Oncostatin-M Stimulates Tyrosine Protein Phosphorylation in Parallel with the Activation of p42^{MAPK}/ERK-2 in Kaposi's Cells

Evidence That This Pathway Is Important in Kaposi Cell Growth

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Abstract

Oncostatin-M (OSM) is a potent mitogen for Kaposi's sarcoma (KS) cells. We studied signaling by the OSM receptor in three AIDS-related KS lines and show induction of tyrosine phosphorylation of 145-, 120-, 85-, and 42-kD substrates. The 42-kD substrate was identified as p42^{MAPK} (mitogen-activated protein kinase), also known as ERK-2. This serine/threonine kinase relays mitogenic signals from receptor tyrosine protein kinases (TPKs) or receptor-associated TPKs to transcriptional activators. The OSM dose dependence for MAP kinase activation and induction of KS cell growth were almost identical, suggesting functional linkage. MAP kinase activation was dependent on tyrosine phosphorylation, and both OSM-induced MAP kinase activity and KS cell growth could be suppressed by TPK inhibitors, genistein and geldanomycin. OSM also stimulated tyrosine phosphorylation of similar substrates and MAP kinase activity in human vein endothelial cells. While it has been proposed that the OSM receptor may include the gp130 subunit of the IL-6 receptor and α-chain of leukemia inhibitory factor (LIF) receptor, neither LIF nor r.IL-6 induced tyrosine protein phosphorylation or p42^{MAPK} activation in KS cells. However, r.IL-6 did stimulate tyrosine phosphorylation and p42^{MAPK} activity in the human B cell line, AF-10, while OSM and LIF exerted no effects. Our results indicate that, although the OSM and IL-6 receptors share a common signaling pathway, this pathway is selectively activated by OSM in Kaposi's cells. (*J. Clin. Invest.* 1993. 92:848–857.) Key words: kinase • AIDS • signaling • mitogenesis • inhibitor

Introduction

Kaposi sarcoma (KS)[1] is the most common malignancy seen in patients with HIV infection (1). Recent laboratory studies have provided important clues to the pathogenesis of this tumor and its relationship to HIV infection (2–9). One observation is that Oncostatin-M (OSM), a 28-kD T cell–derived cytokine that is made in large quantities by retrovirus-infected T cells and monocytes (10, 11) acts as a potent mitogen for KS cells in culture (12, 13). Since OSM can either stimulate or inhibit a variety of normal and malignant cells, the near universal stimulation of KS cells in culture appears to be unique (12). Moreover, this stimulation appears to be different from the effects of OSM on cultured human endothelial cells (HEC), which are thought to share a common ancestry with KS cells (2, 14). A fundamental question is whether KS cells have a different OSM receptor complex or utilize a different postreceptor pathway than HEC.

While OSM interacts with a 150–160-kD cell surface protein in HEC, the exact nature of the OSM receptor is unknown (15, 16). It is likely, however, that the OSM receptor belongs to the so-called cytokine or hemapoietin receptor family because of the structural similarity of the former cytokine with leukemia inhibitory factor (LIF) and IL-6 (17), both of which ligate receptors belonging to this family (17). Moreover, the LIF and IL-6 receptors share a common signaling subunit, gp130, which was originally identified as the β subunit of the IL-6R (18, 19). OSM binds to high affinity LIF receptors and OSM-induced KS cell growth is diminished in the presence of anti-gp130 antibodies (20). One suggestion, therefore, is that the OSM receptor is composed of gp130 and the α-chain (ligand-binding subunit) of the LIF receptor (18, 19). Recently, it has been reported that OSM stimulates tyrosine protein phosphorylation in HEC in association with the activation of an *src*-like tyrosine protein kinase (TPK), p62^{yes} (16). OSM also induces tyrosine protein phosphorylation in a hepatoma ($HepG_2$) cell line (21). It seems appropriate, therefore, to postulate that OSM utilizes tyrosine protein phosphorylation in induction of KS cell growth. Much remains to be learned, however, about the specific kinases and signaling intermediaries involved in the OSM receptor pathway.

We and others have recently described activation of the serine/threonine protein kinase, p42 MAP kinase (*m*itogen *a*ctivated *p*rotein kinase), also known as p42^{MAPK} or ERK-2 (*e*xtracellular *r*eceptor signal regulated *k*inase type 2), by members of the cytokine receptor family, namely, the GM-CSF, IL-3, IL-5, and IL-6[2] receptors (22–24). MAP kinase is a 42-kD phosphoprotein that is activated by dual phosphorylation on threonine and tyrosine residues in response to ligation of diverse mitogenic receptors (25–31). p42^{MAPK} and related isoforms in the ERK family appear to provide an important molecular switch mechanism whereby extracellular growth signals are transmitted via intrinsic TPK or TPK-associated receptors to an intracellular cascade of serine/threonine kinases

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1. *Abbreviations used in this paper:* APT, anti-phosphotyrosine; HEC, human endothelial cells; HUVEC, human umbilical vein endothelial cell; IMDM, Iscoves modified Dulbecco's medium; KS, Kaposi sarcoma; LIF, leukemia inhibitory factor; OSM, Oncostatin-M; r.IL-6, recombinant IL-6; [^{3}H]TdR, [^{3}H]thymidine; TPK, tyrosine protein kinase.

2. Daeipour, M., G. Kumar, M. C. Amaral, and A. E. Nel, 1993. *J. Immunol.* In press.

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involved in growth (29–32). For example, p42^{MAPK}/ERK-2 phosphorylates and activates p90^{RSK} (*r*ibosomal *S*6 *k*inase), which in turn phosphorylates c-*jun* and c-*fos* as well as ribosomal S6 protein (31, 32). In addition, ERKs also phosphorylate transcriptional activators such as c-*myc* and c-*jun* (33, 34). Inasmuch as the OSM receptor is a member of the cytokine receptor family (17), ERK-2 may play an important role in the induction of KS cell growth.

In light of the above, we were interested to determine whether OSM activates a TPK pathway that participates in Kaposi's cell growth and to compare tyrosine phosphoproteins in this pathway with those induced by LIF and IL-6. In addition, we were interested to determine whether p42^{MAPK}/ERK-2 plays a role in this pathway and in KS proliferation. We demonstrate here that OSM, but not LIF or r.IL-6, stimulated tyrosine protein phosphorylation in KS cells. Included in the OSM-induced tyrosine phosphoprotein profile was a 42-kD protein that was identified as p42^{MAPK}/ERK-2. OSM, but not LIF or r.IL-6, activated MAP kinase in KS cells in a dose-dependent fashion. The TPK inhibitors, genistein and geldanomycin, prevented p42^{MAPK}/ERK-2 activation by OSM and also blocked KS cell growth.

Methods

Antibodies and other reagents. Immobilon-P was obtained from Millipore Corp. (Milford, MA) and Hybond ECL nitrocellulose was from Amersham Corp. (Arlington Heights, IL). Antibodies for immunoblotting included monoclonal anti-phosphotyrosine (APT) antibody, 4G.10, from Upstate Biotechnology Inc. (Lake Placid, NY), monoclonal anti-ERK-2 from BIODESIGN International (Kennebunkport, ME), and peroxidase-conjugated sheep anti-mouse IgG (S&M IgG-HRP) from Amersham Corp. A peptide, APRTGGRR, which includes a consensus phosphorylation site for MAP kinase in bovine myelin basic protein was bought from Upstate Biotechnology Inc. Geldanomycin, an *src* kinase inhibitor, was obtained from GIBCO BRL (Gaithersburg, MD). Genistein, a general TPK inhibitor, was obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). OSM was generously provided by Dr. Peter Linsley (Bristol-Myers-Squibb, Seattle, WA) and LIF and recombinant IL-6 (r.IL-6) were bought from Amgen Biologicals (Thousand Oaks, CA). PMA, sodium heparin, and endothelial growth supplement were obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was from ICN Biomedicals, Inc. (Costa Mesa, CA) and the ^{125}I-protein A from Amersham Corp. FCS and other tissue culture reagents were from GIBCO BRL or Irvine Scientific (Irvine, CA). All other reagents were of the highest purity grade available and were obtained from Sigma Chemical Co.

Derivation, characterization, and maintenance of AIDS-KS-derived cells and human umbilical vein endothelial cells. The techniques for the isolation and characterization of the cell lines (KSL-1, KSL-8, EDS) from pleural effusions and skin of three different patients with AIDS-related KS have been previously described (12). Early passage lines were maintained in continuous monolayer culture on gelatinized flasks in IMDM containing 10% FCS, 30 μg/ml endothelial cell growth supplement, 1% penicillin/streptomycin with fungizone, 100 USP units/ml sodium heparin, and supplemental *l*-glutamine (growth medium A) with or without the addition of 500 pg/ml of recombinant OSM. Human umbilical vein endothelial cells (HUVECs) were obtained by trypsin treatment of umbilical veins. Each isolate was subcloned, characterized by immunohistochemical stains for endothelial markers, and then frozen at an early passage (less than 4). The cells for these assays were grown in DME containing 10% FCS, 30 μg/ml endothelial cell growth supplement, 1% penicillin/streptomycin with fungizone, 100 USP units/ml sodium heparin, and supplemental *l*-glutamine (growth medium B). AF-10 cells were cloned from the human IgE myeloma cell line, U266, and responded to r.IL-6 by enhanced proliferation. These cells were grown in RPMI 1640 supplemented with 10% FCS and antibiotics.

Proliferation assays. Subconfluent monolayers of AIDS-KS-derived cells were washed twice with PBS. The monolayers were trypsin/EDTA harvested, cells were counted, viability was determined by trypan blue exclusion, and the cells were replated at 75×10^3 cells in 1 ml of growth media in 24-well plates previously coated with 1% gelatin (12). 24 h after plating, the supernatant media were removed and serum-free IMDM containing 100 USP units/ml sodium heparin, 1% penicillin/streptomycin with fungizone, and ITS+ (Collaborative Research, Inc., Cambridge, MA) were added together with the indicated doses of geldanomycin and OSM (12). Control cultures received neither of these additions or OSM only. After growing the cells for 18 h at 37°C in a 5% CO_2 atmosphere, [^{3}H]thymidine ([^{3}H]TdR) was added at 1 μCi/ml (1 μCi = 37 kBq) for an additional 18 h. The cells were released with trypsin/EDTA and harvested onto glass wool filters, and radioactivities were measured in a scintillation cocktail. Each assay was done in quadruplicate.

Cellular stimulation for kinase assays/Western blotting. Subconfluent monolayers of KS were OSM starved for 48 h. Kaposi's cultures as well as HUVECs were harvested in PBS containing 10 mM EDTA without trypsin. After washing in growth medium A or B, respectively, duplicate aliquots (2×10^6) of KS cells and HUVECs were transferred into Eppendorf vials in 450 μl medium. AF-10 cells (5×10^6) were transferred in duplicate in 450 μl RPMI 1640 + 10% FCS. Cells were treated with 1–200 ng/ml OSM, 100 ng/ml LIF, or 100 U/ml r.IL-6 for time periods as indicated. For testing the effect of geldanomycin, Kaposi's cultures were previously (16 h) treated with 1–100 ng/ml of the drug in the absence of OSM before harvesting and stimulation as above. The effect of genistein was determined by prior exposure of OSM-deprived KS cells for 30 min with 5–30 μM of this drug before OSM treatment.

Assays for MAP kinase activity. Soluble cell extracts were generated by lysing cells through sonication in 20 mM Tris, pH 7.4, 1 mM EGTA, 20 mM PNP, 100 μM sodium vanadate, 1 μg/ml leupeptin, and 10 KU/ml aprotinin at 4°C (22, 26). The lysates were centrifuged at 12,000 *g* for 3 min and the soluble cell supernatants were equalized for protein. MAP kinase activity was determined in reaction vials that included 5 μg cellular supernatant together with 0.5 mM of the peptide substrate, APRTPGGRR, 20 μM ATP containing 2.5 μCi [γ-^{32}P]ATP (2–8 μCi/nmol), 10 mM $MgCl_2$, and 4.8 mM DTT in a volume of 50 μl for 5 min at 30°C (35). The reaction was stopped by adding 30 μl 20% TCA and 10 μg BSA for 15 min at 4°C. Vials were centrifuged at 12,000 *g* and supernatants spotted onto P81 paper squares (2×2 cm). These squares were washed four times in 75 mM H_3PO_4, dried, and counted by the Cerenkov method in a β-scintillation counter.

Immunoblotting. Soluble cell extracts (50 μg protein/lane) were boiled with SDS sample buffer and resolved by 10% SDS-PAGE. Electrophoretic transfer onto Immobilon-P or Hybond nitrocellulose membranes was conducted in a transblotting cell (Bio-Rad Laboratories, Richmond, CA). Membranes were then blocked for 2 h at RT in 6% BSA (Fraction V), 0.15 M NaCl, and 50 mM Tris, pH 7.4. The membranes were washed in PBS-Tween and incubated with 4G.10 (APT) (1:1,000 dilution) or anti-ERK-2 (1:5,000 dilution) for 1 h (22, 26). After washing in PBS-Tween, membranes were incubated with the 2° antibody sheep-α-mouse IgG-horseradish peroxidase (1:5,000 in dilution) for 45 min. Membranes were washed and developed with a chemiluminescence kit from Amersham Corp. (ECL kit).

Results

OSM induces tyrosine protein phosphorylation in Kaposi's cells: contrasting features with IL-6 and LIF stimulation. The cell line, KSL-1, was established from a Kaposi's tumor in a patient suffering from AIDS (12). One of us (S. Miles) has previously shown that the growth of this cell line can be enhanced in tissue culture by OSM (12). KSL-1 cells were incu-

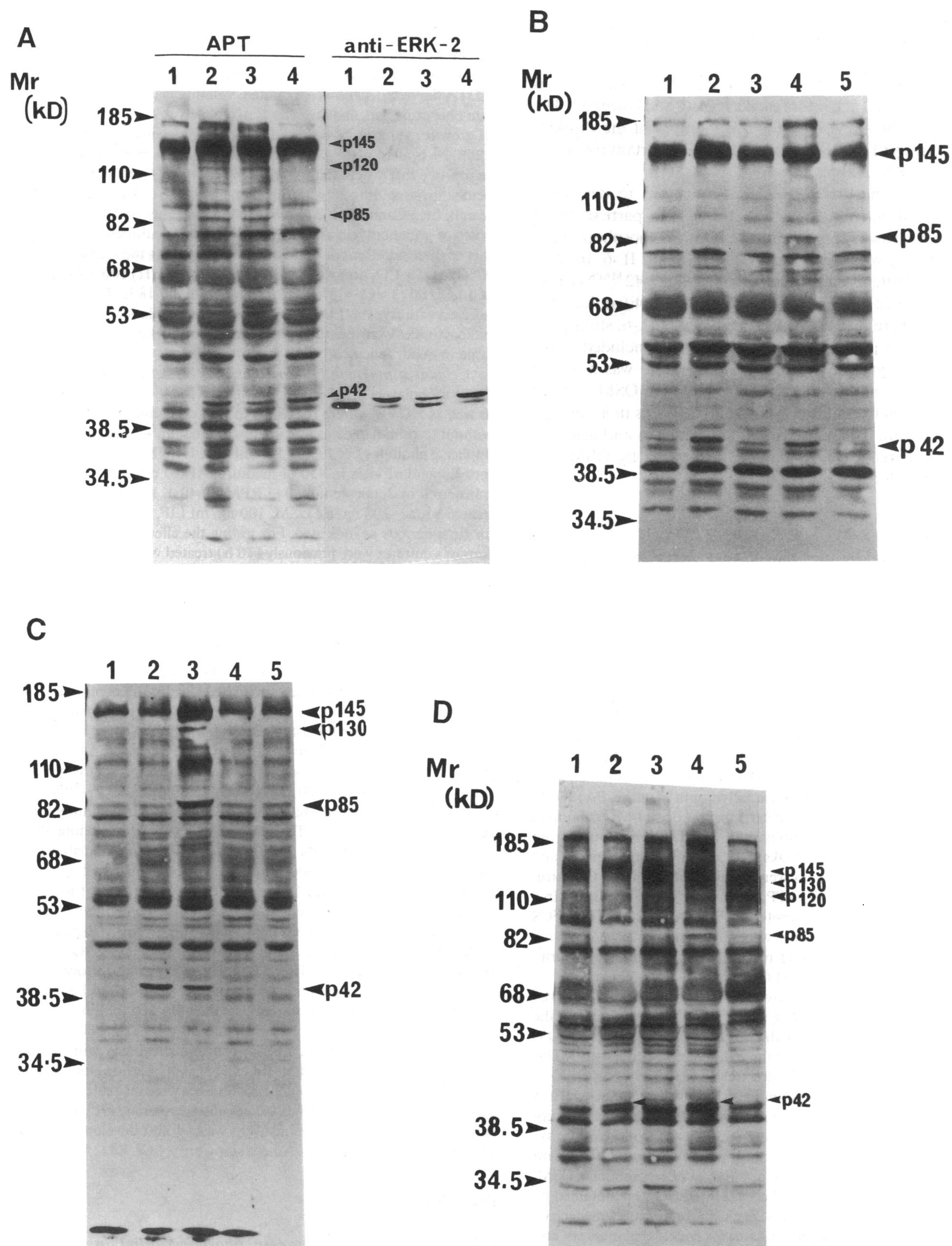


Figure 1. Comparison of the effects of OSM, LIF, and r.IL-6 on induction of tyrosine protein phosphorylation as assessed by immunoblotting. (*A*) KS-1 cells were OSM starved for 48 h in gelatinized flasks. Cells were harvested in PBS containing 10 mM EDTA without trypsin. After washing in IMDM plus 10% FCS, 2×10^6 cells were transferred into Eppendorf vials before stimulation with 100 ng/ml OSM for 10 (lane *2*) or 20 (lane *3*) min, and PMA for 10 min (lane *4*). Control cells (lane *1*) received no stimulus. After lysis, 50 μg supernatant protein from each aliquot was resolved by 10% SDS-PAGE. The gel was transferred to Immobilon P filters, blocked, and overlayed with 1:1,000 of the APT anti-

bated with 100 ng/ml OSM for 10 and 20 min, and soluble supernatants were resolved by 10% SDS-PAGE. APT immunoblotting of this gel showed enhanced phosphorylation of substrates with molecular masses of 145, 120, 85, and 42 kD compared with the control lane (Fig. 1 *A, left panel,* lanes *1–3*). The phorbol ester, PMA, stimulates tyrosine phosphorylation of a 42-kD substrate in most culture cell lines and was used as the positive control (25–30). PMA induced the appearance of p42 in KSL-1 cells together with p145 but did not affect the phosphorylation of 120- and 85-kD substrates (Fig. 1 *A, left panel,* lane *4*). Similar results were obtained with OSM and PMA in two additional AIDS-related KS cell lines, KSL-8 (not shown) and EDS (see Fig. 5 *D*). Although IL-6 has been implicated as a possible growth factor for KS cells (5) and the β subunit (gp130) of this cytokine receptor exhibits low affinity binding for OSM (18), r.IL-6 did not induce tyrosine phosphorylation in KSL-1 (Fig. 1 *B,* lane *3*), KSL-8, or EDS cells (not shown). It is interesting, however, that r.IL-6 induced tyrosine phosphorylation of 145-, 130-, 85-, and 42-kD substrates in a B cell line, AF-10 (Fig. 1 *C,* lane *3*). In contrast, OSM did not induce tyrosine protein phosphorylation in AF-10 cells (Fig. 1 *C,* lane *4*), while PMA induced the appearance of p42 only (Fig. 1 *C,* lane *2*). LIF, which is structurally related to OSM and binds to a receptor that also interacts with OSM, failed to stimulate tyrosine protein phosphorylation in KSL-1 (Fig. 1 *B,* lane *5*) or AF-10 cells (Fig. 1 *C,* lane *5*).

Although the exact origin of KS cells is unknown, it has been suggested that they may be derived from the same mesenchymal precursor as endothelial cells. It is therefore interesting that OSM induced tyrosine phosphorylation of the same range of substrates (p145, p130, p120, p85, and p42) in HUVECs as in KSL-1 cells (Fig. 1 *D,* lane *4*). In contrast to the lack of effect in KSL-1 cells, r.IL-6 induced tyrosine phosphorylation of 145- and 120-kD proteins in HUVECs without inducing the appearance of p85 and p42 (Fig. 1 *D,* lane *3*). HUVECs also differed from KSL-1 cells in their response to LIF; while not eliciting a biochemical response in KSL-1 (Fig. 1 *B*), LIF stimulated tyrosine phosphorylation of p145 and p120 in HUVECs (Fig. 1 *D,* lane *5*). PMA induced the appearance of p42 in HUVECs (Fig. 1 *D,* lane *2*) but did not induce phosphorylation of other proteins (Fig. 1 *A,* lane *4*). Taken together, these results show that OSM induced the same profile of tyrosine phosphoproteins in KSL-1 and HUVECs, but these cell types differed in their responsiveness to r.IL-6 and LIF.

OSM stimulates a mobility shift of ERK-2 in KSL-1 in parallel with MAP kinase activation. In Fig. 1 *A,* we have demonstrated the appearance of a 42-kD tyrosine phosphoprotein during OSM and PMA treatment (Fig. 1 *A, left*). Since we have identified a 42-kD PMA-inducible TPK substrate as p42 MAP kinase ($p42^{MAPK}$), also known as ERK-2, in other cell types, we were interested to determine whether OSM-responsive p42 in KSL-1 is $p42^{MAPK}$ (22, 26, 36–38). During activation, $p42^{MAPK}$ undergoes a conformational change which is reflected as a mobility shift of the kinase on SDS-PAGE (22, 39). Overlay of the immunoblot shown in the left panel of Fig. 1 *A* with an antibody that recognizes ERK-2 revealed the presence of a 41-kD protein in control cells (lane *1*), which shifted to a 42-kD position after treatment with PMA as well as OSM (Fig. 1 *A, right panel,* lanes *2–4*). The electrophoretically retarded form of ERK-2 (42 kD) coelectrophoresed with p42, suggesting that they are identical proteins (Fig. 1 *A,* lanes *2–4, left and right panels*). This notion was strengthened by coincidental induction of MAP kinase activity (Fig. 2) with the appearance of p42 in KSL-1 (Fig. 1, *A* and *B*), AF-10 (Fig. 1 *C*), and HUVECs (Fig. 1 *D*) during cellular stimulation. While OSM induced MAP kinase activity in KSL-1 and HUVECs, no kinase activation was seen in AF-10 cells (Fig. 2). In contrast, r.IL-6 failed to activate $p42^{MAPK}$ in KSL-1 and HUVECs but activated this kinase in AF-10 cells (Fig. 2). LIF failed to activate $p42^{MAPK}$ in KSL-1 or HUVECs, while PMA activated this kinase in all three cell types (Fig. 2). Although AF-10 cells were not treated with LIF in the experiment shown in Fig. 2, no MAP kinase activation was seen in a separate experiment (not shown).

Results similar to KSL-1 were obtained when EDS cells (see Fig. 5 *B*) and KSL-8 (Table I) were exposed to OSM and r.IL-6. Basal MAP kinase levels were consistently higher in KSL-8 than in KSL-1 cells, with the result that the magnitude of increase in kinase activity was less during OSM stimulation in KSL-8 (1.5–2-fold) than in KSL-1 (3–6-fold). Although not proven, the variance in background may relate to differences in the amounts of endogenous OSM produced by KS cell lines (12).

Confirmation that p42 is a MAP kinase was obtained during immunoaffinity chromatography, which demonstrated that ERK-2 protein in the supernatants of OSM-treated cells bound to immobilized APT mAb (Fig. 3). In contrast, no immune adsorption of ERK-2 was obtained when the supernatants of control (OSM-starved) cells were passed over the same column (Fig. 3). Likewise, a relative abundance of MAP kinase activity was eluted from the column loaded with OSM-treated cell supernatants compared with control (OSM-starved) cell supernatants (Fig. 3).

$p42^{MAPK}$/ERK-2 activation by OSM involves tyrosine phosphorylation of the kinase. $p42^{MAPK}$/ERK-2 activation occurs by way of a kinase cascade which includes one or more afferent MAP kinase kinase(s) that induce phosphorylation of $p42^{MAPK}$/ERK-2 (29, 40–42). While the exact sequence of this event is still a matter of debate, $p42^{MAPK}$ requires phosphorylation on both threonine 183 (Thr-183) and tyrosine 185 (Tyr-

body, 4G.10. The primary antibody was detected by SαM IgG–horseradish peroxidase (1:5,000 dilution), utilizing a chemiluminescence kit. The left panel shows the autoradiogram of the APT immunoblot. Notice enhanced phosphorylation of p145, p120, p85, and p42 during OSM treatment. The same membrane was stripped and overlayed with a primary mAb that recognizes ERK-2 protein. The secondary antibody and further development of the blot was as for 4G.10. The resulting autoradiogram (*right panel*) shows mobility shift of ERK-2 in OSM- and PMA-treated cells (lanes *2–4*). Notice that electrophoretically retarded ERK-2 (*right*) migrated with p42 (*left*). (*B*) Autoradiogram of APT immunoblot in KSL-1 cells after stimulation for 10 min with control (lane *1*), 100 nM PMA (lane *2*), 100 U/ml r.IL-6 (lane *3*), 100 ng/ml OSM (lane *4*), and 100 ng/ml LIF (lane *5*). (*C*) Autoradiogram of APT immunoblot on AF-10 cells (B cell line) stimulated for 10 min with control (lane *1*), 100 nM PMA (lane *2*), 100 U/ml r.IL-6 (lane *3*), 100 ng/ml OSM (lane *4*), and 100 ng/ml LIF (lane *5*). (*D*) Autoradiogram of APT immunoblot on HUVECs. HUVECs were obtained by trypsin treatment of umbilical veins and were grown in DME plus 10% FCS as described in Methods. Lane *1,* control; lane *2,* 100 nM PMA; lane *3,* 100 U/ml r.IL-6; lane *4,* 100 ng/ml OSM; lane *5,* 100 ng/ml LIF.

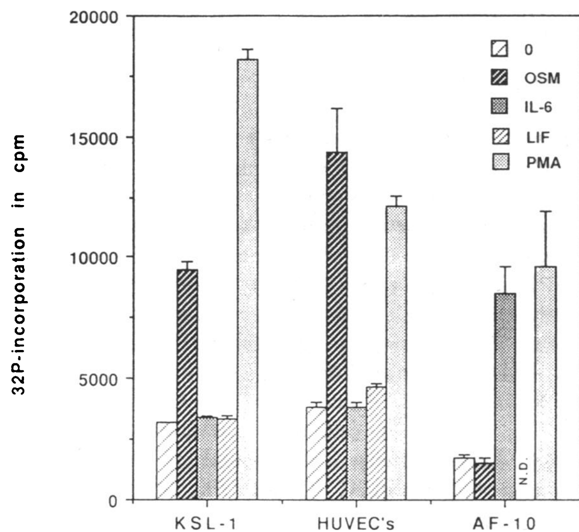


Figure 2. Comparison of MAP kinase activation by OSM, IL-6, LIF, and PMA in KSL-1 cells, HUVECs, and AF-10 cells. Cells were grown and harvested as described in Fig. 1. Duplicate aliquots of 2 $\times 10^6$ KSL-1 cells, 2 $\times 10^6$ HUVECs, and 5 $\times 10^6$ AF-10 cells were treated with 100 ng/ml OSM, 100 U/ml r.IL-6, 100 ng/ml LIF, or 100 nM PMA for 10 min. Cells were lysed in a Tris buffer and soluble supernatants were obtained after 12,000-*g* centrifugation. MAP kinase activity was determined by utilizing 5 μg supernatant protein to phosphorylate 0.5 mM MBP peptide, APRTPGGRR, in 50-μl reaction vials (35). Results depict ^{32}P incorporation into the peptide±SEM.

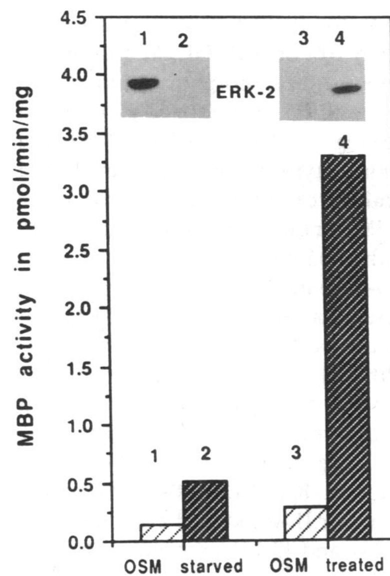


Figure 3. An APT immune affinity column binds ERK-2 and MAP kinase in supernatants from OSM-treated but not OSM-starved cells. KSL-1 cells were grown in tissue culture flasks to near-confluency in the absence of OSM. After harvesting in PBS plus 10 mM EDTA, cells were divided into two aliquots of 25 $\times 10^6$ cells each. One aliquot was treated with 100 ng/ml OSM for 10 min, while the other (OSM starved) served as control. Cells were lysed in 20 mM Tris, pH 7.4, containing 10 mM EDTA, 100 mM NaCl, protease and phosphatase inhibitors, and ultracentrifuged at 100,000 *g* at 4°C for 30 min. An equal amount of protein from each supernatant was loaded over an APT immunoaffinity column (Upstate Biotechnology Inc.) and washed with cell lysis buffer. All nonbound material was collected and the protein concentration determined (lanes *1* and *3*). Both columns were then eluted with a buffer containing 100 mM phenylphosphate and the protein concentration determined (lanes *2* and *4*). MAP kinase activity and presence of ERK-2 protein were determined in each of the nonbound eluate fractions by peptide assay and ERK-2 immunoblotting as described in Methods. The immunoblot result is displayed at the top of this figure. Lanes *1* and *3*, nonbound material; lanes *2* and *4*, phenylphosphate eluates.

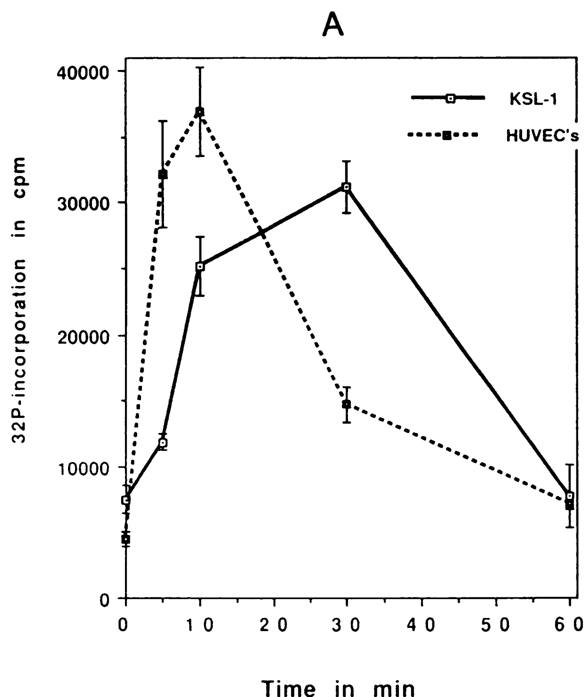
185) residues to be rendered active (25, 26). Likewise, in KSL-1 cells, the kinetics of appearance of p42 (Fig. 4 *A*) and activation of MAP kinase (Fig. 4 *B*) by OSM were identical. Clearly recognizable p42 and enhanced MAP kinase activity were seen 10 min after OSM addition and lasted 30 min before declining (Fig. 4, *A* and *B*). This time course is somewhat slower than the evanescent MAP kinase response that we have observed in other cell types (25, 36, 37), as well as in OSM-treated HUVECs (Fig. 4 *A*). Confirmation of the importance of tyrosine phosphorylation in ERK-2 activation in KSL-1 was sought by using TPK inhibitors. Geldanomycin is a potent inhibitor of *src*-like kinases (43), but does not directly inhibit already active MAP kinase (not shown). Incubation of intact KSL-1 cells with this drug decreased p42^{MAPK} activation by OSM (Fig. 5 *A*). The IC_{50} of inhibition was calculated as 30 ng/ml geldanomycin, which is in agreement with its inhibitory range for *src*-kinases (43). Another more general TPK inhibitor, genistein, also suppressed MAP kinase activation by > 55% at a dose of 30 μM (Fig. 5 *B*). Confirmation that geldanomycin acted by inhibiting tyrosine phosphorylation of ERK-2 rather than through a toxic effect was obtained by APT immunoblotting. The autoradiogram in Fig. 5 *C* shows that at 10 ng/ml, geldanomycin suppressed OSM-induced tyrosine phosphorylation of several substrates, including p42. Similar observations were made with genistein in EDS cells (Fig. 5 *D*), confirming the functional importance of TPK activation toward MAP kinase activation by OSM.

Table I. OSM Activates MAP Kinase in KSL-8 Cells

	^{32}P incorporation in MBP peptide
	cpm±SD
Control	6,345±629
OSM	12,212±465
r.IL-6	6,865±533

Duplicate aliquots of KSL-8 (2 $\times 10^6$) were treated with 100 ng/ml OSM at 100 U/ml r.IL-6 for 10 min. After cellular lysis and collection of 12,000-*g* supernatants, MAP kinase activity was determined by in vitro phsophorylation of MBP peptide as described in Methods.

p42^{MAPK}/ERK-2 activation by OSM is critical for KS cell growth. OSM stimulated KSL-1 cell growth (Table II) with similar dose dependence as activation of MAP kinase in this cell type (Table III). Optimal cellular and biochemical responsiveness in KSL-1 occurred with OSM concentrations ranging from 50 to 100 ng/ml. HUVECs exhibited a similar dose-responsive profile during OSM treatment (Table III). This suggested that p42^{MAPK} plays a critical role in KS cell growth, and we hypothesized that inhibition of MAP kinase activity correlated with disruption of cell growth. Indeed, geldanomycin inhibited KSL-1 growth, as assessed by [^{3}H]TdR uptake, in a dose-dependent fashion (Table IV). In the presence of 100 ng/ml OSM, geldanomycin inhibited KSL-1 growth with an IC_{50} of 30 ng/ml, which is identical to the IC_{50} for inhibiting p42^{MAPK} activation. Genistein also inhibited, in a dose-dependent manner, growth of EDS cells in the presence of 100 ng/ml OSM (not shown). Taken together, these results strongly suggest that p42^{MAPK} is critical for KSL-1 growth stimulation by



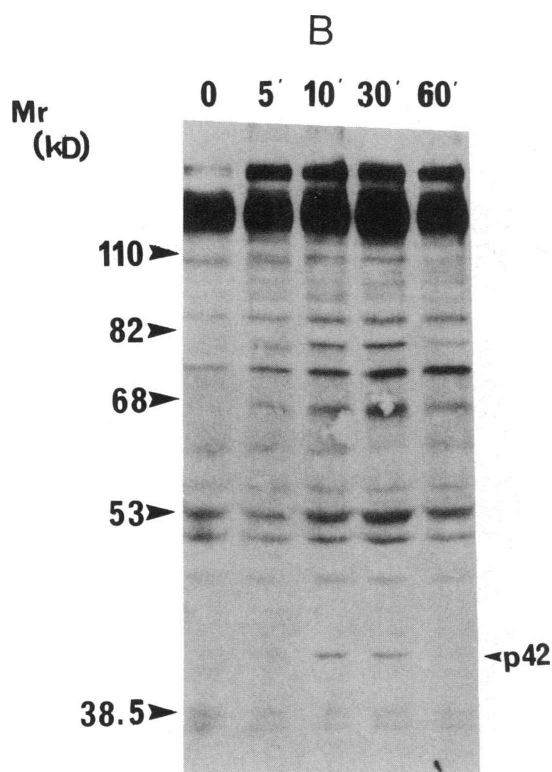


Figure 4. Kinetics of MAP kinase activation by OSM is identical to kinetics of appearance of p42 on APT immunoblots. OSM-starved KSL-1 cells (2×10^6/aliquot) were stimulated with 100 ng/ml OSM for 5–60 min. (*A*) Assays for MAP kinase activity were conducted in the supernatants as described in Fig. 2. Error bars represent SEM (duplicate measurements). Similar measurements were made in HUVECs during treatment with 100 ng/ml OSM. (*B*) Autoradiogram of APT immunoblot on KSL-1 material, showing appearance of p42 at 10 and 30 min after addition of OSM.

OSM. Inasmuch as other growth factors for KS cells exist (7, 9), we do not imply, however, that the MAP kinase cascade is the sole pathway responsible for cellular growth.

Discussion

The data in this paper show induction of tyrosine protein kinase (TPK) activation by OSM in three KS cell lines along with the activation of $p42^{MAPK}$/ERK-2. The stimulation of cell growth and activation of MAP kinase by OSM had similar dose dependence. Moreover, OSM-induced cell growth was inhibited in parallel with the $p42^{MAPK}$ activation by TPK inhibitors, geldanomycin and genistein. r.IL-6 and LIF did not activate TPK or MAP kinase activity in KS cells.

OSM is a member of a family of related cytokines that include LIF, IL-6, and granulocyte colony-stimulating factor (17). Its cellular effects are pleiotropic and include growth inhibition of various tumor cells, growth stimulation of fibroblasts and smooth muscle cells, induction of IL-6 production in human endothelial cells, and induction of acute phase protein synthesis in the hepatoma cell line, Hep G2 (10, 15, 16, 21). Recently, it was demonstrated that OSM is a potent growth factor for human KS cell lines (12, 13). In addition, KS cells adopt a spindle shape during OSM treatment and are induced to secrete IL-6 (10, 12). Although OSM has been cross-linked to a membrane protein of 150–160 kD in HECs, we do not know the specific composition and molecular mechanism of action of this receptor (16). We suspect, however, that the OSM receptor may belong to the cytokine receptor family because its ligand is structurally related to LIF and IL-6 (17). The 130-kD subunit of the IL-6R serves as a signal transducer for IL-6, LIF, and OSM (17, 19, 44). Since the OSM receptor complex can include the gp130 subunit of the IL-6 receptor, and either the α-chain of the LIF receptor (18, 19, 44) or a putative α-chain of the OSM receptor, the differential expression of these receptor complexes on KS cells could help distinguish their response from that of other cell types to OSM. One way in which this can be accomplished is by expression of different α subunit combinations (i.e., the α-chain of LIF, OSM, or IL-6 receptor) with gp130 on the cell surface (44). Alternatively, the post-OSM receptor pathways may differ between KS cells and their normal mesenchymal counterparts. Results in this paper are in favor of the former notion.

In looking for a signaling pathway related to the OSM receptor, we were influenced by previous findings which indicated that OSM induces tyrosine protein phosphorylation in hepatocytes and HECs (16, 21). In addition, we have previously shown that two members of the cytokine receptor family, GM-CSF-R (22) and IL-6R,[2] stimulate tyrosine protein phosphorylation in association with activation of the Ser/Thr kinase, $p42^{MAPK}$, also known as ERK-2 (24). In this paper, we show that OSM stimulates tyrosine phosphorylation of a 42-kD protein in all three KS cell lines tested (Fig. 1, *A* and *B*). The 42-kD substrate was identified as ERK-2 on the basis of its coelectrophoresis with activated ERK-2 on SDS-PAGE (Fig. 1 *A*). Moreover, an immobilized anti-phosphotyrosine antibody removed ERK-2 and MAP kinase from the supernatants of OSM-treated but not control cells (Fig. 3).

In keeping with its intimate relationship with mitogenic responses in other cell types (26, 36–38), ERK-2/$p42^{MAPK}$ appears to play an important role in OSM-mediated KS cell

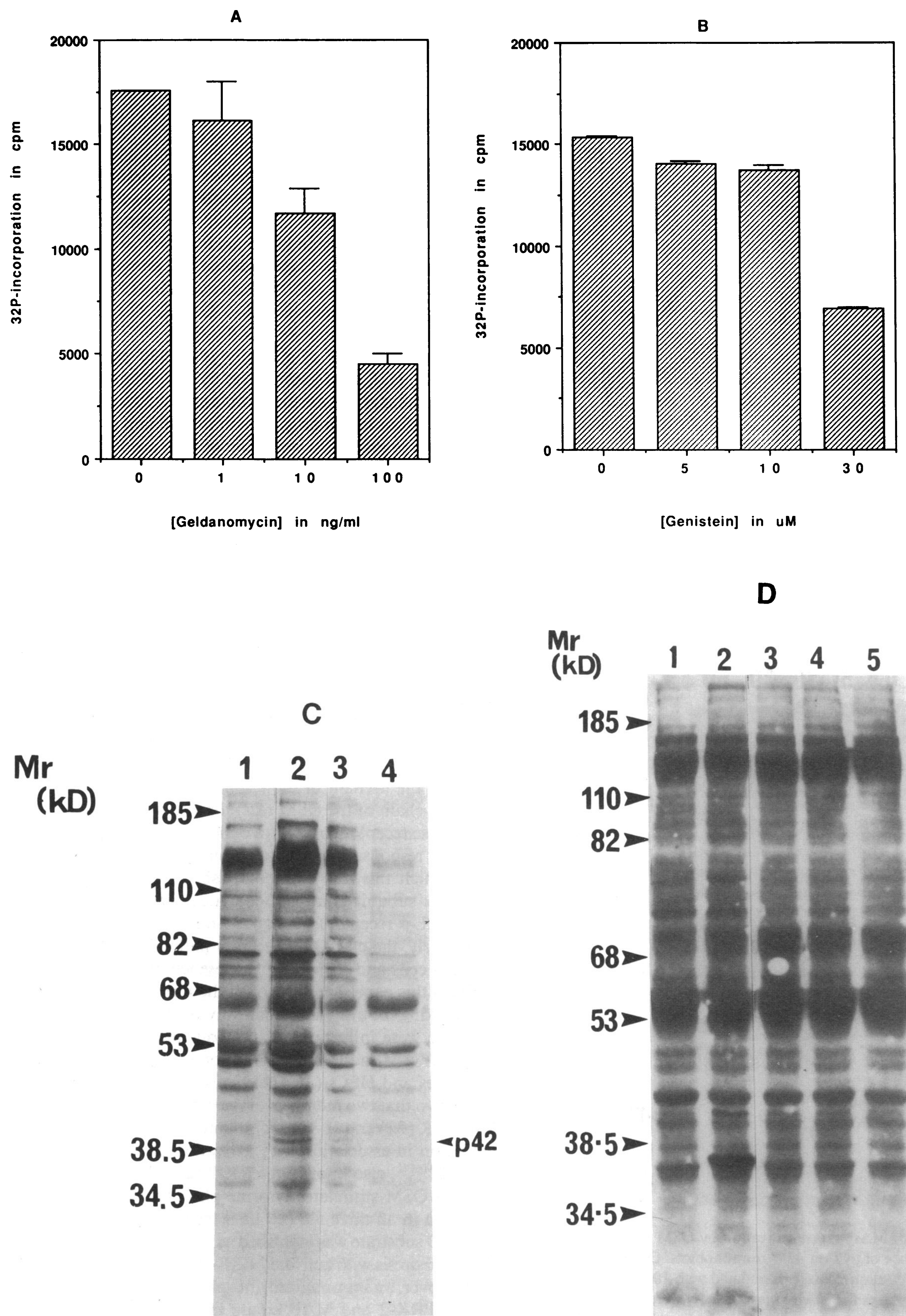


Figure 5. Inhibition of MAP kinase activity in parallel with the inhibition of tyrosine phosphorylation of p42^{MAPK} by geldanomycin and genistein. KSL-1 and EDS cells were OSM starved for 48 h. Geldanomycin was added to KSL-1 cultures for 16 h at final concentrations of 1, 10, and 100 ng/ml, respectively, before stimulation with 100 ng/ml OSM for 10 min. Genistein was added to EDS cells at a final of 5, 10, or 30 μM for 30 min before stimulation with 100 ng/ml OSM for 10 min. Cellular supernatants were used for performance of MAP kinase assays

Table II. OSM Stimulates a Dose-dependent Growth Response in KSL-1 Cells

	^{3}H-TdR uptake
	cpm±SD
Control	1,179±214
OSM, 2 ng/ml	2,844±38
OSM, 10 ng/ml	4,172±881
OSM, 100 ng/ml	5,398±1,447
OSM, 250 ng/ml	5,438±1,195

75 × 10^{3} KSL-1 cells were plated in quadruplicate in each well of a 24-well culture plate and OSM was added at the indicated concentrations for 36 h. 1 μCi [^{3}H]TdR was added for the last 18 h in culture before cell harvest and scintillation counting.

Table III. OSM Activates MAP Kinase in a Dose-dependent Fashion in KSL-1 Cells and HUVECs

	^{32}P incorporation in MBP peptide	
	KSL-1	HUVECs
	cpm ± SEM	
Control	7,486±1,075	3,245±168
OSM, 1 ng/ml	8.654±18	3,783±242
OSM, 10 ng/ml	10,241±1,028	8,605±103
OSM, 100 ng/ml	28,989±1,898	28,684±2,430
OSM, 200 ng/ml	29,258±2,218	30,518±2,511

Duplicate aliquots of KSL-1 (2 × 10^{6} cells) or HUVECs (3 × 10^{6} clls) were treated with the indicated concentrations of OSM for 10 min. MAP kinase activity was determined by in vitro assay as described in Methods.

growth. First, there was almost similar dose dependence for induction of cell growth and MAP kinase activation by OSM (Tables II and III). Second, interference with p42^{MAPK} tyrosine phosphorylation and activation occurred in parallel with diminished KS cell growth (Table IV and Fig. 5). Also, the IC$_{50}$ for geldanomycin inhibition of cell growth and kinase activation was identical (30 ng/ml). While we cannot exclude the possibility that inhibition of tyrosine phosphorylation of other substrates by genistein and geldanomycin contribute to KS growth arrest, it is reasonable to suggest that the effect of these drugs is partially mediated through MAP kinase inhibition. Although its exact role in KS growth still remains to be elucidated, it is attractive to postulate that p42^{MAPK}/ERK-2 phosphorylates transcriptional activators and/or other growth-associated Ser/Thr kinases (e.g., p90^{RSK}) that are involved in OSM-induced KS cell growth (32–34). Since we have only tested three KS cell lines, it is possible that some heterogeneity may be found with respect to the growth pathway in KS.

The stimulation of tyrosine protein phosphorylation and activation of p42^{MAPK}/ERK-2 by OSM appear to be related events. p42^{MAPK} activation closely followed the OSM-induced appearance of p42 on APT immunoblots (Fig. 4). Moreover, interference of OSM-induced tyrosine phosphorylation by the TPK inhibitors, geldanomycin and genistein, diminished p42^{MAPK} activation (Fig. 5). p42^{MAPK} activation by mitogenic receptors is dependent on the dual phosphorylation of this kinase on Thr-183 and Tyr-185 residues by a complicated afferent kinase cascade (27, 40–42). During its posttranslational modification, p42^{MAPK}/ERK-2 undergoes conformational change as evidenced by gel mobility shift during OSM treatment (Fig. 1 *A*) (39). It is generally accepted that an afferent "MAP kinase kinase" is responsible for the phosphorylation and activation of p42^{MAPK}/ERK-2 (40–42). The gene for such a kinase, designated MEK (*M*AP kinase/*E*RK *k*inase), has recently been cloned, and, although it resembles a Thr/Ser kinase, its product can phosphorylate MAP kinase on both threonine and tyrosine residues (42). Another possibility, however, is that MEK phosphorylates p42^{MAPK} on Thr-183 and induces it to autophosphorylate on Tyr-185 (45). Whatever the exact mechanism of MAP kinase tyrosine phosphorylation, recent studies have shown that receptor-associated TPKs, including members of the *src* family, can participate in the MAP kinase activation cascade. We and others have previously shown that activation of p42^{MAPK} by the T cell antigen receptor involves a *src* family member, p56^{lck} (46, 47). Moreover, in endothelial cells, OSM activates another *src*-like kinase, p62^{yes} (16). From the perspective of the *src*-kinase family, it is also interesting to note that these kinases functionally interact with the *ras* oncogene product to the extent that dominant negative *ras* can disrupt MAP kinase activation in v-*src* transfected cell lines (48). It may therefore be relevant that *ras* oncogene expression is increased in the majority of KS lesions (49).

Since it has previously been suggested that KS cells and HEC share a common ancestry, it is interesting that OSM activated p42^{MAPK} in HUVECs (Figs. 1 *D* and 2). Another cell type of the same putative lineage, aortic smooth muscle cells, also responded to OSM treatment with p42^{MAPK} activation (not shown). The biochemical responses of KS and HUVECs differed, however, during r.IL-6 and LIF treatment. Both cytokines induced tyrosine protein phosphorylation in HUVECs but not KS cells (Fig. 1). The lack of a biochemical response to LIF in KSL-1 cells agrees with failure to proliferate. The most likely explanation is that the α-subunit of the LIF receptor is not expressed in KS cells. Although there has been good circumstantial evidence for a possible role for IL-6 in KS cell growth (5), our published data show that there is no effect of exogenous IL-6 in the absence of inhibiting of endogenous IL-6 expression by IL-6 specific antisense oligonucleotides (5). It is possible, however, that IL-6 may contribute to KS lesions in some alternate way because of its abundant expression in tumor sites and the anti-proliferative effect of antisense mRNA

and APT immunoblotting as described in Methods. *A* shows the effect of geldanomycin on MAP kinase activity in KSL-1 cells. ^{32}P incorporation into MBP peptide in control cells, not exposed to OSM, was 5,423±158 cpm. *B* shows the effect of genistein on MAP kinase activity in EDS cells. ^{32}P incorporation into MBP peptide in unstimulated cells amounted to 3,766±486 cpm, while in r.IL-6-treated cells the value was 4,676±846. *C* shows the autoradiogram of the APT immunoblot conducted on KSL-1 material. Lane *1*, control cells, no geldanomycin exposure; lane *2*, 100 ng/ml OSM in absence of geldanomycin; lanes *3* and *4*, 100 ng/ml OSM in cells preloaded with 10 and 100 ng/ml geldanomycin, respectively. *D* shows the autoradiogram of the APT immunoblot performed on soluble EDS extracts. Lane *1*, control cells, no genistein exposure; lane *2*, 100 ng/ml OSM in the absence of genistein; lanes *3–5*, 100 ng/ml OSM after preloading with 5, 10, and 30 μM genistein, respectively.

Table IV. Effect of Geldanomycin on OSM-stimulated KSL-1 Growth

Geldanomycin	Control	[^{3}H]TdR uptake OSM 10 ng/ml	OSM 100 ng/ml
ng/ml		*cpm*	
0	7,160±2,224	9,391±1,657	9,240±966
1	5,750±1,377	9,560±3,270	8,160±1,713
10	5,980±2,575	7,435±1,694	6,520±2,222
100	1,300±148	1,785±355	2,810±1,123

[^{3}H]TdR was determined as in Table I. Geldanomycin was added at t = 0 together with OSM at the indicated concentrations. [^{3}H]TdR was added at t = 18 h and cells were harvested at t = 36 h.

in KS cells (5). We propose that the OSM receptor on KS cells consists of a specific OSM α-subunit which interacts with gp130, and that this receptor complex constitutes an important growth pathway. Other growth pathways must, however, also be considered because it has clearly been reported that the development of KS lesions involves a complex cascade of cytokines and other growth factors (2). For instance, other members of the cytokine receptor family, such as IL-11 and ciliary neurotrophic factor (CNTF) receptors, may play a role. In addition, the growth-promoting effects of HIV-1 TAT protein (9) and platelet-derived growth factor (7) on KS cells require consideration. Whether these growth factors, like OSM, activate a TPK and MAP kinase cascade in KS cells is unknown and worthy of further study.

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