3-fold increase in the levels of phosphorylated PAK-2 but a negligible increase in PAK-1 activation (Fig. 1). We next determined the activation of PAK-1 and PAK-2 by autophosphorylation in their respective immunoprecipitates under these conditions (Fig. 2A). Stimulation of cells with α2M* caused a 2- to 3-fold increase in the autophosphorylation of PAK-2 (Fig. 2A) similar to that observed by Western blotting (Fig. 1). However, autophosphorylation of PAK-1 was negligible under these conditions (Fig. 2A). Because binding of GTP-bound forms of Rac/Cdc42 induces autophosphorylation and activation of PAK-2, these results indirectly demonstrate activation of these GTPases in macrophages stimulated with α2M*.

α2M* stimulates the kinase activity of PAK-2 in macrophages

In the next series of experiments, we studied the activation of PAK-1 and PAK-2 by measuring its kinase activity toward MBP (Fig. 2B). Treatment of cells with α2M* elevated phosphorylation of MBP by severalfold in the PAK-2 immunoprecipitate, but phosphorylation was negligible in the PAK-1 immunoprecipitate (Fig. 2B). These results are similar to those observed by Western blotting but are at a variance in the magnitude of increase in the two cases. A reasonable explanation for differences in the magnitude of their activation may be the differences in the amount of the proteins present in the cells used for immunoprecipitation and PAGE of lysates. Given the results obtained with respect to PAK-1, the remaining studies were focused only on PAK-2.
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

αM* activates Rac-1 in macrophages

PAKs 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.

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).
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 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 α5M* 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 α5M* regulates cytoskeletal rearrangement via LIM kinase and cofilin, which are downstream targets of activated PAK-2.

**Modulation of α5M*-induced activation of PAK-2**

In the preceding sections, we have shown that stimulation of cells with picomolar concentrations of α5M* caused a marked stimulation of PAK-2 activation and that this event was dependent upon GRP78 activation, which was tyrosine phosphorylated upon α5M* binding (Fig. 1). In our earlier studies, we have shown that binding of α5M* to cells causes activation of Ras, PI3K/Akt signaling and MAPK signaling (21–44). 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-dependent mechanism are also involved in PAK-2 activation (Fig. 6).

**Discussion**

In this study, we have examined the role of PAK-2 in α5M*-stimulated mitogenic signaling and cellular responses by quantifying its activation and regulation in macrophages. The principal findings are as follows: 1) binding of α5M* to cell surface-associated GRP78 caused its tyrosine phosphorylation and recruitment

**FIGURE 4.** Effect of silencing GRP78 gene expression on phosphorylated PAK-2 and NCK. Experimental details are described in section in Materials and Methods. Shown are the levels of phosphorylated PAK-2 (■) and NCK (□) in GRP78 gene-silenced cells. The bars are as follows: 1) LipofectAMINE buffer; 2) LipofectAMINE + α5M* (50 pM/10 min); 3) LipofectAMINE + GRP78 dsRNA + α5M*; and 4) LipofectAMINE + scrambled dsRNA (25 µg/ml) (48 h) + α5M* as above. The data shown are the mean ± SE from three individual experiments. Representative immunoblots are shown below. *, Significantly different at the 5% level from α*-stimulated cells where GRP78 was silenced.

**FIGURE 5.** Up-regulation of Ras-GAP, phosphorylated LIM kinase (LIMK), and phosphorylated cofilin upon α5M* stimulation. A, Effect of time of incubation of macrophages with α5M* on Ras-GAP levels. Corresponding immunoblots of Ras-GAP and the protein loading control actin are shown below the bar graph (see also Table I). B, Effect of time of incubation on phosphorylation of LIMK and cofilin in macrophages stimulated with α5M*. Protein loading controls are also shown (see also Table I). Values are expressed in arbitrary units and are mean ± SE from three to four independent experiments.
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) \(\alpha_2 M^*\) 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) \(\alpha_2 M^*\) 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 \(\alpha_2 M^*\)-stimulated murine peritoneal macrophages. The mechanism by which \(\alpha_2 M^*\) 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 \(\alpha_2 M^*\)-induced calcium signaling, increased inositol 1,4,5-triphosphate synthesis and \([^{3}H\text{]thymidine incorporation}\) (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 \(\alpha_2 M^*\)-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 \(\alpha_2 M^*\)-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 stimulants such as TNF-\(\alpha\), 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
domain of PAK-1 (53). In macrophages, we find that the inhibition of P3K greatly reduces activation of PAK-2. This may occur either by attenuated interaction of P3K with small G proteins upstream of PAK-2 or downstream effectors of P3K.

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 Wiskott 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 phosphatidylinositol 4,5-bisphosphate (PIP2) (56–58). 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 phosphatidylinositol-specific phospholipase C and Ca2+/H9251,
drour experimental conditions. Increased intracellular calcium is also involved in PAK-2 activation under our experimental conditions, both PIP2 and NCK may mediate actin polymerization stimulated cells as substrate for phosphatidylinositol-specific phospholipase C and Ca2+/H9251,
der our experimental conditions. Increased intracellular calcium is also involved in PAK-2 activation un-
drour experimental conditions. Increased intracellular calcium is also involved in PAK-2 activation un-

References

6. Aepfelbacher, M., F. Vauti, P. C. Weber, and J. A. Glomsset. 1994. Spreading of differentiating human monocytes is associated with a major increase in mem-
16. Archer, S., F. A. Babayannis, H. Hansen, C. Schneider, C. A. Stanyon, O. Bernardo, and P. Caroni. 1998. Regulation of actin dynamics through phos-
tors and the newly described αM signaling receptor. J. Biol. Chem. 269: 18303–18306.
duction: evidence from RNA interference that the low density lipoprotein recep-
30. James, K. 1980. αM-Macroglobulin and its possible importance in immune sys-
tems. Trends Biochem. Sci. 5: 143.

Disclosures
The authors have no financial conflict of interest.

Abstract activated αM and PAK-2 activation.

ACTIVATED αM AND PAK-2 ACTIVATION


