vCLAP, a Caspase-recruitment Domain-containing Protein of Equine Herpesvirus-2, Persistently Activates the IκB Kinases through Oligomerization of IKKγ*

Received for publication, November 8, 2000, and in revised form, December 6, 2000
Published, JBC Papers in Press, December 11, 2000, DOI 10.1074/jbc.C000792200

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vCLAP, the E10 gene product of equine herpesvirus-2, is a caspase-recruitment domain (CARD)-containing protein that has been shown to induce both apoptosis and NF-κB activation in mammalian cells. vCLAP has a cellular counterpart, Bcl10, which is also an activator of apoptosis and NF-κB. Recent studies demonstrated that vCLAP activates NF-κB through an IκB kinase (IKK)-dependent pathway, but the underlying mechanism remains unknown. In this report, we demonstrate that vCLAP associates stably with the IKK complex through direct binding to the C-terminal region of IKKγ. Consistent with this finding, IKKγ was found to be essential for vCLAP-induced NF-κB activation, and the association between vCLAP and the IKK complex induced persistent activation of the IKKs. Moreover, enforced oligomerization of the isolated C-terminal region of vCLAP, which interacts with IKKγ, can trigger NF-κB activation. Finally, substitution of the C-terminal region of IKKγ, which interacts with vCLAP, with the CARD of vCLAP or Bcl10 produced a molecule that was able to activate NF-κB when ectopically expressed in IKKγ-deficient cells. These data suggest that vCLAP-induced oligomerization of IKKγ, which is mediated by the CARD of vCLAP, could be the mechanism by which vCLAP induces activation of NF-κB.

Equine herpesvirus-2 (EHV-2) is a gammaherpesvirus related to other lymphotropic herpesviruses such as herpesvirus saimiri and Epstein-Barr virus. EHV-2 contains 79 reading frames that encode 77 distinct molecules, several of which show significant similarity to cellular genes. One of these molecules, vCLAP (also called vCIPER/E10/vCARMEN) (1–4), a CARD-containing apoptotic protein, was recently found to induce both apoptosis and activation of the transcription factor NF-κB in mammalian cells. vCLAP, like its cellular counterpart Bcl10, contains two domains, an N-terminal CARD that can oligomerize via homotypic interactions and a C-terminal domain that probably functions as the NF-κB activation domain. Because NF-κB activation is considered to be a survival signal, virally encoded proteins, such as vCLAP, may be utilized by viruses as a strategic tool to initiate self-replication or to suppress apoptosis in infected cells (5).

In most resting cells, NF-κB is sequestered in the cytoplasm through interaction with the IκB inhibitory proteins. IκBs mask the NF-κB nuclear localization signal, thereby preventing its nuclear uptake. Exposure of cells to a wide variety of stimuli, such as viral or bacterial infection, inflammatory cytokines, or UV irradiation leads to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of the IκBs (6–9). This allows the activated NF-κB to translocate to the nucleus and activate the transcription of several NF-κB target genes.

The kinase activity responsible for phosphorylation of IκBs is present in a large (700–900 kDa) cytoplasmic complex composed of two catalytic subunits, IKKα and IKKβ (10–14), and a noncatalytic subunit termed IKKγ (also called NEMO, IKKAP1, or FIP-3) (15–18). We and others have recently demonstrated that activation of the IKK complex could be achieved through IKKγ-mediated oligomerization of the IKK kinases, indicating that IKKγ functions as an adaptor to link the IKKs with the upstream regulators of NF-κB (19, 20). Here we show that vCLAP associates directly and specifically with IKKγ through its C-terminal glycine-rich domain and may regulate the activity of the IKK complex through CARD-mediated oligomerization of IKKγ.

MATERIALS AND METHODS

Cell Culture, Transfection, and Treatment—Cells were cultured either in Dulbecco’s modified Eagle’s medium (DMEM) (HeLa, Rat-1, or 5R cells) or DMEM/F12 (293T cells; Life Technologies, Inc.), supplemented with 10% fetal bovine serum, 200 μg/ml penicillin, and 100 μg/ml streptomycin sulfate. Transfections were carried out using LipofectAMINE (Life Technologies, Inc.). Cells were stimulated with either 20 ng/ml recombinant human TNF-α (Sigma) or 0.1 μg/ml AP1510 (Arriad) for the indicated times. NEMO/IKKγ-deficient Rat-1 cells (5R) are a gift from S. Yamaoka.

Expression Vectors and Antibodies—Constructs encoding full-length IKKγ, IKKα, and IKKβ, or vCLAP or truncated mutants have been described previously (1, 20). The FKBP12 fusion of vCLAP C-terminal domain (CTD) was constructed in a modified pcDNA3-T7 vector, which contains a T7 tag sequence, by fusing three tandem repeats of FKBP12 cDNA in frame with the C-terminus of vCLAP-CTD (residues 108–311) as described previously (1). The plasmids expressing the green fluorescent protein (GFP) (pEGFP-CN) and the red fluorescent protein (RFP) (pDsRed1-N1) were from CLONTECH. FLAG-M5 antibody was from Sigma. T7-horseradish peroxidase conjugate antibody was from Novagen. IKKα and IKKγ polyclonal antibodies were from Santa Cruz.

Biochemical Analysis—Immunoprecipitations were performed as described previously (20), and the precipitated proteins were analyzed by PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.

* This work was supported by National Institutes of Health Grant CA85421 (to E. S. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviations used are: EHV, equine herpesvirus; TNF, tumor necrosis factor; CARD, caspase-recruitment domain; IKK, IκB kinase; RIP, receptor-interacting protein; GST, glutathione S-transferase; GFP, green fluorescent protein; RFP, red fluorescent protein; CTD, C-terminal domain; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.
transfected cells, indicating that vCLAP activates endogenous expression was comparable in vCLAP-transfected and non-

interaction of vCLAP with the IKK complex. The inability of vCLAP to induce NF-

RESULTS
vCLAP Interacts with the IKK Complex and Persistently Activates the IKK Kinases—Whereas in resting cells the IKK kinases are inactive, potent activators, such as TNF-α, interleukin-1, or lipopolysaccharide, induce a very rapid IKK activation, detectable within minutes. However, numerous studies have shown that this activation is only transient and after ~30 min decreases to about 25% of its peak value (12, 14, 23). Using a luciferase reporter assay, we and others demonstrated that expression of vCLAP results in a robust activation of NF-κB (1–4). Because of the very high level of this activation, we asked whether vCLAP could persistently activate the IKKs, resulting in a sustained rather than transient activation of NF-κB. To answer this question, endogenous IKKα was isolated by immunoprecipitation from extracts prepared from vCLAP-transfected or TNF-α-treated HeLa cells and assayed for IKK catalytic activity using a GST-IκBα fusion protein as a substrate. Consistent with previous observations, TNF-α stimulation of HeLa cells induced high but transient IKKα kinase activity (Fig. 1A); the activity, which was maximum after 10 min of stimulation, declined sharply with time and was barely detectable after 90 min of stimulation. However, compared with TNF-α stimulation, overexpression of vCLAP in HeLa cells induced a robust and sustained IKKα kinase activity in the absence of any external stimulation (Fig. 1B). IKKα protein expression was comparable in vCLAP-transfected and non-transfected cells, indicating that vCLAP activates endogenous IKKα by a post-translational mechanism. To determine whether vCLAP associates stably with the IKK complex, immunoprecipitates obtained using anti-IKKα or anti-FLAG antibodies were assayed for the presence of vCLAP or the IKK components, respectively. As shown in Fig. 1B, vCLAP was readily detected after precipitation of endogenous IKKα. Moreover, IKKα and IKKγ were also detected in immunocomplexes obtained after precipitation of vCLAP (Fig. 1C). The vCLAP immunoprecipitates also possessed IKKα kinase activity (Fig. 1C). These results provide direct biochemical evidence that vCLAP associates stably with the IKK complex and is able to persistently activate the IKK kinases.

vCLAP Interacts Directly with and Requires IKKγ for Activation of NF-κB—To determine which component of the IKK complex interacts with vCLAP, 293T cells were transfected with expression vectors for FLAG-tagged vCLAP and T7-IKKβ with or without T7-IKKγ. As shown in Fig. 2A, a small amount of IKKβ was coimmunoprecipitated with vCLAP in the absence of ectopic IKKγ. However, a remarkably higher amount of IKKβ was coimmunoprecipitated with vCLAP in the presence of coexpressed IKKγ (Fig. 2A). The ectopic T7-IKKγ was also detected in these complexes (Fig. 2A). No IKKβ or IKKγ were precipitated with the FLAG antibody in the absence of FLAG-vCLAP (Fig. 2A). This result shows that IKKγ mediates the interaction of vCLAP with the IKK complex.

To rule out the possibility that other proteins were necessary for the vCLAP-IKKγ interaction, we analyzed the ability of a GST-IKKγ fusion protein to associate with an in vitro-translated 35S-labeled vCLAP. In agreement with a direct interaction between vCLAP and IKKγ, 35S-labeled vCLAP bound to the GST-IKKγ-FLAG protein but not the GST control (Fig. 2B).

To address the physiological relevance of this finding, we transiently expressed vCLAP in wild type or IKKγ-deficient Rat-1 cells (15). In contrast to wild type Rat-1 cells, no NF-κB activation was elicited in the IKKγ-deficient 5R cells after transfection with the vCLAP construct or treatment with TNF-α (Fig. 2C). This result provides genetic proof for the requirement of IKKγ in vCLAP-induced activation of NF-κB, confirming its role as a molecular adaptor in the assembly of the vCLAP-IKK complex. The inability of vCLAP to induce NF-κB activation in 5R cells cannot be attributed to defects in the NF-κB pathway downstream of IKKγ, because transfection of these cells with IKKγ can restore NF-κB activation by Tax, which is expressed stably in this cell line (Ref. 15 and data not shown).

In contrast to vCLAP, Bcl10 was unable to interact with IKKγ in vitro (data not shown). However, like vCLAP, Bcl10 was able to induce NF-κB activation in Rat-1, but not in 5R cells (Fig. 2C), suggesting that Bcl10 could relay its signal to IKKγ indirectly.

Mapping of the Interaction Domains of vCLAP and IKKγ—We next mapped the regions of vCLAP and IKKγ that are required for their interaction. FLAG-tagged IKKγ was expressed in 293T cells with T7-tagged full-length domain, CARD (residues 1–107), or CTD (residues 108–311) of vCLAP. Extracts prepared from the transfected cells were immunoprecipitated with an anti-FLAG antibody, and the resulting immunocomplexes were analyzed by Western blotting with an anti-T7 antibody that recognizes the T7-vCLAP variants. Both the full-length domain and the CTD of vCLAP were able to bind to IKKγ (Fig. 3A). In contrast, the CARD did not interact with IKKγ (Fig. 3A). The same results were obtained using a GST-IKKγ pull-down assay, which showed that the recombinant

FIG. 1. vCLAP associates stably with the IKK complex and persistently activates the IKK kinases. A, HeLa cells were either left untreated or were incubated with TNF-α for the indicated times and then lysed. The lysates were immunoprecipitated with anti-IKKα antibody, and the immunoprecipitates were assayed for IKK activity by an immune complex kinase assay and analyzed by SDS-PAGE and immunoblotting (IB). B and C, HeLa cells were transfected with expression constructs for vCLAP-FLAG or anti-FLAG (C) antibody. The immunoprecipitates were assayed for IKK activity by immune complex kinase assay and analyzed by SDS-PAGE and immunoblotted with anti-FLAG antibody (B) or anti-IKKα and anti-IKKγ antibodies (C). The cellular extracts were also immunoblotted with anti-IKKα and anti-FLAG (B) or anti-FLAG (C) antibodies.

SDS polyacrylamide gel electrophoresis followed by immunoblotting. GST pull-down assays, luciferase reporter gene assays, and IKK kinase assays were performed as described (20, 22).

Confocal Microscopy—293T cells were grown on coverslips and then transfected with the GFP-tagged IKKγ or RFP-tagged vCLAP separately or together, with the indicated vectors. 24 h after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min. The coverslips were mounted on a glass slide, and the fluorescence was detected by confocal microscopy using an excitation wavelength of 488 nm and a detection wavelength of 522 nm (GFP) or an excitation wavelength of 568 nm and a detection wavelength of 585 nm (RFP). Images were calibrated-averaged with a Kalman filter to increase the signal/noise ratio.
GST-IKKγ fusion protein is able to bind the in vitro-translated 35S-labeled full-length domain or the CTD of vCLAP, but not the CARD of vCLAP (not shown). Combined, these results show that the CTD of vCLAP mediates its interaction with IKKγ.

To extend the characterization of the vCLAP-IKKγ interaction, we expressed T7-tagged vCLAP in 293T cells together with several FLAG-tagged full-length or truncated IKKβ. vCLAP was found to specifically associate with full-length IKKγ but not with the C-terminally truncated IKKγ (1–200) or IKKγ (1–251) (Fig. 3B). Removal of the last 119 amino acids of IKKγ strongly reduced its interaction with the vCLAP (Fig. 3B). Taken together, these data indicate that the interaction between vCLAP and IKKγ involves sequences in the C-terminal region of IKKγ.

To confirm that vCLAP associates intercellularly with IKKγ when the two proteins are coexpressed in the same cell, we cotransfected 293T cells with constructs encoding GFP-IKKγ and RFP-vCLAP fusion proteins and then monitored the subcellular localization of these proteins by confocal microscopy. As shown in Fig. 3C, the two proteins exhibited different patterns of cellular localization when expressed alone. Whereas vCLAP exhibited a clear pattern of discrete and interconnecting cytoplasmic filaments, IKKγ displayed a somewhat punctate cytoplasmic or whole-cell distribution. However, coexpression of the two proteins resulted in redistribution of IKKγ to the vCLAP filaments. This observation is consistent with a direct interaction between vCLAP and IKKγ.
domain that interacts with IKKγ (see above). Interestingly, we
and others have shown that the CARDs of cellular and viral
CLAP proteins are important for homo- and heterotypic interac-
tions (1–4). It is therefore likely that CARD-mediated self-
association of vCLAP could induce oligomerization of IKKγ
resulting in activation of the IKK complex. To test this hypo-
thesis, we generated a fusion protein (CARD-IKKγ-ΔC) composed
of the CARD of vCLAP linked to the N-terminal part (residues
1–200) of IKKγ (IKKγ-ΔC) and determined its ability to induce
NF-κB activation. To rule out the possibility that the CARD-
IKKγ-ΔC chimera functions through interaction with the en-
dogenous IKKγ protein, we examined its ability to activate
NF-κB in the IKKγ-deficient 5R cells. As shown in Fig. 4A,
transient transfection of the CARD-IKKγ-ΔC chimera resulted
in a large increase of NF-κB activity in a dose-dependent man-
ner. In contrast, neither the separate CARD of vCLAP nor
IKKγ-AC were able to activate the NF-κB when transfected at
either low or high doses (Fig. 4A). Moreover, a single point
mutation of a conserved residue in the CARD of vCLAP that
abrogates homodimerization (L49R) (1) prevented NF-κB acti-
vation by the chimeric protein (Fig. 4A). Similar results were
obtained when the CARD of Bel10 was used instead of vCLAP-
CARD in the above experiments (data not shown).

We then tested whether enforced oligomerization of the CTD
of vCLAP could induce NF-κB activation. For this purpose, the
CTD of vCLAP was fused to a 3-fold repeat of the FKBP12
polypeptide, which oligomerizes when it binds to the cell-per-
meable synthetic organic ligand AP1510 (29). As shown in Fig.
4B, transient tranfection of this construct into Rat-1 cells, but
not in the IKKγ-deficient 5R cells, induced a large NF-κB activa-
tion in a ligand-dependent manner. No NF-κB activation was
detected when the FKBP12-CTD construct was cotrans-
fected with kinase-inactive IKKβ (not shown) or after treat-
ment of empty vector-transfected 293T cells with AP1510 (Fig.
4B). Taken together, these results demonstrate that vCLAP-
induced oligomerization of IKKγ is the triggering event lead-
ting to activation of the IKK complex.

DISCUSSION

Although previous studies have shown that the EHV-2-en-
coded vCLAP protein activates NF-κB in mammalian cells (1,
4), the mechanism by which vCLAP interfaces with the cell’s
NF-κB-activating machinery remains unclear. In this paper,
we report several observations that, when combined, provide
a potential mechanism for vCLAP-induced NF-κB activation.
First, we show that the IKK kinases are persistently activated
in vCLAP-expressing cells, which might explain the robust
NF-κB activity observed in vCLAP-transfected cells (1, 4).
Second, we demonstrate that vCLAP, via its C-terminal domain,
interacts physically with the IKK complex through direct bind-
ing to the C-terminal part of IKKγ. Consistent with this obser-
vation, IKKγ was found to be essential for vCLAP-induced
activation of NF-κB. Third, we demonstrate that vCLAP activ-
ates the IKK complex through oligomerization of IKKγ. In-
deed, CARD-dependent clustering of the N-terminal part of
IKKγ, which we have previously shown to interact with the
IKK kinases (20), was able to activate NF-κB. Moreover, en-
forced oligomerization of the CTD of vCLAP was able to induce
a large increase in NF-κB activity in Rat-1 cells but not in the
IKKγ-deficient 5R cells. This mechanism of NF-κB activation
by vCLAP, namely oligomerization-induced activation of the
IKK kinases via IKKγ, is reminiscent of the model we proposed
for RIP-induced activation of NF-κB after ligation of TNF-R1
(20). Based on this model, TNF-α stimulation induces binding
of RIP to, and concomitant oligomerization of IKKγ, which in
turn passes the oligomerization signal to the effector kinases,
resulting in their activation through autophosphorylation of
their T-loop serines (20). However, in contrast to vCLAP, which
binds stably to the IKK complex, RIP releases the activated
IKK complex after its oligomerization, resulting in transient
rather than persistent activation. Therefore, our results illus-
trate the ubiquitous role of oligomerization in IKK activation.

Activation of NF-κB by expression of a single viral protein
has been described in several studies, indicating that infection
with an intact virus is not always required for NF-κB activa-
tion. One well documented example of this is the human T-cell
leukemia virus-Tax protein (30–33). Tax has been shown to
interact with the IKK complex through direct interaction with
IKKγ (25–27). Moreover, Tax mutants defective in IKKγ bind-
ing failed to activate NF-κB (34). Interestingly, Tax has been
shown to function as a dimerizer that stabilizes dimer for-
ma
induced oligomerization of IKKγ is, as for vCLAP, the triggering event in the activation of the IKK kinases by Tax.

Recently, several independent groups have demonstrated that the cellular homologue of vCLAP, Bcl10 (also called cCLAP/CIPER/hE10/CARMEN), was also able to activate NF-κB when expressed in cells, although at a lesser degree than vCLAP. Bcl10 requires IKKγ for activation of NF-κB (Fig. 2C). Our preliminary results suggest that cCLAP interacts with the IKK complex, as immunoprecipitation of the endogenous cCLAP results in isolation of the IKK components. However, in a GST-IKK pull-down assay, in vitro-translated 35S-labeled cCLAP was not able to bind to recombinant GST-IKK or GST-IKKα (data not shown), indicating that the cCLAP-IKK complex interaction could be indirect or regulated by a post-translational modification of cCLAP, such as phosphorylation. Future studies will reveal the precise physiological function of cCLAP and how it activates the IKK complex.

Acknowledgment—We thank W. C. Green for the IKKβ kinase inactive construct and S. Yamaoka and A. Israel for the NEMO/IKK-deficient Rat-1.

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J. Biol. Chem. 2001, 276:3183-3187.
doi: 10.1074/jbc.C000792200 originally published online December 11, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000792200

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