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MAPK p38 Regulates Transcriptional Activity of NF-κB in Primary Human Astrocytes via Acetylation of p65

Ramendra N. Saha, Malabendu Jana, and Kalipada Pahan

MAPK-p38 plays an important role in inflammation. Several studies have shown that blocking p38 activity attenuates the transcriptional activity of the proinflammatory transcription factor NF-κB without altering its DNA-binding activity. We have also observed that blocking p38 in human primary astrocytes suppresses the transcriptional but not the DNA-binding activity of NF-κB and down-regulates the expression of an NF-κB-dependent gene, inducible NO synthase. However, the molecular mechanism of p38-mediated regulation of NF-κB remains largely unknown. In this study, we delineate that p38 controls the transcriptional activity of NF-κB by regulating acetylation of p65, but not its phosphorylation. The combination of IL-1β and IFN-γ, previously shown to strongly induce inducible NO synthase in human primary astrocytes, induced p38-dependent phosphorylation of acetyltransferase coactivator p300, but not p65, and subsequent association of p300 with p65. Furthermore, immunocomplex-histone acetyltransferase assays demonstrated that cytokine-induced association of p65 with biologically active immunocomplex-histone acetyltransferase assay was dependent on p38. It has been previously reported that acetylation of p65 at K310 residue is important for transcriptional activity of NF-κB. Accordingly, we found that cytokine-induced association of p65 with p300 led to acetylation of p65 at K310. Because p38 regulated the association between p65 and p300, blocking p38 activity also led to attenuation of p65-K310 acetylation in cytokine-stimulated astrocytes. Taken together, this study illuminates a novel regulatory role of p38 during neuroinflammation where this MAP kinase controls acetylation of NF-κB p65 by regulating acetyltransferase activity of coactivator p300.


NF-κB is one such transcription factor. It is a family of five proteins (RelA/p65, RelB/p56, cRel/p75, p52, and p50), which dimerize with self or other members in the family and play an important role in the transcription of proinflammatory molecules (4). Classically, NF-κB refers to the p65:p50 complex. This prototypical complex is retained in the cytoplasm by IκB in an inactive form. The activation process of this transcription factor may be divided into three phases: events in the cytoplasm, nuclear translocation, and events in the nucleus. The first phase involves signal-dependent activation of IκB kinase, which phosphorylates IκB on two N-terminal residues leading to its proteosomal degradation and subsequent NF-κB release (4, 5). In the second phase, liberated NF-κB binds to importins and is ferried across nuclear pore complexes in the nuclear membrane (6). The third phase of nuclear events includes association of NF-κB with coactivators to form the transcription complex. This association depends on and also results in certain posttranslational modifications of NF-κB (7). Acetylation of NF-κB p65 is one such major modification. NF-κB p65 is acetylated at multiple lysine residues, each manifesting site-specific biological outcomes (8, 9). For example, acetylation of K310 enhances transcriptional activity of p65 complexes (10).

Nuclear acetylation of NF-κB depends on coactivators, such as p300 and CREB-binding protein (CBP) (7). These coactivators, in addition to serving as protein bridges between transcription factors like NF-κB and the RNA polymerase II-associated transcriptional machinery, also serve as acetyltransferases. They acetylinate chromatin subunits and members of the transcriptional complex (11). Previously, p300 has been shown to mediate inflammatory responses (12–14), which it achieves in part by acetylating proinflammatory factors like NF-κB (15, 16). Interestingly, acetylation of NF-κB-p65 at K310 occurs after it is phosphorylated (15, 16). In fact, in addition to acetylation, optimal transcriptional output of NF-κB relies on phosphorylation of p65 at several residues including, two key serine residues at S276 and S536 (17). Phosphorylation of p65 permits binding of NF-κB with DNA and p300/CBP.

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Abbreviations used in this paper: iNOS, inducible NO synthase; hr, human recombinant; siRNA, small interfering RNA; DAPI, 4′,6-diamidino-2-phenylindole; HAT, histone acetyltransferase; MSK1, mitogen- and stress-activated protein kinase 1; CBP, CREB-binding protein.

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The phosphate accepting p65 serine residues, which may be phosphorylated in the cytoplasm or nucleus, serve as downstream targets for several upstream kinases in a signal-specific manner (17). In numerous instances, p38 MAPK (p38) has been cited as an upstream NF-κB regulatory kinase.

The p38 MAPK is a family of serine/threonine kinases that form an integral component of proinflammatory signaling cascades in various cell types (19) including, astroglial cells (20). Blocking p38 with specific inhibitors diminishes NF-κB-driven transcriptional activity and attenuates the expression of NF-κB target genes (21–24). However, nuclear translocation or DNA-binding activity of NF-κB is not contingent on p38 activity (24–26). Similar incidents have been reported previously in nonastroglial cells as well (27–29). The molecular mechanism responsible for such selective regulation of NF-κB by p38 is however, yet elusive.

Because p38 regulates transcriptional activity of NF-κB, and because transcriptional activity of NF-κB is dependent on coactivators, we hypothesized that p38 may exert its effect on transcriptional activity of NF-κB by regulating its coactivators. In this study, we tested this hypothesis in human primary astrocytes using cytokines capable of triggering the expression of an NF-κB-dependent gene INOS. In the current article, we report that p38 dictates transcriptional activity of p65 indirectly by regulating its acetylation via phosphorylation-coupled acetyltransferase activity of p300.

Materials and Methods

Reagents

Abs were purchased as follows: Anti-p65 (sc-372X) and anti-LaminB (sc-6217) from Santa Cruz biotechnology, anti-phospho-S276-p65 (P-S276-p65) and anti-phosphoserin from Abcam (ab9332 and ab2615), anti-phospho-S536-p65 (P-S536-p65) and anti-p38α from Cell Signalling Technology (3033 and 9217), and p300 specific monoclonal anti-p300 from Upstate Biotechnology (05–257). SB203580 (SB) was obtained from Calbiochem, while human recombinant (hr) TNF-β from Upstate Biotechnology (3033 and 9217), and p300 specific monoclonal anti-p300 were purchased from R&D Systems. The A3000 expression plasmid was purchased from Upstate Biotechnology. Short interfering RNA (siRNA) against p38α was purchased from Ambion.

Astrocytes

Human primary astrocytes were prepared and maintained in culture as described previously (30). In brief, cells were obtained from fetal brain tissue (Human Embryology Laboratory, University of Washington, Seattle, WA). These cells were grown in a serum-free, defined medium (B16) enriched with 5 ng of basic fibroblast growth factor per milliter for optimal growth of astrocytes and for the suppression of fibroblast growth. By immunofluorescence assay, these cells homogeneously expressed GFAP. Cells plated at 50–60% confluence were transfected with plasmid of interest by Lipofectamine Plus (Invitrogen Life Technologies) following the manufacturer’s protocol. For promoter reporter assays, cells were co-transfected with either pBIX-Luc (NF-κB-driven) or phNOS(7.2)Luc (an 7.2-kb human iNOS promoter-driven) (30) and pRL-TK (Renilla luciferase control) as described previously (31). Firefly and Renilla luciferase activity was determined by Dual Luciferase Kit (Invitrogen Life Technologies) as described previously (30, 31). In brief, cells were taken out of the culture dishes directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories) according to the manufacturer’s instructions. For Northern blot analysis, 20 μg of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels, electro-transferred to Hybond-Nylon Membrane (Amersham Biosciences) and hybridized at 68°C with [32P]-labeled cDNA probe using Express Hyb hybridization solution (Clontech) as described by the manufacturer. The cDNA probe was made by PCR amplification using two primers (forward primer, 5′-CTC CTT CAT AGA GCC AAA AAT A 3′; reverse primer, 5′-CAC TTC CTC CAG GAT GTT GT-3′). After hybridization filters were washed two or three times in solution I (2 × SSC, 0.05% SDS) for 1 h at room temperature followed by solution II (0.1X SSC, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed to x-ray films (Kodak). The same amount of RNA was hybridized for probe for GAPDH (32, 33).

Immunocprecipitation and Western blotting

Nuclear lysate was prepared from treated cells by first liberating the nuclei in a nonionic detergent buffer) 10 mM HEPES (pH 7.9), 10 mM KCl 2 mM MgCl2, 0.5 mM dithiobitol, 0.1% Nonidet P-40) and subsequently lysing them in NETN buffer (0.5% Nonidet P-40, 1 mM EDTA, 50 mM Tris, 120 mM NaCl (pH 7.5)) freshly supplemented with 0.5% protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor mixtures (Sigma-Aldrich). Samples with equal protein load were precleared using slurry of protein A/G beads (Santa Cruz Biotechnology). Subsequently, 4 μg of desired Abs was added and samples were incubated overnight at 4°C with gentle rocking. Ab-Ag complexes were collected with A/G beads, washed thrice with NETN buffer and separated by electrophoresis on 4–12% gradient gels (Invitrogen). Western blotting was performed as described previously (30). Immunoblots were visualized with Odyssey infrared fluorophore tagged secondary Abs (Jackson ImmunoResearch), and observed under a BioRad MRC1024ES confocal laser-scanning microscope. 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen Life Technologies) was added during the final wash step at a dilution of 1/1000.

Immunocomplex histone acetyltransferase (HAT) assay

For serum starvation, astrocytes were maintained in serum-free medium for 6 h. Then, these cells were treated with SB for 1 h, followed by cytokine stimulation for another hour. Subsequently, nuclear lysate was obtained as stated above and immunoprecipitation was performed with anti-p300 or anti-p65 Abs. After several washes, Ag-Ab complex was diluted in HAT assay buffer and activity of HAT was measured in immunocomplex by a HAT assay kit (Upstate Biotechnology) using [3H] acetyl-CoA and histone H4 peptide.

Immunocomplex metabolic-phospho-labeling assay

Astrocytes were starved of phosphate and serum for 6 h by maintaining them in a phosphate and serum-free medium. Then, the cells were treated with SB for 30 min followed by replenishment with 100 μCi/ml [32P]orthophosphate (PerkinElmer). After another 30 min of incubation, cells were stimulated with the cytokine mixture. After 1 h of stimulation,
nuclear lysate was obtained as stated above and immunoprecipitation was performed with anti-p300 Abs. The immunoprecipitate was then separated by electrophoresis. The gel was dried and exposed to autoradiogram film. To verify equivalent loading, the gel was rehydrated and subsequently immunoblotted with anti-p300.

Results

Transcriptional, but not DNA-binding activity of NF-κB is sensitive to MAPK p38 inhibitor

The nuclear translocation, DNA-binding, and transcriptional activity of NF-κB, despite being mutually dependent, may rely on independent regulatory elements. We investigated the role of p38 MAPK in each of these processes by using SB203580 (SB), a specific inhibitor for the p38. Primary human astrocytes were stimulated with major proinflammatory cytokines, TNF-α, IL-1β, and a combination of IL-1β and IFN-γ (IL-IF), and NF-κB DNA binding was assessed by EMSA. As evidenced from Fig. 1A, all three treatments induced the DNA-binding activity of NF-κB (Promega Probe) and supershift assay. B, Nuclear lysate obtained from serum-starved cells pretreated with 10 μM of SB followed by IL-IF treatment for various time periods was immunoblotted with Abs against p65 and LaminB. C, Cells were cotransfected with 10 ng of pRL-TK and 0.2 ng of pBIIX-Luc using Lipofectamine Plus as described under Materials and Methods. After 24 h of transfection, cells were incubated with different concentrations of SB for 1 h followed by stimulation with IL-IF for 6 h. Firefly and Renilla luciferase activities were analyzed in total cell extract. D, Cells were cotransfected with 0.2 μg of phiNOS(7.2)Luc (an 7.2-kb human iNOS promoter-driven reporter construct) and 10 ng of pRL-TK (transfection efficiency control). After 24 h of transfection, cells were incubated with different concentrations of SB for 1 h followed by stimulation with IL-IF for 12 h. Firefly and Renilla luciferase activities were analyzed in total cell extract. Data are mean ± SD of three different experiments. * p < 0.001 vs IL-IF. E, Cells pretreated with different concentrations of SB were stimulated with IL-IF under serum-free condition. After 12 h of stimulation, total RNA was isolated and Northern blot analysis for iNOS mRNA was conducted as described under Materials and Methods. F, Cells were treated as in (E) and whole cell lysate was obtained after 24 h which was used to detect protein level of iNOS by Western blotting. Blots were reprobed for Actin. G, Astrocytes were transfected either with 100 nM si-C or si-p38α. After 48 h, whole cell lysate was obtained and level of p38α and actin was detected by Western blotting. H, Cells were transfected either with 100 nM si-C or si-p38α. After 48 h, they were treated with IL-IF for an hour and nuclear extract was prepared, which was used to perform EMSA (Licor Biosciences probe). I, Astrocytes were transfected as in H. After 48 h they were treated with IL-IF for an additional 18 h. Then, whole cell lysate was obtained and was used to detect protein level of iNOS by Western blotting. Blots were reprobed for actin. All results are representative of at least three independent trials.
and the DNA-binding activity of NF-κB, SB inhibited TNF-α-, IL-1β-, and IL-IF-induced transcriptional activity of NF-κB as evidenced by NF-κB-driven luciferase activity (Fig. 1C). These results suggest that transcriptional activity but neither nuclear translocation nor DNA-binding activity of NF-κB is sensitive to inhibition of p38.

To reflect this contingency on a bona fide proinflammatory NF-κB-target gene, we selected iNOS as our gene of interest. We have previously reported that, among several proinflammatory cytokines, a combination of IL-1β and IFN-γ (IL-IF) was found to be the most efficient inducer of iNOS in human primary astrocytes (30). Therefore, we have used this cytokine combination throughout this study. Transcriptional activity of the iNOS promoter was assessed with the previously described iNOS promoter reporter construct (ph-iNOS(7.2) luciferase) (30). As described earlier (30), IL-IF induced iNOS promoter-driven luciferase activity in human primary astrocytes (Fig. 1D). However, SB markedly inhibited (IL-IF)-induced activation of iNOS promoter (Fig. 1D). This observation was further corroborated with Northern blotting, which showed a marked reduction in iNOS mRNA level after SB pretreatment in a dose-dependent fashion (Fig. 1E). This further suggests that SB-induced block is a pretranslational phenomenon. To confirm further, we detected protein level of iNOS after cytokine stimulation in the presence or absence of SB. As shown in Fig. 1F, SB dose-dependently inhibited (IL-IF)-induced expression of iNOS protein in astrocytes.

SB, being a chemical inhibitor, may invite criticism regarding its target specificity. Therefore, we performed a similar set of experiments after selective knockdown of p38α using siRNA directed against it. As seen in Fig. 1G, astrocytes showed significantly reduced level of p38α after transfection with siRNA specific for p38α (si-p38α), but not control siRNA (si-C). Next, EMSA was performed with nuclear extract from si-C and si-p38α transfected cells. As shown in Fig. 1H, siRNA knockdown of p38α did not affect the DNA binding activity of NF-κB (Licor Biosciences Probe). However, in agreement with Fig. 1F, siRNA knockdown of p38α resulted in reduced expression of iNOS, an NF-κB target gene, in cytokine-stimulated cells (Fig. 1H). Taken together, interfering with p38 (either by a chemical inhibitor or by siRNA knockdown) attenuates NF-κB-dependent gene expression by specifically hindering transcriptional activity of NF-κB without affecting its nuclear translocation or DNA-binding activity.

MAPK p38 inhibitor does not modulate phosphorylation status of NF-κB p65

Because p38 is known to phosphorylate transcription factors, we next tested whether inhibition of p38 alters the phosphorylation status of NF-κB p65. To detect any change in (IL-IF)-induced total phosphorylation level of p65, we used an anti-phospho-serine Ab to detect the phospho-status of immunoprecipitated p65 from the nuclear lysate of cells treated with IL-IF in the presence or absence of SB. As seen in Fig. 2A, we did not detect any alteration in the total phospho-level of p65.

To substantiate this finding further, we next verified the phosphorylation status of S276 residue. Phosphorylation of S276 is a prerequisite for p65 nuclear translocation and is also phosphorylated by the catalytic subunit of PKA (36). Thus, nuclear p65-S276 residue in the immunoblots probed with an anti-phospho-S276-p65 Ab. Similar observations were made in our immunostaining experiments (Fig. 2C). However, MSK1 is also regulated by the other MAPK ERK (35) and therefore, MSK1 may remain active even in the absence of p38. Furthermore, p65-S276 is also phosphorylated by the catalytic subunit of PKA (36).
to serve as positive controls of our previous observation, we assessed phosphorylation status of p65-S276 after inhibiting these kinases. Cells were pretreated with a combination of U0126 (ERK pathway inhibitor) and H89 (inhibitor of PKA and MSK1). As a result, (IL-IF)-induced p65-S276 phosphorylation was significantly inhibited by the combination of U0126 and H89 (Fig. 2D).

To confirm further that phosphorylation at S276 of p65 is independent of p38, we next tested the signal-induced phosphorylation status of p65-S276 after over-expression of kinase-deficient dominant-negative mutant of p38 (Δp38). Consistent to our previous set of observations, we did not detect any difference in nuclear p65-S276 phosphorylation state between cells transfected with an empty vector and that with Δp38 (Fig. 2E). In addition to S276, p65 activity is also dependent on phosphorylation of S536. To verify any effect of p38 on this residue in response to IL-IF treatment, we knocked down the MAPK using si-p38a. As seen in Fig. 2F, cells transfected with either si-C or si-p38a exhibited a similar level of IL-IF-induced nuclear S536 phosphorylation suggesting that similar to p65-S276 phosphorylation, the phosphorylation of p65-S536 also does not depend on p38.

NF-κB p65 can be phosphorylated both in the nucleus as well as in the cytoplasm (37). Because all the above studies were aimed at assessing the modified status of nuclear p65, we next wondered whether p38 regulates nonnuclear phosphorylation of p65. To answer this query, we expressed a carboxyl truncated p65 construct, FLAG-p65-Δ313 (38), which lacked the nuclear localization signal but housed the phospho-acceptor S276 residue. Protein expressed by this construct would thus be signal-dependently phosphorylated at S276. However, its inability to enter into the nucleus provides the scope to assay phosphorylation of S276 outside the nucleus. Cells that were transfected with FLAG-p65-Δ313 (Fig. 2G, IL-IF column, open arrowhead), showed phosphorylation of p65-S276 after IL-IF treatment both within and outside the nucleus. The extra-nuclear p65-S276 phosphorylation sites are visible as yellow immuno-signals arising from the overlapping red anti-FLAG and green anti-phospho-S276-p65 signals. This is in contrast with the nontransfected cell in the same field (Fig. 2G, IL-IF column, closed arrowhead), where the anti-phospho-p65-S276 signal is restricted to the nucleus. Additionally, IL-IF induced such extra-nuclear colocalization of the ectopically expressed FLAG-p65-Δ313, and p65-S276 phosphorylation was not influenced by SB treatment ((IL-IF)+SB column, Fig. 2G). To rule out any possibility of Ab cross-reactivity in our assays, we transfected cells with a construct expressing an unrelated nuclear protein (FLAG-Brg1). In these cells, we did not notice any cytoplasmic immunoreactivity for p65-S276 phosphorylation (data not shown). Additionally, nuclear phospho-S276-p65 signal did not colocalize with nuclear Flag in (FLAG-Brg1)-transfected cells (data not shown). Taken together, these results suggest that p65 can be phosphorylated at S276 both in the nucleus and cytoplasm and that p65-S276 phosphorylation is not regulated by p38 MAPK.

Signal-dependent NF-κB p65:p300 complex formation is sensitive to inhibitor of MAPK p38

NF-κB p65, once optimally phosphorylated, binds to coactivator p300 or CBP and several other factors in the nucleus to form a complex, which is transcriptionally active (transcriptional complex) (18). These coactivators, endowed with endogenous acetyltransferase activity, allow the p65 transcriptional complex to access promoters by acetylating specific histone lysine residues and p65 itself. Despite adequate phosphorylation, damped transcriptional activity of p65 may arise from its failure to form a functional transcriptional complex with active histone acetyltransferase. Because inhibition of p38 did not hinder phosphorylation of p65, we next assessed acetyltransferase activity of the p65-transcriptional complex upon p38 inhibition. Using nuclear lysates of cells treated with IL-IF in the presence or absence of SB, the p65-associated transcriptional complex was immunoprecipitated with an anti-p65 Ab. This Ag-Ab complex was used to perform an acetyltransferase assay. It is clearly evident from Fig. 3A that IL-IF increased the acetyltransferase activity of the p65 transcriptional complex. However, SB pretreatment was capable of knocking down IL-IF-mediated increase in acetyltransferase activity of the p65 transcriptional complex (Fig. 3A).

Because acetyltransferase activity is inherently possessed by co-activators, not p65, we next focused on the involved coactivator. Among several nuclear acetyltransferases, p300 has been previously linked with expression of several NF-kB-dependent proinflammatory genes, including iNOS in astroglial (12) and nonastroglial cells (14). As seen in Fig. 3B, over-expression of wild type p300 augmented ph-iNOS (7.2) promoter activity in both control and IL-IF-treated human primary astrocytes. However, over-expression of dominant-negative p300 (∆HAT) significantly reduced iNOS promoter-driven luciferase activity (Fig. 3B). Therefore, we hypothesized that the p65 transcriptional complex may lack acetyltransferase activity due to decreased association between p65 and p300 and decided to investigate the effect of SB on the association between p300 and p65. We over-expressed T7-p65 and FLAG-p300 in astrocytes. Subsequently, these cells were treated with IL-IF in the presence or absence of SB. As seen in Fig. 3C, cytokine treatment induced an association between overexpressed T7-p65 and FLAG-p300. However, SB dose-dependently inhibited this association (Fig. 3C). To confirm this observation further, coimmunoprecipitation assays were performed where endogenous p300-associated and p300-associated complexes were immunoprecipitated with respective Abs from nuclear lysates of cells treated with IL-IF in the presence or absence of SB. Fig. 3D clearly shows that IL-IF increased the association between p300 and p65 and that SB was capable of inhibiting such association. This was further corroborated in cells treated with si-p38a to knock-down the MAPK. As seen in Fig. 3E, interaction between p65 and p300 was abrogated in cells transfected with si-p38a but not with si-C. Along the same lines, we detected association of phosphor-S276-p65 with p300 in response to IL-IF treatment (Fig. 3F; upper lane). But this association was abrogated when cells were pretreated with SB (Fig. 3F; upper lane). Interestingly, we also observed that IL-IF stimulated the association of p38 with p300 (Fig. 3F; second panel). However, SB was unable to modulate such association (Fig. 3F; second panel) suggesting that p38 does interact with p300 and that interaction does not depend on the kinase activity of p38. Collectively therefore, blocking p38 activity hinders the association of p300 with p65. As a result, inhibition of p38 attenuates the acetyltransferase activity of the p65-associated transcriptional complex.

MAPK p38 regulates phosphorylation and acetyltransferase activity of p300

The next question in order was as follows: because p38 did not exert any effect on the phosphorylation of p65, does it regulate the phosphorylation of p300 to produce this effect? To detect any effect of p38 inhibition on the phosphorylation of p300, we performed the immuno-metabolic-phospho-labeling assay. In this study, initially phosphate-starved cells were labeled with radio-labeled phosphate and were subsequently treated with either IL-IF or SB+-(IL-IF). Ag-Ab complex, obtained by immunoprecipitating a nuclear extract of these cells with anti-p300, were separated by gel electrophoresis. Any alteration in phosphorylation status of p300 was examined by autoradiography. As seen in Fig. 4A, IL-IF
cells used for IPs above was Western blotted to detect pre-IP level of p300. Whole cell extract from a quarter of similarly transfected cells were treated with Abs against T7. The same blot was stripped and probed for FLAG. Abs against FLAG and the resulting immunoprecipitate was immunoblotted. The same blot was also stripped and probed for p300. The p300 immunoprecipitate was probed for p65 (upper panels) or p300 (lower panels). The p65 immunoprecipitate was gel separated and was probed for p300 (upper panels). The same blot was probed for p65 after stripping. In contrast, the p300 immunoprecipitate was probed for p65 (lower panels). The same blot was also stripped and probed for p300. E. Cells were transfected either with 100 nM si-C or si-p38

markedly induced the phosphorylation of p300. Interestingly, this posttranslational modification was significantly attenuated by SB pretreatment suggesting a critical role of p38 in the phosphorylation of p300. To confirm this finding further, we transfected cells with a kinase-deficient p38 construct (Δp38). This construct has been previously described (39, 40). After transfection, cells were treated with IL-IF. As seen in Fig. 4B, IL-IF markedly induced the phosphorylation of p300 in empty vector-transfected cells. However, over-expression of Δp38 inhibited (IL-IF)-induced phosphorylation of p300 (Fig. 4B). Taken together, these results suggest that p38 is involved in the phosphorylation of p300 in cytokine-stimulated human astrocytes.

Because p38 regulated the phosphorylation of p300, next we investigated the role of p38 in acetyltransferase activity of p300. HAT assays were performed in anti-p300 immunoprecipitates from a nuclear extract of cells treated with IL-IF and SB+ (IL-IF). As depicted in Fig. 4C, blocking p38 with SB significantly diminished IL-IF-induced acetyltransferase activity of p300 and this decrease in activity is coupled with decreased phosphorylation of p300 (Fig. 4, A and B).

MAPK p38 regulates acetylation of p65 via p300

Next, we investigated the functional consequence of compromised acetyltransferase activity of p300 on p65. Because acetylation of K310 is required for optimum transcriptional activity of p65 (10), we verified the acetylation status of p65 at this residue in astrocytes over-expressing dominant-negative p300 (Δp300). Cells transfected with either vehicle or Δp300, were treated with IL-IF and p65 was immunoprecipitated from a nuclear lysate. As detected by Abs specific for the acetylated K310 residue of p65 (Ac-K310-p65), cells transfected with Δp300 showed reduced acetylation in comparison to vehicle-transfected cells (Fig. 5A). Because
blocking p38 activity attenuated acetyltransferase activity of p300 (Fig. 4D), we next verified the effect of SB on p65-K310 acetylation. Cells were treated with IL-IF in the presence or absence of SB, and p65 was immunoprecipitated from a nuclear lysate. The Ag-Ab complex was separated by gel electrophoresis and was probed with an Ac-K310-p65 Ab. Fig. 5B shows that IL-IF induced the acetylation of p65 at K310 and that SB was capable of inhibiting (IL-IF)-induced p65 acetylation. To confirm the inhibitory effect of SB on K310 acetylation of p65, cells were immunostained with an Ac-K310-p65 Ab after similar treatment (Fig. 5C). In this case as well, acetylation of nuclear p65 on K310 was abrogated in cells pretreated with SB. To confirm the role of p38 further, acetylation status of K310 was verified in cells transfected with either vehicle or kinase deficient Δp38. As seen in Fig. 5D, cells transfected with Δp38 showed a lower abundance of Ac-K310-p65 in their nucleus than vehicle-transfected cells. Together, our data suggest that cytokine-induced acetylation of p65 is dependent on p38.

**Discussion**

The current report identifies a novel role of MAPK-p38 in inflammation, where the MAPK mediates inflammatory responses by regulating the coactivator p300. Widely considered as a prime regulator of inflammation, p38 integrates inflammatory responses by regulating several aspects of target gene transcription and translation (19). In certain cases, it facilitates translation of inflammatory moieties by phosphorylating AU-binding proteins (19). Phosphorylation of these proteins leads to their separation from the AU-region of proinflammatory mRNAs, thereby resulting in their liberation, stabilization, and translation. Furthermore, it aids inflammatory transcription by two known mechanisms. First, H3 S10 phosphorylation was shown to depend on p38 MAPK signaling on NF-κB target genes, but a direct role of p38 MAPK in the phosphorylation of H3 has not been demonstrated yet. Such phosphorylation ‘flagmarks’ them as targets of proinflammatory transcription factors (41). Secondly, p38 is known to regulate activity of several proinflammatory transcription factors (20, 42). Among them, several transcription factors, such as CCAAT/enhancer-binding protein and NFAT, are direct substrates of p38 and the kinase modulates their activity by directly phosphorylating them (43).

In contrast, despite being operational downstream of p38, the p38-dependent regulation of NF-κB has always appeared complex. Blocking p38 with potent and specific inhibitors results in attenuation of transcriptional activity of NF-κB in various cell types (21–24). However, nuclear translocation and DNA-binding ability of this transcription factor remains unaffected (24–26). We have also found a similar situation in human primary astrocytes. Being a kinase, p38 is primarily expected to regulate phosphorylation of NF-κB, directly or indirectly. This expectation arises from the fact that functional output of NF-κB requires several rounds of signal-dependent posttranslational phosphorylation at several residues, most importantly S276 and S536 residues of p65 (17). However, as suggested by our data, p38 implements no phospho-alterations on p65. Interestingly, a previous report (34) has shown that blocking MSK1, a potential p38 substrate (35), compromises p65-S276 phosphorylation. This creates a possibility of p38 acting on p65-S276 through MSK1. Because MSK1 is known to be regulated by both ERK and p38 (35), we investigated signal-dependent modification of this residue in presence of H-89, the potent inhibitor of MSK1 (35) and catalytic subunit of PKA, the kinase also known to phosphorylate p65 at S276 itself (36). Although pretreatment with these inhibitors significantly hindered phosphorylation of p65-S276 residue, yet it was not abrogated completely. This observation may be explained by the involvement of some other kinase(s) in the process.

In addition to phosphorylation, transcriptional output of NF-κB is also largely contingent on signal-induced posttranslational acetylation. NF-κB p65 has been shown to be acetylated at multiple lysine residues, including K122, K123, K218, K221, and K310 (8, 9). Acetylation of these residues manifests site-specific biological outcomes. Acetylation of K122 and K123 attenuates DNA-binding ability of p65 and enhances its association with IκB (9). However, acetylation of K221 increases its DNA-binding ability, and together with acetylated K218, disrupts assembly of p65 with IκB (10). In comparison, acetylation of K310 does not impart any effect
FIGURE 6. Signals arising from cytokine receptors (hypothetically shown together), trigger the canonical pathway of NF-κB activation as is shown on the right side. This is facilitated by one or more kinase(s) that phosphorylate p65. Additional signals activate MAPK p38 (left) which then phosphorylates and activates coactivator p300. Thus activated, p300 binds to NF-κB and acetylates the critical K310 residue of p65. As a result, optimal transcriptional ability is achieved by the NF-κB-p300 transcriptional complex.

on DNA- or IκB-binding of p65. Instead, acetylation of this residue enhances transcriptional activity of p65 complexes (10). Undoubtedly therefore, this is a suitable target for any signaling impediment leading to compromised transcriptional activity of p65. Accordingly, our data show that blocking p38 activity effectively ablates acetylation of the p65-K310 residue.

To regulate acetylation of a target, a kinase like p38 is expected to act through an acetyltransferase intermediate. A worthy candidate in this regard is coactivator p300 as it has been previously shown to act through an acetyltransferase intermediate. A worthy candidate ablates acetylation of the p65-K310 residue. As a result, optimal transcriptional ability is achieved by the NF-κB-p300 transcriptional complex.

Considering that not much is known about the signal-dependent regulation of p300 acetyltransferase activity, our current data reveals a novel means of regulating p300 acetyltransferase activity. Additionally, one of the main functional outcomes of p300 phosphorylation is revealed by our immunocomplex-HAT assays. These studies reveal that, in the absence of functional and active p38 (although p38 may remain physically associated with p300), the acetyltransferase activity of p300 and the p65 containing transcriptional complex is compromised. Because, this loss of acetyltransferase activity couples with the hypophosphorylated state of p300, it is highly probable that the acetyltransferase activity of p300 is contingent on p38-dependent phosphorylation of the coactivator. Loss of p300 acetyltransferase activity is subsequently manifested on p65, which remains phosphorylated, but nonacetylated at the crucial K310 residue, in the absence of p38 activity. This may serve as a basic reason to explain sensitivity of the p65 transcriptional activity to the p38-inhibitor. In the absence of p38 activity, the well-phosphorylated p65 is inherently capable of binding DNA, yet it fails to transactivate target promoters probably as a result of weak association with the coactivator. Further hindrance may result from subsequent inadequate acetylation at its critical K310 residue and inaccessibility to genomic DNA due to poor acetyltransferase activity of its transcriptional complex.

In summary (Fig. 6), MAPK-p38 activity is required for effective phosphorylation and activation of coreceptor p300. Consequently, p65:p300 complex formation is facilitated resulting in acetylation of the critical p65-K310 residue and optimum transactivation of p65 itself. Together, this manifests NF-κB-dependent gene transcription.

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Disclosures
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References


