The Atypical Protein Kinase C-interacting Protein p62 Is a Scaffold for NF-κB Activation by Nerve Growth Factor*

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Nerve growth factor (NGF) binding to both p75 and TrkA neurotrophin receptors activates the transcription factor nuclear factor κB (NF-κB). Here we show that the atypical protein kinase C-interacting protein, p62, which binds TRAF6, selectively interacts with TrkA but not p75. In contrast, TRAF6 interacts with p75 but not TrkA. We demonstrate the formation of a TRAF6-p62 complex that serves as a bridge linking both p75 and TrkA signaling. Of functional relevance, transfection of antisense p62-enhanced p75-mediated cell death and diminished NGF-induced differentiation occur through a mechanism involving inhibition of IKK activity. These findings reveal a new function for p62 as a common platform for communication of both p75-TRAF6 and TrkA signals. Moreover, we demonstrated that p62 serves as a scaffold for activation of the NF-κB pathway, which mediates NGF survival and differentiation responses.

The biological responses to neurotrophins such as NGF include neuronal survival and differentiation (1). Two receptors, TrkA and p75, participate in the formation of the high affinity NGF binding site (2). TrkA enhances both NGF responsiveness and cell survival (3). The transcription factor nuclear factor κB (NF-κB) is activated by both TrkA and p75 receptor components (4). Moreover, p75 has been shown to interact with TRAF6 (5), a critical adapter in the activation of NF-κB by interleukin-1 and other cytokines (6). In addition, inhibition of NF-κB increases p75-mediated apoptosis in this system (7), demonstrating a prosurvival requirement for this transcription factor (8, 9). Furthermore, mice deficient in IKK, the enzyme that phosphorylates and targets the inhibitory molecule IκB leading to NF-κB activation, leads to a defect in neureulation (10). Similarly, TRAF6-deficient mice also display a failure of neural tube closure and exencephaly (11). Collectively, these findings underscore the importance of NF-κB in the nervous system. The activation of IKK and NF-κB has been shown to require atypical protein kinase C (aPKC) in both neuronal and non-neuronal systems (reviewed in Ref. 12). Moreover, aPKC over-expression enhances NGF prosurvival signaling through up-regulation of NF-κB (13). In contrast, proapoptotic signaling inhibits aPKC and blocks NF-κB (14). Additionally, the selective aPKC-binding protein, p62 (15, 16), has been shown to interact with TRAF6 and to be essential during interleukin-1 signaling to NF-κB (17). Here we report that p62 plays a novel role as a scaffold for the activation of NF-κB by nerve growth factor, linking both p75 and TrkA receptor components.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Cells of human embryonic kidney 293 (HEK 293) or NIH-3T3 cells were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Subconfluent cells were transfected by the calcium phosphate method. PC12 cells were grown on cultureware coated with rat tail collagen in RPMI containing 10% horse serum and 5% fetal calf serum and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). PC12 or NIH-3T3 cells were routinely transfected using LipofectAMINE 2000 (Life Technologies, Inc.). 2.5 S NGF was purchased from Bioproducts for Science (Indianapolis, IN). The monoclonal 12CA5 anti-HA and anti-Flag antibodies were from Sigma. The rabbit anti-Myc, anti-TRAF6, anti-TrkA, and anti-IKK antibodies were from Santa Cruz Biotechnology. The monoclonal anti-p62 was obtained from BD Transduction Laboratories. The anti-Myc antibody (9E10) was from Babraham Pharmaceuticals.

Immunoprecipitation and Western Blot Analysis—To detect endogenous proteins in PC12 cells or those cotransfected into HEK cells, lyses were prepared from subconfluent cultures of cells grown on 100-mm dishes. Typically cells were transfected for 36–42 h with 5–10 μg of construct and pcDNA3 plasmid to give 30 μg of total DNA. After transfection, cells were stimulated or not with 50 ng/ml NGF. Cells were then harvested and lysed in PD buffer (40 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM phenyl phosphate, 300 μM Na3VO4, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM dithiothreitol). Extracts were centrifuged at 15,000 × g for 15 min. Protein was determined, and equal amounts of whole-cell lysate were diluted in PD buffer and incubated with antibody as indicated for 2 h. Then protein A or G beads were added for an additional hour at 4 °C. The immunoprecipitates were then washed five times with PD buffer. As control an aliquot of the cell lysate (1/10) volume was also analyzed by immunoblotting. Samples were fractionated on 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to Western blot analysis with the corresponding antibodies. Proteins were detected with ECL reagents (Amerham Pharmacia Biotech).

Measurement of NF-κB Activity—NF-κB activation was measured using a reporter gene assay. HEK, 3T3, or PC12 cells were transfected with a κB-luciferase reporter gene plasmid, 2EConA-Luc (17). After 24 or 48 h, the cells were stimulated with NGF and activity determined using a Promega luciferase assay kit. Individual constructs were transfected in duplicate and each assay measured in triplicate. Values are reported as the mean ± S.E. of three individual experiments.
encompassing amino acids 266–446 of p62 binds TrkA, whereas the binding of p62 to TRAF6 has been mapped to amino acids 225–251 (Ref. 17 and Table I). This indicates that p62 may accommodate both TrkA and TRAF6 simultaneously.

Deletion of the TRAF6 binding site did not effect TrkA binding to p62, thus further strengthening the notion that p62 interacts with TrkA and TRAF6 through two independent binding domains (Table I).

TRAF6 has been reported to interact with p75 (5). To determine whether TRAF6 interacts with TrkA as well, both p75 and TrkA were coexpressed in HEK cells along with Flag-TRAF6. Whereas p75 associated with TRAF6 in an NGF-dependent manner (Fig. 2A) as previously reported (5), TRAF6 failed to associate directly with TrkA. The interaction of p75 with TRAF6 was mapped to the C-proximal TRAF-C domain, a region that also accommodates p62 (17). Coexpression experiments in HEK cells revealed that TRAF6 can bind both p75 and p62 Is a Scaffold for NF-κB Activation by NGF
p62 simultaneously (not shown). Because p62 interacts with TRAF6 (17), it is conceivable that p62 may be brought into a p75 complex via TRAF6 serving as a bridge. If this model is correct we should be able to coimmunoprecipitate p62 with p75 only in the presence of TRAF6. The results shown in Fig. 2B strongly suggest that these predictions are correct. Thus, in

HEK cells transfected with different expression vectors, a small amount of p75 was found to associate with p62, likely through endogenous TRAF6. Upon coexpression of TRAF6, recruitment of p75 into the p62 complex was significantly enhanced (~2.5-fold). The ability of TrkA to coassociate with TRAF6 was dramatically and consistently enhanced by the presence of exogenous p62 (Fig. 2B). We next determined whether NGF could stimulate the formation of an endogenous TRAF6-p62 complex in PC12 cells (Fig. 2C). In the absence of NGF little or no association of TRAF6 with p62 or aPKC could be detected. However, the addition of NGF resulted in a rapid interaction of TRAF6 with p62 and consequently with aPKC. Close examination revealed that the kinetics of association between TRAF6 and p62 (maximum 1–5 min) occurs prior to the recruitment of p62 to the TrkA receptor (Fig. 1C, peaks at

Table I

Mapping of p62-TrkA interaction domains

| p62 | TRAF6 | aPKC |
|-----|-------|------|
| AID | +     | +    |
| ZZ  | +     | +    |
| p62 as | + | +   |
| 1-266 | -   | -    |
| 117-439 | - | -    |
| 1-117 | -   | -    |
| ZIP2 | +   | +    |

Fig. 2B

Role of p62 in NF-κB activation. A, HEK cells were cotransfected with pcDNA3-p75 (150 ng) along with either TRAF6 (150 ng) or antisense p62 (ASp62, 1 μg) in the presence of NF-κB reporter. NF-κB activation was determined by luciferase assay (24). B, HEK cells were cotransfected with pcDNA3-p75 along with TRAF6 and increasing concentrations of p62 (1 or 2 μg). NF-κB activity was determined by luciferase assay. RLU, relative luciferase unit. C, PC12 cells were cotransfected with LipofectAMINE 2000 with increasing concentrations of p62 (0.5, 1.25, or 2.5 μg) and NF-κB reporter followed by stimulation with NGF (50 ng/ml) for 3 h. D, PC12 cells or NIH-3T3 cells expressing either p75 or TrkA were transiently transfected with increasing concentrations of antisense p62 construct (0.5, 1.25, or 2.5 μg) or antisense p62 alone (2.5 μg) with LipofectAMINE along with NF-κB reporter. After 48 h the cells were stimulated with NGF (50 ng/ml) followed by assay of luciferase activity.

Fig. 3

FIG. 3. Role of p62 in NF-κB activation. A, HEK cells were cotransfected with pcDNA3-p75 (150 ng) along with either TRAF6 (150 ng) or antisense p62 (ASp62, 1 μg) in the presence of NF-κB reporter. NF-κB activation was determined by luciferase assay (24). B, HEK cells were cotransfected with pcDNA3-p75 along with TRAF6 and increasing concentrations of p62 (1 or 2 μg). NF-κB activity was determined by luciferase assay. RLU, relative luciferase unit. C, PC12 cells were cotransfected with LipofectAMINE 2000 with increasing concentrations of p62 (0.5, 1.25, or 2.5 μg) and NF-κB reporter followed by stimulation with NGF (50 ng/ml) for 3 h. D, PC12 cells or NIH-3T3 cells expressing either p75 or TrkA were transiently transfected with increasing concentrations of antisense p62 construct (0.5, 1.25, or 2.5 μg) or antisense p62 alone (2.5 μg) with LipofectAMINE along with NF-κB reporter. After 48 h the cells were stimulated with NGF (50 ng/ml) followed by assay of luciferase activity.

Fig. 4

FIG. 4. p62 regulates both p75 and TrkA functional properties. A, HEK cells were cotransfected with pcDNA3-p75 (150 ng) along with either TRAF6 (150 ng) or antisense p62 (ASp62, 1 μg) in the presence of NF-κB reporter. NF-κB activation was determined by luciferase assay (24). B, HEK cells were cotransfected with pcDNA3-p75 along with TRAF6 and increasing concentrations of p62 (1 or 2 μg). NF-κB activity was determined by luciferase assay. RLU, relative luciferase unit. C, PC12 cells were cotransfected with LipofectAMINE 2000 with increasing concentrations of p62 (0.5, 1.25, or 2.5 μg) and NF-κB reporter followed by stimulation with NGF (50 ng/ml) for 3 h. D, PC12 cells or NIH-3T3 cells expressing either p75 or TrkA were transiently transfected with increasing concentrations of antisense p62 construct (0.5, 1.25, or 2.5 μg) or antisense p62 alone (2.5 μg) with LipofectAMINE along with NF-κB reporter. After 48 h the cells were stimulated with NGF (50 ng/ml) followed by assay of luciferase activity.

TABLE I

Mapping of p62-TrkA interaction domains

| p62 | TRAF6 | aPKC |
|-----|-------|------|
| AID | +     | +    |
| ZZ  | +     | +    |
| p62 as | + | +   |
| 1-266 | -   | -    |
| 117-439 | - | -    |
| 1-117 | -   | -    |
| ZIP2 | +   | +    |
15 min), suggesting that it is a two-step process. Collectively, these results reveal that p62 interacts with TRAF6 in response to NGF and may likely serve as a bridge between both p75 and TrkA receptor components.

As TRAF6 interaction with p75 results in activation of NF-κB (7), it was of interest to determine whether the downregulation of p62 with a p62 antisense construct (17) would block the induction of NF-κB as measured by a luciferase reporter system. The results shown in Fig. 3A demonstrate that this is the case, because there was a dramatic reduction of NF-κB activity by the expression of p75 and TRAF6 in cells transfected with the p62 antisense construct as compared with the nontransfected cells. On the other hand, the transfection of a p62 expression vector, that by itself does not activate NF-κB in this system (17), dramatically enhanced p75-TRAF6 or TrkA-TRAF6 activation of NF-κB (Fig. 3B). In contrast, p62/ZIP2, which lacks the TRAF6 binding site, failed to activate NF-κB (Fig. 3B). Altogether this indicates that the recruitment of p62 to the NGF receptor signaling complex is critical for the activation of NF-κB. Consistent with this notion, overexpression of p62 in PC12 cells resulted in a dose-dependent enhancement of both basal as well as NGF-stimulated activation of NF-κB (Fig. 3C). We next conducted experiments to investigate whether the antisense construct of p62 would block NGF-induced activation of NF-κB in cells expressing either one or both NGF receptors. Interestingly, antisense p62 blocked NGF-induced activation of NF-κB in PC12 cells expressing both receptors (Fig. 3D). Likewise, p62 down-regulation in NIH-3T3 cells expressing either p75 or TrkA receptor (20) (Fig. 3D) also abrogated NGF-induced activation of NF-κB. Collectively these findings demonstrate that p62, like aPKC (13), is essential in the activation of NF-κB by NGF and that it serves to scaffold proximal NGF receptor components in this pathway.

The functional relevance of the presence of p62 in these complexes was addressed further in the following series of experiments. Overexpression of p75 in HEK cells results in ligand-independent cell death that is prevented by TRAF6 (7, 8). Consistent with the role of NF-κB in cell survival signaling (4), the expression of antisense p62 enhanced p75 mediated-cell death (Fig. 4A), whereas expression of TRAF6 or p62 blocked cell death. The activation of NF-κB is likewise required for neuronal differentiation (9) and TrkA responsiveness (4). Transfection of antisense p62 significantly impaired NGF-induced neurite outgrowth, whereas overexpression of p62 enhanced NGF responsiveness (Fig. 4A). The mechanism whereby p62 regulates activation of NF-κB likely involves recruitment of TRAF6 and aPKC onto the p62 scaffold, thus enabling aPKC-mediated phosphorylation of IKK (21). To provide evidence for the involvement of p62 in this process, we assessed the activity of endogenous IKK activity in an in vitro kinase assay using GST-IKKα as the substrate (4, 21). Transfection of antisense p62 suppressed NGF-stimulated activation of IKK (Fig. 4B).

The findings reported here provide new insight into the proximal components of the NGF/NF-κB pathway and demonstrate formation of a p62 bridge that scaffolds together both p75 and TrkA receptors for the activation of NF-κB (Fig. 4C). Our results stress the role of p62 as a common and critical intermediary that channels different signaling pathways toward IKK activation. Understanding the mechanism whereby the TRAF6-p62 complex is regulated in vivo is an area of ongoing study. Together, these findings underscore the importance of p62 as a scaffold for NF-κB and as a common platform for communication of both p75 and TrkA receptor signals.

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