Regulation of Interleukin Receptor-associated Kinase (IRAK) Phosphorylation and Signaling by Iota Protein Kinase C*

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We have previously shown that the activity of the interleukin-1 (IL-1) receptor-associated kinase (IRAK) is required for nerve growth factor (NGF)-induced activation of NF-κB and cell survival (2002) J. Biol. Chem. 277, 28010–28018). Herein we demonstrate that NGF induces co-association of IRAK with atypical protein kinase C iota (PKC) and that the iota PKC-IRAK complex is recruited to the p75 neurotrophin receptor. Recruitment of IRAK to the receptor was dependent upon the activity of the iota PKC. Moreover, transfection of kinase-dead iota PKC blocked both NGF- and IL-1-induced IRAK activation and the activity of NF-κB. Hence, iota PKC lies upstream of IRAK in the NF-κB pathway. Examining the primary structure of IRAK, we identified three putative PKC phosphorylation sites; iota PKC selectively phosphorylated peptide 1 (RGG) within the death domain at Thr66, which is highly conserved among all IRAK family members. Mutation of Thr66 to Ala impaired the autokinase activity of IRAK and reduced its association with iota PKC but not TRAF6, resulting in impaired NGF- as well as IL-1-induced NF-κB activation. These findings provide insight into the underlying mechanism whereby IRAK regulates the NF-κB pathway and reveal that IRAK is a substrate of iota PKC.

Like interleukin-1 (IL-1),¹ the neurotrophin receptor p75 NTR utilizes the interleukin-1 receptor-associated kinase (IRAK), for activation of the NF-κB pathway (1). In neurons, the functional outcome of this pathway is trans-activation of numerous genes that play a role in central nervous system survival. The molecular events at the p75 receptor complex leading to induction of NF-κB have been characterized recently. The adapter protein MyD88 recruits IRAK to the p75 receptor complex where IRAK subsequently interacts with TRAF6 and p62 scaffold, which bridges them to I KKβ (NF-κB kinase). However, the mechanism whereby IRAK is activated in the NF-κB pathway has not yet been fully elucidated. The identification of a kinase responsible for the signal-induced phosphorylation of IRAK has been the subject of intense study (2). In this regard, mutation of the catalytic domain reveals that IRAK is still phosphorylated upon IL-1 stimulation (3), suggesting that IRAK is likely to be the substrate of another kinase lying upstream proximal to the receptor. However, in the IL-1 pathway, the catalytic activity of IRAK is not required for mediating for activation of the NF-κB pathway (4). In contrast, in both the p75 NTR and TNF pathways, IRAK catalytic activity is required for mediation of the NF-κB response (5). Hence, in some systems, upstream phosphorylation by another kinase may regulate not only the catalytic activity of IRAK but also the enzyme’s ability to interact with effectors of the NF-κB pathway; thus phosphorylation of IRAK may serve as a bifurcation point in the NF-κB pathway.

NGF, IL-1, and TNF are potent activators of the atypical PKCs. Moreover, the highly homologous atypical PKC (aPKC) isoforms zeta and lambda/iota have been shown to play a critical role during NF-κB activation (6, 7). NGF binding to the p75 NTR receptor results in production of second messenger metabolites such as ceramide (8), which may lead to activation of iota aPKC (9). In addition, the p75 NTR receptor employs p62, the atypical protein kinase C-interacting protein, as a selective scaffold for activation of NF-κB (1). Thus, we hypothesized that iota PKC may be recruited into the p75 receptor complex to serve as an IRAK. Herein, we report the discovery of a novel pathway revealing that iota PKC phosphorylates IRAK within the death domain (DD) at the conserved residue Thr66. Mutation of this site impairs IRAK autophosphorylation, interaction with iota PKC, and NF-κB activation. Altogether these findings reveal that IRAK is a substrate of iota PKC and that the Thr66 phosphorylation site serves to enhance interaction between kinase and substrate.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—IRAK-deficient 11A cells were obtained as a gift from Dr. Xiaoxia Li, Lerner Research Institute (10) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Subconfluent IRAK-deficient 11A cells were transfected with His/Myc mPLK/IRAK (gift of Dr. Maureen Harrington) and/or GST-aPKC active/inactive (gift of Dr. Jorge Moscat, Madrid, Spain) employing the Mammalian Cell Transfection Kit (Cell & Molecular Technologies, Inc.). All transfected cDNAs were normalized by using empty vector, pcDNA. PC12 cells were grown in Dulbecco’s modified Eagle’s medium with 10% horse serum and 5% fetal calf serum. They were transfected employing LipofectAMINE 2000 (Invitrogen). Purified IRAK enzyme was provided by Tularik, Inc., San Francisco. Polyclonal antibody to p75 NTR was a gift of Dr. Alfonso Ross (University of Massachusetts) or were purchased from Promega.

Site Mutagenesis—The cDNA of His/Myc-mPLK/IRAK or His/Myc-cimPLK, a mutant of IRAK that has no autophosphorylation activity, was site-mutated Thr66 to Ala using the QuikChange site-directed mutagenesis kit from Stratagene by GeneMed Synthesis (San Francisco). Two complimentary oligonucleotides containing the desired mutation and flanked by unmodified nucleotide sequences were synthesized (5’-GCTTCCCGGAGCGCCCGGCCCAGCCTGTC-3’ and 5’-GAC-GCTGCGCCGGGCTGCGCCGGGCGG-3’). Following transformation,
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Inclusion of phosphatase 2A abolished immunoreactivity to IRAK, thus revealing that this antibody detects the phosphorylated/activated form of the enzyme. Because we previously observed that IRAK interacts with iota PKK-interacting protein/p62 upon stimulation with NGF (1), we next asked whether IRAK could also directly interact with iota PKK (Fig. 1B). Treatment of PC12 cells with NGF followed by immunoprecipitation of iota PKK confirmed that NGF stimulated a rapid but transient co-association of iota PKK with IRAK. Moreover, both IRAK and iota PKK were recruited to the p75 receptor in a coincident time frame (Fig. 1B). Likewise, IRAK immunoprecipitates contained both p75 and iota PKC. The interaction of both IRAK and iota PKC with p75 occurred prior to the interaction of iota PKK and IRAK, thus suggesting that the iota-IRAK interaction took place once recruited to the receptor. As control the lysates were blotted with each antibody and revealed equal amounts of protein (Fig. 1C). In addition, at a time when iota PKK was recruited to the p75 receptor (2 min), an immune complex kinase assay using

RESULTS AND DISCUSSION

NGF Induces Association of IRAK with Iota PKK in a p75 Receptor Complex—In previous experiments we observed that IRAK migrated as a doublet on Western blots coincident with its activation and increase in enzyme activity (1), thus suggesting that the IRAK antibody may detect the phosphorylated/activated form of the enzyme. To test this possibility, cell lysates were incubated with phosphatase 2A for various times followed by Western blotting with monoclonal antibody to IRAK (Fig. 1A).
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Iota PKC kinase activity is required for recruitment and activation of IRAK. PC12 cells were transfected with a construct of GST-tagged aPKC-iotA (wild type or kinase-inactive mutant). 24 h post-transfection, cells were treated with NGF (50 ng/ml) for 2 min. Cell lysates (750 μg) were immunoprecipitated (IP) with p75 antibody and Western blotted (WB) with IRAK monoclonal antibody. As control, whole cell lysates were also blotted for IRAK and GST (aPKC). B, lysates were immunoprecipitated with IRAK polyclonal antibody and subjected to an immune complex kinase assay with MBP as substrate. C, HEK cells were co-transfected with constructs as shown (250 ng) along with NF-κB reporter pGL3 (25 ng/well). 24 h post-transfection, NF-κB activity was measured by dual luciferase assay and reported as RLU/μg. D, HEK or PC12 cells were transfected as shown. After 24 h transfection, the cells were treated with either IL-1 or NGF for 3 h. NF-κB activity was measured by dual luciferase assay and reported as RLU/μg. The means ± S.E. from three independent experiments are shown (C and D).

hnRNPA1 as a defined iota PKC substrate revealed that the enzyme was active (data not shown).

Recruitment of Phosphorylated IRAK to the p75 Receptor Requires Iota PKC Activity—Within minutes after PC12 cells are exposed to NGF, IRAK is recruited to the p75 receptor where it becomes highly phosphorylated and activated (1). Because our results reveal that iota PKC associates with IRAK, we analyzed whether the activity of iota PKC modulated recruitment of IRAK to the p75 receptor by transfecting a kinase-dead mutant of iota PKC followed by immunoprecipitation of p75 and Western blotting for IRAK (Fig. 2A). Interestingly, transfection of kinase-dead iota PKC diminished recruitment of IRAK to the p75 receptor complex and also blocked immunoreactivity of IRAK toward the monoclonal IRAK antibody, suggesting that the phosphorylation/activity of IRAK may be regulated by iota PKC. To examine this possibility, IRAK was immunoprecipitated, and its activity was measured in an immune complex kinase assay with MBP as substrate (Fig. 2B).

Co-transfection of constitutively active iota PKC enhanced the basal as well as NGF-stimulated activity of IRAK. By comparison, the kinase-inactive form of iota PKC blocked NGF-stimulated activity of the enzyme along with diminished recruitment of IRAK to the p75 receptor complex. However, phosphorylation of MBP did not return to basal levels with transfection of inactive iota PKC. We believe that this effect is due to the co-association of kinases such as Src with iota PKC, which are also capable of phosphorylating MBP and thus contribute to the higher background. To test the effect of iota PKC on the ability of IRAK to activate the NF-κB pathway, HEK293 cells were transfected with either catalytically active iota PKC or a kinase-dead iota PKC construct in the presence or absence of IRAK (Fig. 2C). Whereas overexpression of catalytically active iota PKC enhanced IRAK-induced NF-κB, the kinase-dead form of iota PKC abrogated IRAK-induced NF-κB activation. We also tested the effects of iota PKC in two systems in which receptor interaction results in activation of NF-κB, e.g. NGF and IL-1 (Fig. 2D). In both systems, transfection of kinase-active iota PKC enhanced NF-κB activity, whereas transfection of kinase inactive iota PKC abrogated ligand-induced NF-κB activation. Collectively they reveal that iota PKC lies upstream of IRAK in the NF-κB signaling pathway, strongly suggesting that iota PKC may participate directly in the activation of IRAK.

Thr 66 in the IRAK Death Domain Is Phosphorylated by Iota PKC—Iota PKC is a multidomain protein (2) containing an N-terminal DD (residues 1–103) followed by a domain of unknown function (UD) (residues 104–198), a kinase domain (KD) (residues 199–522), and a two-part C-terminal domain, also of unknown function (residues 523–619 for C1 and residues 619–712 for C2) (Fig. 3A). Analysis of the IRAK sequence revealed the presence of three putative PKC phosphorylation sites (RXxxG) in the DD, UD, and C1/C2 domains of IRAK. As a first step in determining whether iota PKC might phosphorylate IRAK, an in vitro kinase assay employing purified kinases was undertaken (Fig. 3B). Increasing concentration of aPKC directly stimulated the phosphorylation of IRAK. As a separate means to assess site-specific phosphorylation of IRAK by iota PKC, three peptides (RTAS, RPSS, RAHS) corresponding to the putative phosphorylation sites (RXxxG) within IRAK were synthesized and employed in an in vitro kinase assay as substrate with purified iota PKC enzyme (Fig. 3C). Iota PKC preferentially phosphorylated peptide 1 from the DD (Fig. 3C), which possesses a unique threonine within the putative consensus phosphorylation site (RXxxS). Because neither peptide 2 nor 3 was phosphorylated by iota PKC, we deduced that Thr 66 within peptide 1 may be the amino acid targeted by iota PKC. To examine whether this was the case, a similar peptide was...
Threonine 66 Regulates IRAK Functional Properties and NF-κB Activation—Comparative sequence analysis of IRAK family members revealed that Thr\(^{66}\) is highly conserved among IRAK family members: IRAK, IRAK-2, IRAK-M, and IRAK-4 (Fig. 4A). Site-directed mutagenesis of IRAK changing Thr\(^{66}\) to Ala was undertaken to assess the consequences of iota PKC on IRAK phosphorylation and ability to couple with downstream effectors (Fig. 4B). Transfection of IRAK\(^{-}\) HEK cells with His/Myc-tagged IRAK or His/Myc-tagged mutant IRAK (Thr\(^{66}\) to Ala), along with iota PKC-active/inactive and/or TRAF6 was undertaken. The autokinase activity exhibited by wild type IRAK was completely abolished upon mutating Thr\(^{66}\) to Ala. The basal autokinase activity of wild type IRAK was significantly reduced upon inclusion of inactive iota PKC. Mutation of Thr\(^{66}\) not only blocked the auto-kinase activity of IRAK but also diminished its ability to associate with iota PKC without any effect on the interaction with TRAF6. Thus, Thr\(^{66}\) appears not only to serve as a phosphorylation site but also serves a novel role in anchoring enzyme and substrate (iota PKC-IRAK). In this regard, IRAK can be considered to belong to a family of proteins known as STICKs (substrates that interact with C-kinase) (13). It has been shown previously that PKC is able to interact with, phosphorylate, and modify the function of IRAK family members: IRAK, IRAK-2, IRAK-M, and IRAK-4. If iota PKC were responsible for phosphorylation of IRAK at Thr\(^{66}\), we reasoned that we would be able to more clearly discriminate phosphorylation of IRAK over its auto-phosphorylation activity by employing the catalytically active mutant. Under these conditions, iota PKC was able to phosphorylate the kinase-dead mutant of IRAK, and mutation of Thr\(^{66}\) to Ala abolished iota PKC induced phosphorylation of IRAK (Fig. 3E).

The effects of the IRAK Thr\(^{66}\) mutation were also evaluated in a physiological setting. Because IRAK plays a critical role in p75 NTR activation of NF-κB, we next evaluated the effect that the Thr\(^{66}\) mutant had upon NGF-induced NF-κB activation (Fig. 4C). PC12 cells were transfected with vector or mutant IRAK followed by treatment with NGF and the measurement of NF-κB employing dual lucerase reporter assay. We previously reported that constitutively active IRAK increases NGF-induced NF-κB and that the catalytic activity of IRAK is required for activation of this pathway (1). Transfection of the mutant IRAK abrogated the ability of NGF to activate the κB pathway (Fig. 4C) as well as IL-1-induced activation of the κB pathway (Fig. 4D).

The phosphorylation of IRAK was examined by endogenous kinase assay. The whole cell lysates were blotted with anti-GST or Myc antibodies as control. WB, Western blot; KD, kinase dead.
IRAK with iota PKC and autophosphorylation. A, anti-GST and FLAG antibodies as shown. For expression control, whole WB examine IRAK autophosphorylation or Western blotting (T66A mutant as shown) and NF-

HEK cells were transfected with transfected IRAK (wild type or kinase-inactive mutant) and FLAG-tagged TRAF6 as His/Myc-tagged IRAK (wild type or T66A mutant), GST-tagged aPKC

with IRAK (wild type or T66A mutant) and NF-

activity was measured and reported as RLU/H9262

respectively. NF-

media for 4 h followed by NGF treatment (50 ng/ml) for 0, 1, 2, and 4 h, (25 ng/well). 24 h post-transfection, cells were placed into serum-free

FIG.4 . Mutation of Thr 66 to Ala affects the co-association of

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activity was measured and reported as RLU/G9262

g) were Western blotted with GST and Myc

IP

B reporter gene pGL3 (25 ng/well).

D, HEK cells were transfected with transfected with IRAK (wild type or T66A mutant as shown) and NF-xB reporter gene pGL3 (25 ng/well). 24 h post-transfection, cells were treated with IL-1 for 3 h. NF-xB activity was measured and reported as RLU/pg.

Conclusions—Our findings reveal that iota PKC regulates the NF-xB pathway at the level of Thr66 within IRAK. Interestingly, it has recently been shown that zeta PKC, a closely related homolog of iota, phosphorylates p65 RelA and regulates its transactviation potential (14). Collectively, these findings suggest that regulation of the NF-xB pathway by the aPKCs takes place by two distinct mechanisms: 1) iota PKC regulation of IRAK at the membrane (herein) and 2) zeta PKC regulation of transcription within the nucleus (14).

In other systems removal of the KD of IRAK still produces an enzyme that is phosphorylated (3), thus suggesting that another kinase phosphorylates the enzyme. Although IRAK4 has been identified as an IRAK (11), IRAK4 expression is restricted to liver and kidney, thus suggesting that other kinases also participate in the phosphorylation/regulation of IRAK in other tissues (12). Herein, we identify iota PKC as an IRAK kinase. In addition, deletion of the DD, which contains a Thr66 site, impairs IRAK autophosphorylation and the ability of IRAK to couple with downstream effectors, and it blocks IL-1- as well as c-Jun N-terminal kinase-induced NF-xB activation (3, 15). Thus, consistent with previous findings suggesting that the DD serves as a bifurcation hub for the NF-xB pathway, the identification of Thr66 as the site within the DD sheds light on the mechanism underlying this effect. Thus, considering the conserved nature of the Thr66 within IRAK family members, the regulation IRAK by iota PKC may be a common mechanism employed by diverse stimuli for regulation of IRAK function (12). Employing a totally different rationale and approach, it has been observed recently that Thr66 of IRAK plays a critical role in regulation of IL-1-induced NF-xB (16), without considering the possibility that this is a site targeted for transphosphorylation by another kinase. The authors therein (16) conducted modeling studies revealing that Thr66 may play a role in the stabilization of the fourth and first a-helices of IRAK; furthermore, their study suggested that Thr66 to Ala mutation may effect IRAK autophosphorylation, although without direct measure of phosphorylation. Our findings reveal that Thr66 to Ala mutation totally impairs the basal autokinase activity of IRAK and hence provides further insight into the mechanism whereby this site regulates enzyme activity. Altogether, these results reveal that Thr66 plays a conserved role in several signaling pathways to fine-tune the IRAK's function and signaling abilities and underscore the importance of iota PKC in the regulation of the xB pathway.

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