Phosphorylation of Threonine 290 in the Activation Loop of Tpl2/Cot Is Necessary but Not Sufficient for Kinase Activity*

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Tpl2/Cot is a serine/threonine kinase known to activate the ERK, p38, and JNK kinase pathways. Studies of Tpl2 knock-out mice reveal a clear defect in tumor necrosis factor-α production, although very little detail is known about its regulation and the signaling events involved. In the present study we demonstrated that phosphorylation of Cot was required for its maximal activity as phosphatase treatment of Cot decreased its kinase activity. The Cot sequence contains a conserved threonine at position 290 in the activation loop of the kinase domain. We found that mutation of this residue to alanine eliminated its ability to activate MEK/ERK and NF-κB pathways, whereas a phosphomimetic mutation to aspartic acid could rescue the ability to activate MEK. Thr-290 was also required for robust autophosphorylation of Cot. Antibody generated to phosphorylated Cot identified two wild-type and kinase-dead Cot, suggesting that phosphorylation of Thr-290 did not occur through autophosphorylation but via another kinase. We showed that Cot was constitutively phosphorylated at Thr-290 in transfected human embryonic kidney 293T cells as well as human monocytes as this residue was phosphorylated in unstimulated and lipopolysaccharide-stimulated cells to the same degree. Treatment with herbimycin A inhibited Cot activity in the MEK/ERK pathway but did not inhibit phosphorylation at Thr-290. Together these results showed that phosphorylation of Cot at Thr-290 is necessary but not sufficient for full kinase activity in the MEK/ERK pathway.

Cot/Tpl2/MAP3K8 is a serine/threonine kinase known to activate the ERK, p38, and JNK kinase pathways. Studies of Tpl2 knock-out mice reveal a clear defect in tumor necrosis factor-α production, although very little detail is known about its regulation and the signaling events involved. In the present study we demonstrated that phosphorylation of Cot was required for its maximal activity as phosphatase treatment of Cot decreased its kinase activity. The Cot sequence contains a conserved threonine at position 290 in the activation loop of the kinase domain. We found that mutation of this residue to alanine eliminated its ability to activate MEK/ERK and NF-κB pathways, whereas a phosphomimetic mutation to aspartic acid could rescue the ability to activate MEK. Thr-290 was also required for robust autophosphorylation of Cot. Antibody generated to phosphorylated Cot identified two wild-type and kinase-dead Cot, suggesting that phosphorylation of Thr-290 did not occur through autophosphorylation but via another kinase. We showed that Cot was constitutively phosphorylated at Thr-290 in transfected human embryonic kidney 293T cells as well as human monocytes as this residue was phosphorylated in unstimulated and lipopolysaccharide-stimulated cells to the same degree. Treatment with herbimycin A inhibited Cot activity in the MEK/ERK pathway but did not inhibit phosphorylation at Thr-290. Together these results showed that phosphorylation of Cot at Thr-290 is necessary but not sufficient for full kinase activity in the MEK/ERK pathway.

Cot is a serine/threonine kinase of the mitogen-activated protein kinase kinase kinase (MAP3K) family, designated MAP3K8 (1). It is expressed primarily in spleen, thymus, and lung tissue (2) and is a key component in activation of T cells and macrophages, resulting in TNF-α production from these cell types (3, 4). In tissue culture, overexpressed Cot is constitutively active and can activate the ERK, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase pathways; Cot directly phosphorylates the MAP2Ks for each of these pathways in vitro (5–7).

Cot and its murine homolog, Tpl2, are 93% identical at the amino acid level. Macrophages derived from Tpl22/2 mice exhibit defects only in the MEK/ERK mitogen-activated protein kinase pathway (4). The Tpl22/2 mice are phenotypically normal, yet peritoneal macrophages from these mice do not produce TNF-α in response to bacterial lipopolysaccharide (LPS) treatment.

Little is known about the regulation of Cot activity during cell signaling events. In unstimulated cells, Tpl2 is stabilized by association with NF-κB/p105 and ABIN-2 (8–10). In mouse macrophages, activation of Tpl2 occurs via LPS stimulation of Toll-like receptor 4 (4). Activation of Tpl2 involves its dissociation from p105 and is rapidly followed by its degradation (8), although the steps prior to this separation are not yet known. Human kinase suppressor of Ras 2 (hKSR-2) negatively regulates Cot in the NF-κB and MEK/ERK pathways by direct binding (11). In addition, Cot is phosphorylated by Akt on serine 400 (12), although this phosphorylation event seems to have no regulatory effects. Other phosphorylation events and regulatory components have yet to be determined.

MAP3Ks have been shown to be activated by several mechanisms, such as interaction with a MAP4K (13), autophosphorylation via intramolecular reaction (MEK kinase 1 and Ssk2) (14, 15), or dimerization and subsequent intermolecular autophosphorylation (ASK1 and MLK3) (16–18). Many serine/threonine kinases contain activating phosphorylation sites in the activation loop of the kinase domain, which lies between the conserved DFG and APE motifs. The MAP3Ks MEK kinase 1, Ssk2, and TAK1 are all activated by phosphorylation of conserved residues in this area.

To investigate more fully the extent to which phosphorylation may regulate the activity of Cot, we analyzed mutants of a residue in the activation loop, Thr-290. This threonine corresponds to a conserved phosphorylation site in the activation loop of several MAP3Ks and therefore may be important for Cot regulation or activation. In this report we demonstrate that phosphorylation of Thr-290 is necessary but not sufficient for Cot kinase activity.

EXPERIMENTAL PROCEDURES

Phosphatase Assay—λ-protein phosphatase (New England Biolabs) was incubated with Cot (30–388) with a N-terminal His tag in the buffer provided (50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35) for 1 h at 30 °C. The phosphatase reaction was stopped by addition of a phosphatase inhibitor mixture (1 mM EGTA, 10 mM NaF, 25 mM β-glycerophosphate, 0.1 mM Na3VO4) (14). A portion of this reaction was removed and reserved for the kinase assay. Samples were diluted in kinase reaction buffer (20 mM MOPS (pH 7.2), 50 mM β-glycerophosphate, 3 mM EDTA, 1 mM dithiothreitol, 20 mM MgCl2, 200 μM ATP) and incubated for 0.5 h at room temperature.

Immunoprecipitation and Western Blotting—HEK-293T cells were transfected with wild-type or mutant Cot constructs at 10 μg/1000 μl of

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‡ The abbreviations used are: MAP3K, mitogen-activated protein kinase kinase kinase kinase; MAP2K, mitogen-activated protein kinase kinase; MAP4K, mitogen-activated protein kinase kinase kinase; MEK, mitogen-activated protein kinase/ERK kinase; HEK, human embryonic kidney; TNF, tumor necrosis factor; LPS, lipopolysaccharide; hKSR-2, human kinase suppressor of Ras 2; MOPS, 4-morpholinoepanesulfonic acid.
RESULTS

Phosphorylation Is Necessary for Cot Kinase Activity—To determine whether Cot activity is dependent on phosphorylation, we treated overexpressed and purified Cot with phosphatase and evaluated its activity in an in vitro kinase assay using unactive (unphosphorylated) MEK as the substrate. We used a truncated form of Cot, Cot-(30–398), since this construct has robust activity in vitro (19). Treatment with λ-phosphatase significantly reduced the kinase activity of Cot-(30–398) (Fig. 1A). These results were also seen with full-length Cot (Fig. 1B), supporting the conclusion that phosphorylation is necessary for maximal kinase activity.

TpII/Cot Contains a Conserved Threonine at Position 290—Alignment of the Cot activation loop sequence with that of other MAP3Ks suggests that phosphorylation of a threonine residue at position 290 (Thr-290) may be required for Cot activity. Cot Thr-290 aligns with Thr-575 on MEK kinase 1, Ser-192 on TAK1 (20), and Thr-1460 on Ssk2 (Fig. 2). Mutagenesis studies have shown that phosphorylation of each of these residues is essential for activity of their respective kinases (14, 15, 20). The alignment of Cot with these kinases suggests that phosphorylation of residue Thr-290 may similarly important in the activation of Cot.

Mutation of Throneine 290 to Alanine Abolishes Kinase Activity—To test whether Thr-290 is important for kinase activity, we mutated Thr-290 to Ala as well as Glu and Asp, both of which can mimic phosphorylated threonine. We then measured the activity of these proteins in the MEK/ERK pathway. Wild-type Cot, a kinase-dead Cot K167R (21), and the three Thr-290 mutants were FLAG-tagged and transfected into HEK-293T cells along with a mock-transfected control. Fig. 3A shows the activity of each construct as measured by Western blot analysis of endogenous phospho-MEK and phospho-ERK. Wild-type Cot drives the phosphorylation of MEK and ERK, but the substitution of threonine 290 with alanine effectively eliminates most of the MEK phosphorylation as does the mutation of threonine 290 to glutamic acid. The threonine to aspartic acid mutation partially rescues kinase activity as measured by MEK phosphorylation, and there is nearly complete recovery of phospho-ERK. These results confirm the importance of Thr-290 for kinase activity and suggest that a negative charge in this position, as provided by the T290D mutation, is necessary for activity. Negative charge would also be provided by phosphorylation at this site.

To demonstrate that the various mutants did not lose activity due to a structural change we evaluated the ability of the constructs to bind hKSR-2. hKSR-2 interacts with TpII/Cot and attenuates Cot-mediated ERK and NF-κB activation (11). We co-transfected each of the Myc-tagged Cot constructs into HEK-293T cells with FLAG-hKSR-2 (data not shown). All five mutants were able to associate with hKSR-2, suggesting that the point mutations at Thr-290 did not result in a global structural change of the Cot protein and that the loss of activity of the Cot mutants was due to loss of function normally conferred by phosphorylation.

Phosphorylation of Cot Thr-290 Is Required for Autophosphorylation—FLAG-tagged Cot, kinase-dead Cot K167R, and the three Thr-290 mutants were transfected into HEK-293T cells. Immunoprecipitated, eluted Cot proteins were used in an in vitro kinase assay using MEK as a substrate. Samples were analyzed by Western blotting. As seen in the cascade activity assay only wild-type Cot was active against MEK (Fig. 4A). To evaluate the ability of the mutants to autophosphorylate, eluted Cot samples were assayed in an in vivo kinase assay using [γ-32P]ATP. Wild-type Cot showed significant incorporation of 32P, while, as expected, the kinase-dead Cot was not able...
to autophosphorylate. The Cot T290D mutant retained about 15% (by densitometry of bands) of the autophosphorylation activity by mimicking the phosphorylated state, whereas the autophosphorylation of the T290A and T290E mutants was not far above background (Fig. 4B). These data suggest that not only is Thr-290 phosphorylation required for robust autophosphorylation of Cot but also that autophosphorylation can occur at a site distinct from Thr-290 as the T290D mutant incorporated $^{33}$P.

Thr-290 Is Necessary for Activation of the NF-$\kappa$B Pathway in Vitro—Overexpressed Cot will activate the MEK/ERK pathway and the NF-$\kappa$B kinase cascade (22). We evaluated the activity of these mutant constructs in the NF-$\kappa$B pathway by luciferase reporter assay (Fig. 5). In each test performed, only wild-type Cot activated the NF-$\kappa$B pathway, indicating strongly that Thr-290 phosphorylation is required for kinase activity.

Phosphospecific Cot Thr-290 Antibody Recognizes Wild-type and Kinase-dead Cot—Rabbit polyclonal antibodies were generated using the phospho-Thr-290-specific peptide PKDLRG-pTIEYMSPE where pT is phosphothreonine. To test the antibody for specificity against phospho-Thr-290, we transfected HEK-293T cells with FLAG-tagged full-length Cot, kinase-
dead Cot K167R, and Cot T290A. Immunoprecipitated and eluted recombinant proteins were evaluated by Western blot. The anti-phospho-Thr-290 recognized wild-type and kinase-dead Cot (K167R) but not the T290A mutant, indicating that the antibody was specific for wild-type Cot (Fig. 6A). Phosphospecificity was demonstrated by the addition of a peptide corresponding to the sequence surrounding threonine 290, either unphosphorylated as a control or phosphorylated to block antibody binding. The antibody only recognized wild-type Cot when used with the unphosphorylated peptide. Phosphorylated peptide completely blocked antibody signal, and T290A mutant was not recognized over background, demonstrating the specificity of the antibody.

We investigated the K167R phosphorylation and the specificity of the anti-phospho-Thr-290 antibody further on whole cell lysates from HEK-293T cells transfected with wild-type full-length Cot, Cot K167R, Cot T290A, Cot T290D, and Cot T290E. Lysates were rapidly lysed and boiled to prevent further modification. Anti-phospho-Thr-290 recognized both full-length and kinase-dead Cot. As expected, the antibody did not recognize Cot Thr-290 mutants, confirming the specificity of the antibody (Fig. 6B). As observed with the isolated protein (Fig. 6A), K167R mutant was phosphorylated to the same degree as wild type, suggesting that these proteins are phosphorylated at Thr-290 by another kinase rather than through autophosphorylation. This represents the first identified case in which this conserved residue is phosphorylated by a means other than autophosphorylation.

Thr-290 Is Constitutively Phosphorylated in Transfected HEK-293T Cells—The phosphorylation of kinase-dead Cot at Thr-290 suggests that another kinase may be upstream of Cot activation as this mutant cannot autophosphorylate. Herbinycin A is an irreversible inhibitor of protein tyrosine kinases, and it has been found to block LPS activation of ERK in macrophages (23). Previous work by another group has demonstrated that preincubation of RAW264.7 cells with herbinycin A prevents the activation of Tpl2 by LPS or Taxol (24). We sought to determine whether treatment with herbinycin A would reduce phosphorylation at threonine 290 as this drug may inhibit a putative kinase upstream of Cot. After transfection of wild-type Cot and kinase-dead Cot into HEK-293T cells, cells were then treated with herbinycin A or phorbol 12-myristate 13-acetate, a phorbol ester activator of the MEK/ERK pathway via C-Raf (24). Neither compound inhibited the phosphorylation of Thr-290. However, herbinycin A did inhibit Cot-induced MEK phosphorylation (Fig. 7A), indicating the existence of a herbinycin A-sensitive activator of the Cot/MEK/ERK pathway.

We then repeated this experiment with the addition of staurosporine, a potent inhibitor of most kinases. Staurosporine treatment also did not reduce the phosphorylation at Thr-290
nor did it reduce MEK phosphorylation in cells with wild-type Cot (Fig. 7B). In a control experiment, staurosporine inhibited phorbol 12-myristate 13-acetate-induced phosphorylation of MEK, indicating that it did inhibit activation of the MEK/ERK pathway via Ras/Raf. Therefore, while Thr-290 phosphorylation was resistant to both staurosporine and herbimycin A treatment, Cot activity in the MEK/ERK pathway was inhibited by herbimycin A.

Thr-290 Is Constitutively Phosphorylated in Human Monocytes—Cot kinase is constitutively active when overexpressed in HEK-293T cells and consequently activates the MEK/ERK pathway. However, in monocytes and macrophages, activation of this pathway requires extracellular stimulation. LPS stimulation of mouse macrophages results in Tpl2 (mouse homolog of Cot)-dependent MEK1 phosphorylation, and $\text{Tpl2}^{-/-}$ macrophages are defective in LPS-stimulated activation of MEK1 and ERK1/2 (4). Additionally we have observed that LPS activates Cot in primary human monocytes. Cot immunoprecipitated from untreated human monocytes will not phosphorylate MEK in vitro, whereas Cot immunoprecipitated from LPS-treated monocytes phosphorylates MEK (data not shown). In these cells, LPS may be inducing downstream phosphorylation of Cot and thus increasing its kinase activity.

To determine whether phosphorylation of Cot threonine 290 increases in response to LPS treatment, we prepared primary monocytes from human whole blood and treated the cells with LPS. As expected, LPS treatment activated Cot phosphorylation of MEK (Fig. 8A). In contrast, we observed no increase in phospho-Cot-Thr-290 in response to LPS; phosphorylation was observed on both LPS-treated and untreated samples. We explored this further by examining both the MEK/ERK and p38 pathways in a time course of LPS treatment using total leukocytes from human whole blood (Fig. 8B). Human whole blood was treated with LPS, and samples were removed at 20-min intervals. Cot activity was measured by phosphorylation of p38 and MEK, which showed response to LPS treatment with peak times of 20 min and 1 h, respectively. The phosphorylation of Cot Thr-290, however, was steady throughout, and quantitation of bands correlated with that of the loading control (total MEK). We conclude from these data that, while necessary for kinase activity, threonine 290 phosphorylation of Cot is constitutive in human monocytes.

**DISCUSSION**

Inflammation is a key process in the host defense system. LPS, the predominant endotoxin produced by Gram-negative bacteria, activates cells involved in innate immunity, such as T cells and macrophages, resulting in the production of the proinflammatory cytokine TNF-α. However, excessive production of
activity. Dephosphorylation of Cot with the overall phosphorylation state of Cot is important for kinase activity. We first demonstrated that largely unknown.

The mechanism of Cot activation by LPS, which presumes inflammatory drugs for therapy of human autoimmune diseases, and psoriasis (35–37). Components of the TNF-α and psoriasis (28), systemic lupus erythematosus (29), multiple sclerosis, diabetes (30, 31), Crohn disease (inflammatory bowel disease) (32), and rheumatoid arthritis (33, 34). To date, anti-TNF-α therapy has been successful in treating rheumatoid arthritis, inflammatory bowel disease, and psoriasis (35–37). Components of the TNF-α production signaling pathway are potential targets for further development of therapeutic agents.

Analyses of Tpl2−/− mice have revealed that Tpl2/Cot acts downstream of LPS signaling and mediates TNF-α production (4). Thus, Cot has emerged as an interesting target for anti-inflammatory drugs for therapy of human autoimmune diseases. The mechanism of Cot activation by LPS, which presumably occurs via the LPS receptor Toll-like receptor 4, is still largely unknown.

Our results demonstrate that phosphorylation of Cot at Thr-290 is critical for kinase activity. We first demonstrated that the overall phosphorylation state of Cot is important for kinase activity. Dephosphorylation of Cot with λ-phosphatase significantly decreases kinase activity, although some residual kinase activity remains. In a similar experiment Caivano et al. (24) were unable to remove any phosphate from Cot using various protein phosphatases 1 or 2A or protein tyrosine phosphatase 1B, although they did not use λ-phosphatase. We observed the same result with these phosphatases as well as calf intestinal phosphatase (data not shown). It may be that λ-phosphatase has only limited access to phosphorylated residues on Cot, and thus λ-phosphatase is only partially effective in removing phosphates from this kinase. Similar results have been shown for other MAP3Ks, which only show a partial reduction of activity after dephosphorylation (14, 20). However, the observed reduction of kinase activity suggests that the phosphorylation state is a key component of Cot activity.

We showed that mutation of threonine 290 to alanine (T290A) eliminated kinase activity in vitro and in the MEK/ERK and NF-κB pathways when overexpressed in HEK-293T cells. The phosphomimetic mutation of Thr-290 to aspartic acid (T290D) rescued a portion of the kinase activity in HEK-293T cells and the ability to autophosphorylate in vitro. These data demonstrate that phosphorylation of this residue is necessary for full activity. The residual activity retained by the T290D construct seen in HEK-293T cells was not measurable in the NFxB pathway. This discrepancy could perhaps be explained by a difference in assay sensitivity, accessibility of substrate, or a threshold effect requiring more Tpl2 activity for NF-κB activation than MEK activation. Nevertheless the fact that Thr-290 mutants lost their kinase activity suggests that phosphorylation of Thr-290 is essential for Cot kinase activity.

One of our most significant findings was that kinase-dead Cot K167R is phosphorylated at Thr-290. We conclude from this observation that another kinase must phosphorylate Cot at this site. This finding represents the first case in which a MAP3K is phosphorylated at this conserved residue in the activation loop via another kinase. This situation is in contrast to that of the other MAP3Ks for which phosphorylation at this residue is required for kinase activity. For MEK kinase 1, TAK1, and Sak2, phosphorylation at this residue occurs via autophosphorylation either intra- or intermolecularly (13–15).

The phosphorylation at Thr-290 appears to be constitutive in monocytes and total blood leukocytes, perhaps occurring concomitantly with translation. The fact that endogenous Cot from human monocytes and whole blood leukocytes is constitutively phosphorylated even in unstimulated cells strongly suggests that phosphorylation at Thr-290 precedes other regulatory events.
Constitutive phosphorylation of a site critical for activity has been demonstrated in the case of protein kinase C (38) and B-Raf (39), and this phosphorylation event occurs during transduction of these proteins. While A-Raf and Raf-1 require interaction with Ras-GTP and subsequent phosphorylation at two distinct sites for full activity (40), B-Raf is constitutively phosphorylated at one of the two critical sites and has a phosphomimetic amino acid substituted at the other. In the case of protein kinase C, PDK-1 phosphorylation of the activation loop is required for protein kinase C to autophosphorylate. In all but the novel protein kinase Cs, this phosphorylation is constitutive (38).

A kinase upstream of Cot is suggested by our results using the tyrosine kinase inhibitor herbimycin A. Neither herbimycin A nor staurosporine inhibited Cot in an in vitro assay measuring MEK phosphorylation directly (data not shown). However, treatment of cells with herbimycin A abolished Cot activity in the MEK/ERK pathway. Staurosporine did not affect the activity of Cot in this pathway. These results indicate the existence of a kinase upstream of Cot that is staurosporine-insensitive but is inactivated in the presence of herbimycin A.

We have begun to elucidate the mechanisms by which Cot activation occurs by identifying Thr-290 as being a critical residue in the activation of Cot. Herbimycin A inhibited activation of the MEK/ERK pathway by Cot, while Thr-290 phosphorylation of Cot was unaffected. It is therefore clear that Thr-290 phosphorylation is necessary but not sufficient for kinase activity in this pathway. It is obvious from the drug sensitivity of this pathway that there are additional upstream phosphorylation events required for Cot activity. In contrast to the constitutive phosphorylation of Thr-290, these events may be regulated in response to extracellular stimuli such as LPS. We identified several other phosphorylated residues of Cot and are currently investigating their potential role in Cot activation. Regulation of Cot is of great interest to the signaling field since the Cot/MEK/ERK pathway potentially plays a role in the etiology of inflammatory autoimmune diseases. Our results represent a new insight into the phosphorylation events that activate this kinase.

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