TNF-α induced CD38 expression in human airway smooth muscle cells: role of MAP kinases and transcription factors NF-κB and AP-1

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TNF-α is a pleiotropic protein that has both enzymatic (34–36) and receptor functions (8, 9, 19, 38). It is an ~45-kDa glycosylated transmembrane protein, with a large extracellular domain that has an enzymatic role capable of generating cyclic ADP-ribose (cADPR) and ADP-ribose from nicotinamide adenine dinucleotide phosphate from NADP. In airway smooth muscle (ASM) cells, CD38 is predominantly localized in the plasma membrane (57), and the CD38/cADPR signaling has been shown to play a role in the regulation of intracellular calcium concentration ([Ca2+]i) (12) following activation of G protein-coupled receptors (12, 13, 58). In prior studies, we have shown that CD38 expression and its enzyme activities are increased by TNF-α, IFN-γ, IL-1β, and the T helper 2 (Th2) cytokine IL-13 (11, 12). TNF-α-induced CD38 expression is transcriptionally regulated and involves NF-κB-dependent and -independent mechanisms (30). The increase in CD38 expression is associated with augmented [Ca2+]i (12) responses, largely attributable to cADPR. In ASM cells obtained from CD38-deficient mice, the [Ca2+]i (12) responses to agonists are attenuated compared with responses in cells obtained from wild-type mice (14). CD38-deficient mice also exhibit attenuated methacholine-induced airway responsiveness. Following repeated IL-13 intranasal challenge, the CD38-deficient mice develop reduced airway hyperresponsiveness, a hallmark of asthma, compared with the wild-type mice (22). These results implicate CD38 in normal airway function and in airway hyperresponsiveness.

Proinflammatory agents such as cytokines (IL-4, IL-13, TNF-α, and IL-1β), growth factors, and contractile agonists are known to activate mitogen-activated protein kinases (MAPKs) (20, 28, 39, 42, 44, 55, 61). MAPKs are a family of serine/threonine kinases that are activated by a variety of stimuli and are involved in inflammation, cell cycle regulation, cell proliferation, and differentiation (17, 20). Three distinct members of MAPKs are known: the extracellular signal-regulated kinase (ERK, also called ERK MAPK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) (5, 7, 31). The activation of members of MAPKs is known: the extracellular signal-regulated kinase (ERK, also called ERK MAPK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) (5, 7, 31). The activation of the MAPKs results from a phosphorylation cascade starting from MAPK kinase kinase (MKK) and MAPK kinase (M KK or MEK), culminating in MAPK. Once activated, MAPKs regulate gene expression at transcriptional, posttranscriptional, and posttranslational levels. The transcriptional regulation through MAPKs involves transcription factors such as NF-κB and AP-1 either directly or indirectly through the activation of other protein kinases (7). Furthermore, they regulate gene expression posttranscriptionally through mRNA processing, modification of mRNA stability, and control of translation initiation (4, 10, 24, 56).

Our previous studies using a peptide inhibitor of NF-κB as well as IκB mutants showed a role for NF-κB in TNF-α-induced CD38 expression in human airway smooth muscle (HASM) cells (30). Furthermore, the inhibition of TNF-α-induced CD38 expression by the anti-inflammatory glucocorticoids appears to stem from the inhibition of NF-κB activation and other mechanisms. The signal transduction mechanisms and specifically the role of MAPKs in transcription factor-mediated regulation of CD38 expression are poorly understood. In the present investigation, we sought to determine the link between MAPKs and transcription factors in ASM CD38

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expression. Pharmacological inhibitors and molecular tools were used to modulate the various MAPK pathways in an attempt to delineate their role in this regulation. The results show a major role for the p38 and JNK MAPKs in transcriptional regulation through NF-κB and AP-1. The regulation through the ERK and p38 MAPKs also involves transcript stability.

MATERIALS AND METHODS

**Materials.** Tris base, glucose, HEPES, TNF-α, U0126, SB-203580, SP-600125, and other chemicals were purchased from Sigma Chemical (St. Louis, MO). HBSS and DMEM were purchased from Invitrogen-GIBCO-BRL (Carlsbad, CA). TRizol, Lipofectamine 2000, SuperScript III reverse transcriptase, and the 100-bp DNA ladder were purchased from Invitrogen. Lane marker sample buffer was obtained from Pierce (Rockford, IL). The SYBR Green Master Mix was purchased from Stratagene (Cedar Creek, TX). Dual-luciferase reporter assay system, pRL-cytomegalovirus (CMV) plasmid, GoTaQ Green Master Mix, and EMSA kit were obtained from Promega (Madison, WI). The nuclear extraction kit was purchased from Active Motif (Carlsbad, CA). pAP-1-luc plasmid was obtained from Clontech Laboratories (Mountain View, CA). pmux-green fluorescence protein (GFP) plasmid was obtained from Amaxa (Gaithersburg, MD). Dominant negative MEK (MEK2A) and wild-type ERK were provided by Dr. M. B. Hershenson (Univ. of Michigan, Ann Arbor, MI), and the dominant negative c-Jun (TAM67, pten plasmid vector, Invitrogen) was a kind gift of Dr. T. Korudula (Virginia Commonwealth Univ., Richmond, VA).

**HASM cell culture.** HASM cells maintained in culture were used in this study (with Institutional Review Board approval). HASM cells were isolated from trachealis muscle and propagated as described in earlier publications (2, 12, 43). The cells were plated at a density of 1.0 × 10⁶ cells/cm² and were cultured in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, 2 mM l-glutamine, and 0.25 μg/ml amphotericin B. For studies described below, HASM cells were growth arrested at G₀ in the cell cycle by maintaining for at least 48 h in arresting medium containing no serum, but in the presence of transferrin and insulin before TNF-α treatment for 24 h.

In preliminary experiments to delineate the role of the various MAPKs, growth-arrested cells were exposed to a range of inhibitor concentrations (1–25 μM) 30 min before treatment with TNF-α (40 ng/ml). Cytotoxicity was monitored by the MTT dye reduction assay (25). In subsequent experiments, we used the following concentrations of the inhibitors that resulted in no significant cytotoxicity based on the MTT assay: 15 μM U0126, a selective ERK inhibitor; SB-203580, a p38 MAP kinase inhibitor; and 25 μM SP-600125, a selective JNK inhibitor. These concentrations of the inhibitors are similar to those described in the literature (33, 39, 45). Preliminary experiments confirmed lack of MAPK activation in the presence of the inhibitors, as measured by Western blotting for the phosphorylated protein.

**DNA transfections.** Transient transfections were performed with Lipofectamine 2000 according to the manufacturer’s instructions. Cells (0.5–2 × 10⁶) in 500 μl of growth medium without antibiotics were plated a day before transfection. For each transfection, 0.8 μg of the vector DNA and 2 μl of Lipofectamine 2000 in 50 μl of Opti-MEM were mixed gently and incubated for 5 min at room temperature. Complexes were formed by mixing the diluted DNA and Lipofectamine and incubating for an additional 20 min at room temperature. The complexes were added to each well and incubated at 37°C, and the medium was changed after 6 h. The cells were growth arrested 24 h following transfection for 24 h before being exposed to TNF-α. The cells were collected for total RNA extraction, RT-PCR, and real-time quantitative PCR (see below).

**RT-PCR.** CD38 expression was determined by RT-PCR, as described in earlier publications (29, 30). Briefly, total cellular RNA was isolated from control, TNF-α-treated cells, and cells treated with TNF-α in the presence of the inhibitors, or transiently transfected cells with TRizol following the manufacturer’s protocol. RNA was quantified using a Nanodrop Spectrophotometer (Wilmington, DE), and equal amounts from the different samples were used for the RT-PCR. The RT reaction was carried out using SuperScript III reverse transcriptase as per the manufacturer’s instructions. Human CD38 and GAPDH were amplified by using the following primer sets: CDP99F (5'-TAAAGCCCTGTGCGGTCTC-3') and CDP99R (5'-GACATCGGCGGAGCAGGTCTC-3'), GAPDHF (5'-GAAGAGGA-AGTCCGAGTC-3') and GAPDHR (5'-GAAGATGTTCGTCG- GATTTC-3'), respectively, with GAPDH as internal controls. The PCR was performed under the following conditions: 94°C for 3-min denaturing, 30 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products for the transient transfection and transcript stability experiments were separated on 1.2% agarose gel and identified by ethidium bromide staining. The band intensities of scanned gel photographs were determined by using LABWORKS Image acquisition software (UVP, Upland, CA). Normalization of CD38 expression in all experiments was achieved by comparing the expression of GAPDH to the corresponding sample.

**Quantitative real-time PCR.** Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix as described previously (29, 30). cDNA obtained by reverse transcription of total RNA from the different samples was amplified using the SYBR Green Master Mix and CD38 or GAPDH specific primers. The reactions were performed in the Stratagene Mx3000p sequence detection system under the following conditions: 95°C for 10 min, 45 cycles at 95°C for 30 s, 59°C for 30 s, and 72°C for 45 s. All samples were run in duplicates, and the readings were normalized using non-template control and passive reference dye included in the SYBR Green Master Mix. Normalized fluorescence was plotted against the cycle number (amplification plot), and the threshold suggested by the software was used to calculate Ct (cycle at threshold). Real-time PCR controls were expressed as fold change (2⁻ΔΔCt) in expression in the cells after subtraction of internal GAPDH control and expressed relative to the levels in untreated control cells. The expression levels following TNF-α stimulation in the different preparations were normalized to 100%, and the expression in the presence of the inhibitors were shown as % decrease compared with TNF-α treatment.

**ADP-ribosyl cyclase assay.** The ADP-ribosyl cyclase activity of HASM cell lysates was quantified using a fluorescent cycling assay that measures the production of NAD from cADPR and nicotinamide with modifications (21). Briefly, HASM cells were harvested in Tris-sucrose buffer (pH 7.2) with protease inhibitors on ice and sonicated, and the total protein content of the cell lysates was determined by a Bio-Rad protein assay kit. Cell lysates containing 20 μg of total protein were incubated for 2 h at 37°C with 10 mM or without nicotinamide in the presence of 0.45 mM cADPR in a total volume of 50 μl. The reaction was stopped by the addition of 25 μl of 1 M HCl, vacuum filtered through protein-binding membrane (Immobilon, 0.45 μm, Millipore), and neutralized with 15 μl of 2 M Tris-base. The NAD in the filtrate was quantified by a cycling reaction that generates a fluorescent product. Forty microliters of the neutralized filtrate was incubated with 40 μl of reagent mix (2 μM reasurin, 0.76% vol/vol ethanol, 4 μM flavin mononucleotide, 40 μg/ml alcohol dehydrogenase, and 0.04 U/ml diaphorase in NaH₂PO₄/Na₂HPO₄ buffer, pH 8.0) at room temperature. The fluorescence was quantified (excitation at 544 nm and emission at 590 nm) in a fluorometer (FLUOstar Galaxy, BMG Biotechnologies), and the rate of emission of fluorescence was calculated. A standard curve generated from known NAD standards was used to derive the quantity of NAD generated in the reverse cyclase reaction. The ADP-ribosyl cyclase activity in femtomoles of NAD per hour per milligram of total protein is expressed as % of activity in TNF-α-treated cells.
mRNA stability determination. HASM cells grown arrested for 48 h were treated with 40 ng/ml TNF-α for 12 h in the presence or absence of the MAPK inhibitors at the concentrations mentioned above and incubated for an additional 12 h in the presence of 5 μg/ml actinomycin D in the absence of TNF-α. The cells were collected for RNA extraction and reverse transcribed with oligo-dT, and CD38 expression was determined as described above at various time points (0, 3, 7, and 12 h) following the addition of actinomycin D.

Luciferase reporter gene transactivation assay. Transient transfection with pAP-1 luciferase reporter plasmid was performed with Lipofectamine 2000 as described above. HASM cells were transfected with a 50:1 ratio of the luciferase reporter plasmid and pRL-CMV plasmid. Reporter gene assays were performed 24 h after transfection. Cell lysates were subjected to dual-luciferase reporter assay system, and luciferase activities were measured with a luminometer (Lumat LB9507; Berthold, Bad Wildbad, Germany). Firefly luciferase activities were normalized to Renilla luciferase activity to normalize for transfection efficiency. In each experiment, samples were analyzed in triplicate, and each experiment was repeated at least twice.

Nuclear protein extraction. Nuclear extracts were prepared from growth-arrested HASM cells grown to confluence in a 100-mm dish using the nuclear extraction kit from Active Motif (Carlsbad, CA). Cells were pretreated with the MAPK inhibitors for 30 min and exposed to TNF-α for 1 h. The media was aspirated and washed with ice-cold PBS containing phosphatase inhibitors, and the cells were scraped in 5 ml of the same buffer. The cells were pelleted at 1,000 g for 5 min, and the supernatant was discarded. The cells were resuspended in 500 μl of 1× hypotonic buffer by pipetting several times, transferred to a prechilled microcentrifuge tube, and incubated for 15 min on ice. Detergent (25 μl) was added, vortexed for 10 s, and centrifuged at 14,000 g for 30 s at 4°C. The supernatant was removed, and the nuclear pellet was resuspended in 50 μl of complete lysis buffer and vortexed for 10 s. The mixture was incubated on ice for 30 min, vortexed briefly, and centrifuged at 14,000 g for 10 min at 4°C. The supernatant (nuclear fraction) was aliquoted, and protein content was measured and stored at –80°C until use.

EMSA. Protein concentration in the nuclear extract was quantitated using the Bradford protein assay (Bio-Rad, Hercules, CA). EMSA was performed as described earlier (30). The double-stranded oligonucleotide containing the consensus binding sites for NF-κB (5’-AGGGTACCGCCGAGGTCA-3’) and AP-1 (5’-GAGCTTCTCGAGGAGTCAGCCGGAA-3’) were labeled with [γ-32P]ATP (3,000 Ci/mmol) by T4 polynucleotide kinase (Promega). Nuclear extracts (5 μg) were incubated for 30 min at room temperature with 0.4 pmol of double-stranded 32P-labeled oligonucleotide containing the consensus binding sites in a total volume of 10 μl in a buffer containing 20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5), and 0.25 mg/ml poly(dI-dC). After 30 min at room temperature, samples were separated on a nonreducing 4% polyacrylamide gel using TBE buffer (0.5 M, containing 107.8 g Tris-base, 55 g boric acid, and 7.44 g disodium EDTA-2H2O in 1 l of water). The gels were dried and autoradiographed with intensifying screens at ~70°C. To confirm specificity of the EMSA, competition assays were performed with a 100-fold excess of unlabeled NF-κB or AP-1 probe or the SP1 probe as a nonspecific competitor.

Statistical analysis. HASM cells isolated from at least three different donors were used in the experiments. The experiments involving EMSA and transient transfections of the constructs were repeated three times. Statistical analyses were performed using the GraphPad PRISM statistical software. The quantitative PCR results and ADP-ribosyl cyclase activities in the various samples were compared by one-way ANOVA with Bonferroni’s test for multiple comparisons. Two means were considered significantly different when P value was <0.05. Half-lives (in minutes) of mRNA were determined by nonlinear regression analysis of mRNA remaining vs. time data.

RESULTS

TNF-α-induced activation of ERK, p38, and JNK MAPK and CD38 expression in HASM cells. Prior investigations have demonstrated that TNF-α-induced CD38 expression in HASM cells involves NF-κB-dependent and -independent mechanisms of transcriptional regulation (30). In the present study, we designed experiments to explore the signaling mechanisms involved in this regulation. Following treatment of growth-arrested HASM cells with TNF-α, CD38 expression increased at 24 h (Fig. 1A). Exposure of growth-arrested HASM cells to TNF-α caused activation of the MAPKs as detected in Western blots of their phosphorylated forms in the cell lysates (not shown), confirming previous observations (42). To determine the role of MAPKs in the regulation of CD38 expression, cells were pretreated with selective inhibitors of the three major MAPKs and exposed to TNF-α, and CD38 expression was measured by quantitative real-time PCR. There was a significant decrease in CD38 expression in the presence of 15 μM U0126 or SB-203580 or 25 μM SP-600125, inhibitors of ERK, p38, and JNK MAPKs, respectively (Fig. 1, B and C). In the combined presence of subinhibitory concentrations (5 μM) of U0126 and SB-203580, there was a decrease in CD38 expression comparable to that observed in the presence of the higher concentration of the inhibitors (data not shown).

Earlier observations indicated that in HASM cells, TNF-α-induced increased ADP-ribosyl cyclase activity is maintained for a long duration following removal of the cytokine, suggesting a slow turnover of the CD38 protein (30). To determine whether MAPKs have an effect on CD38 protein content, we measured ADP-ribosyl cyclase activity by the reverse cyclase assay in lysates obtained from cells following treatment with TNF-α in the presence of the MAPK inhibitors. Pretreatment with U0126, SB-203580, or SP-600125 significantly decreased ADP-ribosyl cyclase activity in the cell lysates compared with enzyme activity in cells treated with TNF-α alone (Fig. 1D). The decrease in the CD38 enzyme activity in the presence of the inhibitors paralleled the decrease in CD38 transcript levels.

To demonstrate specificity in the regulation of CD38 expression by the ERK MAPK, we used a dominant negative MEK2A or wild-type ERK constructs. Cells were transiently transfected with the plasmids, and CD38 expression was measured 24 h following treatment with TNF-α. Figure 2A shows the typical transfection efficiency of MEK2A and wild-type ERK in HASM cells as evidenced by GFP (using the pmax-GFP vector). The transfection efficiency assessed by counting the number of GFP-positive cells in a given field was ~70% of the total cell population. Western blot analysis of cell lysates for phospho-42/44 levels revealed a decrease in the cells transiently transfected with the MEK2A and an increase in the cells transfected with the wild-type ERK upon exposure to TNF-α (Fig. 2B). There was a significant and comparable increase in CD38 expression in cells transfected with the wild-type ERK and vector controls on exposure to TNF-α (Fig. 2C). In MEK2A-transfected cells, TNF-α-induced CD38 expression was significantly attenuated (Fig. 2D), confirming a role of ERK in this process.
MAPK-mediated activation of NF-κB and AP-1. MAPKs are known to mediate their effects in a variety of cell types through the activation of NF-κB and AP-1 (5, 7). In a previous study, we provided evidence for NF-κB activation and NF-κB-dependent transcriptional regulation of CD38 expression in HASM cells (30). In the present study, we measured AP-1 activation in HASM cells following exposure to TNF-α. Cells were transfected with a pAP-1-luciferase plasmid and exposed to TNF-α, and AP-1 activation was measured at different time points. AP-1 activation increased in response to TNF-α to a peak level at 6 h (Fig. 3A). To determine whether AP-1 activation is required for TNF-α-induced CD38 expression, HASM cells were transfected with a dominant negative c-Jun (TAM67). TAM67 transfection attenuated TNF-α-induced CD38 expression compared with expression in vector-transfected cells (Fig. 3C).

We next examined the role of ERK, p38, and JNK MAPKs in mediating the activation of NF-κB and AP-1. EMSAs were carried out with nuclear extracts obtained from cells following exposure to TNF-α and the MAPK inhibitors (Fig. 4). TNF-α-induced NF-κB activation was decreased in cells treated with p38 and JNK inhibitors (Fig. 4A). TNF-α-induced AP-1 activation also decreased in the presence of the JNK inhibitor, and to a lesser extent in the presence of the p38 inhibitor (Fig. 4B). In cells treated with the ERK inhibitor U0126, there was no decrease in the activation of NF-κB or AP-1 following exposure to TNF-α (Fig. 4). In fact, we often found an increase in the activation of the two transcription factors in the presence of U0126. The inhibition of CD38 expression by U0126 in the absence of changes in NF-κB and AP-1 activation in response to TNF-α suggests that ERK-mediated regulation of CD38 expression involves other mechanisms (see below).

MAPKs and mRNA stability. In addition to their role in the transcriptional regulation of gene expression, MAPKs have been implicated in posttranscriptional regulation involving transcript stability (4, 10, 24, 27). We designed experiments to assess the role of MAPKs in CD38 transcript stability following induction with TNF-α. Cells were exposed for 12 h to TNF-α in the absence or presence of the MAPK inhibitors, washed, and replaced with fresh medium containing actinomycin D to inhibit further transcription. CD38 expression was determined at 0, 3, 7, and 12 h. In TNF-α-treated cells, the CD38 expression was maintained significantly above the basal levels over the 12-h period following removal of cytokine (Fig. 5). In cells treated with U0126 and SB-203580, there were significant and rapid decreases in the transcript levels. However, in cells treated with the JNK MAPK inhibitor, the decline in message level was similar to that seen in cells treated with TNF-α alone (Fig. 5). The approximate half-lives of the CD38 transcripts calculated by nonlinear regression of the mRNA remaining vs. time plots were 100, 30, 3, and 100 min for cells treated with TNF-α alone or TNF-α in the presence of U0126, SB-203580, and SP-600125, respectively. These results suggest a differential role of MAPKs involving transcriptional and posttranscriptional mechanisms of regulation of CD38 expression.
DISCUSSION

In the present study, we demonstrate that TNF-α-induced CD38 expression in HASM cells involves all three MAPKs and the transcription factors NF-κB and AP-1. Inhibition of NF-κB and AP-1 activation results in decreased CD38 expression, implicating both transcription factors in this regulation. The mechanisms by which the MAPKs mediate their effects on the regulation of CD38 expression are quite distinct. The regulation of CD38 expression through activation of NF-κB and AP-1 involves both the p38 and JNK MAPKs. We also demonstrate that the ERK and p38 MAPKs have a role in the stability of CD38 transcripts. Thus in HASM cells there is evidence for both transcriptional and posttranscriptional regulation of CD38 expression by the inflammatory cytokine TNF-α.

Prior investigations have shown that TNF-α is present in very high levels in human asthmatics and appears to be a marker of inflammation (6, 48, 50). The pleiotropic effects of TNF-α in HASM cells are mediated principally through the TNFR1, although there is also evidence for the expression of TNFR2 (2). TNF-α mediates the different synthetic properties of ASM cells such as secretion of proinflammatory molecules.
growth factors, and adhesion molecules through the TNFR1 (2, 42). Studies from our laboratory and others have shown that TNF-α potentiates the \([\text{Ca}^{2+}]_i\) responses of ASM cells to a variety of agonists (12, 52). The CD38/cADPR signaling has a major contribution in the augmentation of the calcium responses to agonists in HASM cells following exposure to TNF-α, other proinflammatory cytokines, and IL-13 (11, 12, 51). Proinflammatory cytokines, growth factors, and contractile agonists are known to activate the MAPKs (20, 28, 39, 42, 44, 55, 61). In animal models of allergic airway inflammation, the activities of the MAPKs appear to be increased, and inhibition of the MAPK signaling is therefore expected to ameliorate airway inflammation (37). In this context, previous investigations have shown that ovalbumin-induced eosinophil infiltration, mucus production, Th2 cytokine production, and airways, and airway hyperresponsiveness (AHR) are attenuated in mice with transgenic overexpression of a dominant negative Ras, suggesting a role for the Ras-Raf-MEK-ERK signaling in airway inflammation (17, 40, 41, 46, 47). Other investigators have shown that the MEK1/2 inhibitor U0126 abrogated ovalbumin-induced airway eosinophilia, mucus production, Th2 cytokine and eotaxin production, serum IgE levels, airway expression of VCAM-1, and AHR in a mouse model of allergic asthma (16). In lung lysates obtained from U0126-treated mice, there is downregulation of ERK1/2 tyrosine phosphorylation (16). Similar findings in terms of attenuation of allergen-induced airway inflammation and AHR have been reported following treatment with the p38 and JNK MAPK inhibitors and p38α MAPK oligonucleotides (15). These results together provide evidence for MAPKs in regu-
lating the activation and function of ASM cells and other airway resident cells during inflammation. Earlier investigations showed that HASM cell proliferation induced by growth factors, thrombin, or phorbol esters is mediated through activation of ERK, whereas the proinflammatory cytokines such as TNF-α and IL-1β induce the activation of the JNK and p38 MAPKs (42). Studies in bovine tracheal myocytes also confirm the role of ERK in the regulation of expression of cyclin D1, which is involved in cell-cycle progression (44). The role of ERK and p38 MAPKs in TNF-α-induced secretion of ICAM-1, RANTES, and IL-6 from HASM cells (1–3) and IL-6 gene expression (53) has also been demonstrated. These studies showed that inhibition of the ERK resulted in a significant reduction of ICAM-1 expression, with negligible effects on the secretion of RANTES or IL-6. However, inhibition of the p38 MAPK resulted in decreases in the secretion of RANTES and IL-6 following exposure to TNF-α. These results demonstrate MAPK-dependent and -independent signaling mechanisms in the secretory effects of TNF-α in HASM cells. MAPKs appear to mediate the synthetic effects of not only inflammatory cytokines, but also G protein-coupled receptor agonists (20, 32, 42). Evidence for the differential activation of the various MAPKs by inflammatory cytokines also comes from studies on the regulation of expression of the CXC chemokine, GRO-α, in HASM cells (28). This study by Issa et al. (28) clearly showed that the ERK and JNK, but not the p38 MAPKs, activate

Fig. 4. MAPK inhibitors decrease NF-κB and AP-1 activation following exposure to TNF-α. EMSAs demonstrating binding of NF-κB and AP-1 to consensus oligonucleotide probes. Nuclear proteins were extracted from HASM cells treated with TNF-α in the presence of SP-600125 (JNK inhibitor), SB-203560 (p38 inhibitor), or U0126 (ERK inhibitor). The specific complexes formed can be competed by 100-fold molar excess of unlabeled specific oligonucleotide probes (NF-κB consensus or AP-1). A nonspecific oligonucleotide probe (SP1) was used as an internal control. A: representative EMSA for NF-κB binding to consensus oligonucleotide sequences. Note the increased NF-κB activation by TNF-α, which is decreased in the presence of the p38 and JNK inhibitors. Note no significant decrease in binding in the presence of the ERK inhibitor. B: representative EMSA for AP-1 binding to consensus oligonucleotide sequences. Left shows increased AP-1 activation by TNF-α, which is decreased by the JNK inhibitor and to a lesser magnitude by the p38 inhibitor. Right shows the effect of ERK inhibitor on TNF-α-induced AP-1 activation. Note no change in the presence of U0126. These results are representative of 6 experiments.
Fig. 5. MAPKs and CD38 transcript stability. HASM cells were treated with TNF-α in the presence of U0126, SB-203580, or SP-600125 for 12 h. Cells were washed, replaced with fresh media containing 5 μg/ml actinomycin D to inhibit further transcription, and collected at 0, 3, 7, and 12 h for the determination of CD38 mRNA expression. A: gel images showing CD38 mRNA expression following addition of actinomycin D in control cells or in cells treated with TNF-α in the presence of the MAPK inhibitors. GAPDH expression is used as an internal control. B: plot of CD38 mRNA remaining vs. time following addition of actinomycin D. The band intensities of the control samples were subtracted from the treated samples at different time points, and the resulting values were normalized to GAPDH levels. Note the rapid decline in the mRNA levels in the presence of p38 and ERK inhibitors, with short half-lives determined by nonlinear regression. The half-life of the transcript in the cells treated with SP-600125 was similar to that in cells treated with TNF-α. Inset shows mRNA remaining over time as point-to-point line diagram with initial mRNA expression normalized to 100%. Representative of 3 experiments.
decreased nuclear translocation of the transcription factor in the presence of SB-203580 and SP-600125, respectively. The decreased activation of AP-1 in the presence of SP-600125 and SB-203580 indicates the role of JNK and p38 MAPKs as well. However, other potential mechanisms such as decreased expression, dimerization, or decreased transcriptional activities of AP-1 and NF-κB may also contribute to the decreased CD38 expression. A recent study by Iqbal et al. (27) on the regulation of CD38 expression in osteoclasts showed protein kinase C as an important upstream activator of NF-κB in the response to TNF-α. Regulation through the JNK, but not the other MAPKs, appears to have a major role in TNF-α-induced CD38 expression in this system. Our results also suggest that the p38 and JNK MAPKs are upstream activators of the transcription factors, although direct evidence for this has not been presented. The present study showed a significant decrease in CD38 expression on ERK inhibition, although there was increased NF-κB or AP-1 activation. Furthermore, any potential increase in activation of the other MAPKs resulting in increased CD38 expression would have been masked by the effect of U0126 on mRNA stability. The role of other transcription factors in mediating the effects of ERK cannot be discounted. The possibility that ERK may modulate NF-κB and AP-1 transactivation at a different step, as has been suggested for VCAM-1 expression in HASM cells (55), cannot be ruled out either. Our results are indicative of regulation of CD38 expression through posttranscriptional mechanisms.

There is evidence for the control of gene expression in eukaryotic cells through MAPKs involving stability of mRNA (26). The kinetics of mRNA decay involves the interaction of adenylate/uridylylate-rich elements (AREs) in the 3'-UTR with a number of ARE-binding factors (59, 60, 62). In HASM cells, previous investigations have shown that IL-17A via the p38 MAPK increases the stability of TNF-α-induced IL-6 and IL-8 mRNA (23, 24). The authors demonstrated that IL-17A alone did not induce detectable levels of IL-8, but potentiated TNF-α-induced IL-8 gene expression and secretion through stabilizing the transcripts, which were attributed to activation of the p38 MAPK. In silico analysis of the cloned 3'-UTR of CD38 mRNA reveals similar AREs capable of influencing transcript stability (unpublished observations). The decrease in CD38 expression without concomitant changes in transcription factor activation clearly supports a role for ERK in transcript stability. In addition, our results also demonstrate a role for p38 MAPK in transcript stability. The results of the present investigation provide evidence for both transcriptional and posttranscriptional regulation of CD38 expression in HASM cells. The transcriptional regulation involves NF-κB and AP-1 through the p38 and JNK MAPKs. The regulation through ERK and p38 MAPKs also involves transcript stability. A model describing the role of the MAPKs and the mechanisms by which they regulate CD38 expression is presented in Fig. 6. The role of other transcription factors in mediating effects of MAPKs remains to be determined.

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