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A Retroviral-Derived Immunosuppressive Peptide Activates Mitogen-Activated Protein Kinases

Akio Takahashi, Noorbibi K. Day, Voravich Luangwedchakarn, Robert A. Good, and Soichi Haraguchi

The highly conserved region within the retroviral transmembrane envelope proteins has been implicated in a number of retrovirus-associated mechanisms of immunosuppression. CKS-17, a synthetic peptide representing the prototypic sequence of the immunosuppressive domain, has been found to suppress numerous immune functions, disregulate cytokines, and elevate intracellular cAMP. In this report we show that using a human monocytic cell line THP-1, CKS-17 activates mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase 1 and 2 (ERK1/2). Kinetic studies show that CKS-17 induces an acute increase of ERK1/2 activity followed by a rapid decrease and then a second sustained increase of ERK1/2. CKS-17 also activates MAP kinase/ERK kinase (MEK) with a similar induction pattern. Mutant THP-1 cells isolated in our laboratory, in which CKS-17 exclusively fails to activate cAMP, did not show the transient decrease of CKS-17-induced ERK1/2 phosphorylation. Pretreatment of THP-1 cells or mutant THP-1 cells with cAMP analog or forskolin followed by treatment with CKS-17 showed no activation of MEK or ERK1/2. These results indicate that CKS-17 activates the MEK/ERK cascade and that there is a cross-talk between CKS-17-mediated MEK/ERK cascade and cAMP in that the MEK/ERK cascade is negatively regulated by cAMP. These data present a novel molecular mechanism(s) by this highly conserved retroviral immunosuppressive component. The Journal of Immunology, 2001, 166: 6771–6775.

The highly conserved region within the retroviral transmembrane glycoproteins of numerous animal and human retroviruses share conserved structural features (1–3). One highly conserved region of the transmembrane proteins contains a leucine zipper-like domain comprising an α helical secondary structure that may play an important role in the processes of virus fusion, infectivity, and entry (4–7). Of interest, this leucine zipper-like domain overlaps a unique region that has strong immunosuppressive potential (5, 7, 8). CKS-17, a synthetic peptide, represents the prototypic amino acid sequence of this immunosuppressive domain (9) and has been found to exhibit potent immunosuppressive activities in numerous immune reactions both in vitro and in vivo (reviewed in Ref. 8).

We have also reported that CKS-17 induces intracellular cAMP using a human monocytic cell line THP-1 and PBMCs (10). cAMP, an important intracellular second messenger, controls various immune functions, particularly of suppression of the Th1 type of immune responses (11–14). Currently we are trying to elucidate another signal transduction pathway, i.e., mitogen-activated protein (MAP) kinase, induced by CKS-17. MAP kinase, also known as extracellular signal-regulated kinase (ERK) 1 and ERK2, cascades have been widely studied in view of their role as signal transduction pathways through receptor activation by ligand binding (15). Recently, it has been reported that activation of MAP kinase pathway plays a role in enhancing viral infection and replication (16) or in suppressing Th1-related cytokine production (17).

In this study, we show that CKS-17 activates MAP kinases, ERK1 and ERK2. CKS-17 also activates MEK. Kinetic studies demonstrate that CKS-17 induces an acute activation followed by a rapid, transient inactivation and a second activation of ERK1/2 or MEK. This provocative finding and the considerations described above prompted us to further assess the cross-talk between CKS-17-induced MAP kinase and cAMP. We show that pretreatment of THP-1 cells with cAMP-elevating agents suppresses significantly CKS-17-induced activation of ERK1/2 and MEK, indicating that cAMP negatively regulates MAP kinase activation by CKS-17 upstream of MEK.

Materials and Methods

Reagents and Abs

PD98059 (18, 19) (Alexis Biochemicals, Pittsburgh, PA), U0126 (20), forskolin, N6,2′-O-dibutyryladenosine-3′,5′- cyclic monophosphate, and sodium salt monohydrate (dibutylryl-cAMP) (Biomol, Plymouth Meeting, PA) were used to treat cells. Mouse anti-phospho-ERK1/2 (p-ERK1/2) mAb (E10), rabbit anti-ERK1/2 Ab, rabbit anti-phospho-MEK1/2 (p-MEK1/2) Ab, rabbit anti-anti-MEK1/2 Ab, rabbit anti-phospho-Erk1 Ab, inactive ERK2 full length recombinant protein, and inactive Elk-1 fusion protein were purchased from New England Biolabs (Beverly, MA). Goat anti-mouse IgG Ab conjugated to HRP and goat anti-rabbit IgG Ab conjugated to HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of synthetic peptide

A dimer of CKS-17, termed MN10021, [LQNRRGLDILFLKEGGGLC2] was prepared by inclusion of the naturally occurring cysteine at the carboxyl terminus and dimerization via a cysteine-disulfide linkage. The monomer of CKS-17, termed MN10022 (MN22), [LQNRRGLDILLFLKEGGGLC] and the reverse peptide dimer, termed MN20050 (MN50),
[LGGEKLFFDLLGRRNQLC] were prepared similarly. These peptides are the gifts of George Cianciolo (Duke University Medical Center, Durham, NC).

**Cell line**

THP-1, a human acute monocytic leukemia cell line (21), was obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were routinely maintained in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 5 x 10^{-5} M 2-ME at 37°C in a 5% CO₂ incubator.

**Establishment of mutant THP-1 cell lines**

To select mutant cells that are unable to induce cAMP by treatment with CKS-17, THP-1 cells were mutagenized with 400 μg/ml of ethyl methanesulfonate and cloned under limiting dilution conditions. Mutant cells that did not induce intracellular cAMP by treatment with CKS-17 were screened by cAMP assays and further cloned under limiting dilution conditions. Mutant cell lines, THP-1:mCl1.1 (C1) and THP-1:mG7.1 (G7), were selected for this study.

**Treatment of cells**

Subconfluent cells were cultured in RPMI 1640 without FCS for 24 h before treatment. Serum-starved cells were washed once with PBS and resuspended in serum-free and protein-free hydridoma medium (Sigma, St. Louis, MO), then treated with reagents as indicated in figure legends at 37°C using 1.5-ml microcentrifuge tubes.

**Western blotting**

Stimulated cells were lysed with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (octylphenoxy)polyethoxyethanol CA-630, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor mixture (Sigma, St. Louis, MO), then treated with reagents as indicated in figure legends at 37°C using 1.5-ml microcentrifuge tubes.

**Immunoprecipitation**

The cells were lysed with ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml leupeptin, and protease inhibitor mixture. The lysates were centrifuged, and the resulting supernatants were adjusted to equal amounts of protein. The supernatants were incubated for 1 h on ice with anti-p-MEK1/2, and then incubated with 20 μl of protein A-Sepharose 4B beads (Zymed Laboratories, South San Francisco, CA) overnight at 4°C with gentle rocking. To immunoprecipitate active ERK, the supernatants were incubated with anti-p-ERK1/2 (C-terminal antibody) for 1 h on ice with anti-CEP1/2, and then incubated with 20 μl of protein A-Sepharose 4B beads (Zymed Laboratories, South San Francisco, CA) overnight at 4°C with gentle rocking. The beads were washed twice with lysis buffer and subjected to in vitro kinase assay or Western blotting.

**Immunocomplex in vitro kinase assays**

The kinase activities of ERK1/2 and MEK were determined by nonradioactive protein kinase assay system (New England Biolabs). The precipitated immunocomplexes were washed with kinase buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml leupeptin, and protease inhibitor mixture. The supernatants were subjected to in vitro kinase assay or Western blotting.
CKS-17 phosphorylates MAP kinases ERK1 and ERK2

The influence of CKS-17 on MAP kinase (ERK1 and ERK2) activation using THP-1 cells is shown in Fig. 1. THP-1 cells were cultured in serum-starved medium for 24 h and then stimulated with 30 μM of CKS-17 (dimer), MN22 (monomer), or MN50 (reverse dimer). Kinetic studies showed that CKS-17 induced an acute increase of p-ERK levels followed by a rapid decrease and a second activation that sustains elevated levels of p-ERKs determined by Western blotting and scanning densitometry (Fig. 1, A and B). This increase of p-ERK levels was observed within 30 s of treatment and at least 24 h after treatment (data not shown). The control monomer peptide MN22 and reverse peptide dimer MN50 did not induce the increase of p-ERK levels. Fig. 1, C and D, shows the influence of various concentrations (3, 10, and 30 μM) of CKS-17 on MAP kinase phosphorylation. As shown, p-ERK levels were remarkably induced by CKS-17 in a dose-dependent manner both at 2 and 60 min after treatment. MAP kinase phosphorylation by CKS-17 was inhibited by PD98059 and U0126, specific inhibitors of MAP kinase/ERK kinase (MEK) (Fig. 1, C and D).

CKS-17 activates kinase activity of MAP kinase and MEK

We next examined ERK and MEK kinase activity using an in vitro kinase assay. Immunoprecipitated active ERKs and MEK were incubated with an inactive Elk-1 fusion protein, a substrate of ERKs (22), and an inactive recombinant ERK2 protein, a substrate of MEK, respectively. Phosphorylation of Elk-1 or ERK2 was determined by Western blotting using a phospho-Elk-1 (serine 383)-specific Ab or p-ERK1/2 Ab, respectively. As shown in Fig. 2, A and B, the in vitro kinase assay shows that CKS-17 activates MAP kinase and MEK in a similar pattern to the induction of p-ERK levels indicated in Fig. 1, A and B. In addition, both PD98059 and U0126 inhibit MAP kinase phosphorylation by CKS-17 (see Fig. 1, C and D). These results suggest that CKS-17-induced activation of MAP kinase is mediated by MEK activation.

Mutant THP-1 cell lines in which CKS-17 fails to increase cAMP levels do not exhibit initial decrease of p-ERK1/2 levels induced by CKS-17

Because previous experiments in our laboratory have shown that CKS-17 induces increased intracellular levels of cAMP in THP-1 cells (10), we next investigated the influence of cAMP on MAP

Quantitation of cAMP levels

Intracellular cAMP levels were determined as described previously (10). Briefly, cells (1 × 10⁶) were treated with CKS-17 at 37°C and centrifuged. Five hundred microliters of ice-cold 65% ethanol were added to the pellet, which was vortexed and centrifuged at 2000 × g for 15 min at 4°C. The supernatant was transferred to a new tube, dried in a SpeedVac concentrator (Savant, Holbrook, NY), and stored at −20°C. Just before use, the dried extracts were dissolved in 1 ml assay buffer, and cAMP levels were measured by using a cAMP enzyme immunoassay system (Amersham Pharmacia Biotech).

Results

CKS-17 phosphorylates MAP kinases ERK1 and ERK2

The influence of CKS-17 on MAP kinase (ERK1 and ERK2) activation using THP-1 cells is shown in Fig. 1. THP-1 cells were cultured in serum-starved medium for 24 h and then stimulated with 30 μM of CKS-17 (dimer), MN22 (monomer), or MN50 (reverse dimer). Kinetic studies showed that CKS-17 induced an acute increase of p-ERK levels followed by a rapid decrease and a second activation that sustains elevated levels of p-ERKs determined by Western blotting and scanning densitometry (Fig. 1, A and B). This increase of p-ERK levels was observed within 30 s of treatment and at least 24 h after treatment (data not shown). The control monomer peptide MN22 and reverse peptide dimer MN50 did not induce the increase of p-ERK levels. Fig. 1, C and D, shows the influence of various concentrations (3, 10, and 30 μM) of CKS-17 on MAP kinase phosphorylation. As shown, p-ERK levels were remarkably induced by CKS-17 in a dose-dependent manner both at 2 and 60 min after treatment. MAP kinase phosphorylation by CKS-17 was inhibited by PD98059 and U0126, specific inhibitors of MAP kinase/ERK kinase (MEK) (Fig. 1, C and D).
First, we established mutant THP-1 cell lines C1 or G7, which were unable to produce cAMP by CKS-17 (see Materials and Methods). Fig. 3A shows that while CKS-17 significantly induces cAMP in THP-1 cells, CKS-17 completely fails to elevate intracellular cAMP levels in mutant cells, C1 or G7. These mutant cell lines are able to produce cAMP by forskolin, a direct activator of adenylate cyclase (data not shown). Next, both C1 or G7 cells were treated separately with CKS-17 for various times, and p-ERK1/2 levels were determined by Western blotting. As shown in Fig. 3B, the initial decline of p-ERK1/2 levels by treatment of CKS-17 that was observed using THP-1 cells (see Fig. 1, A and B, and Fig. 3B) was abolished using C1 and G7 cells.

cAMP inhibits MAP kinase and MEK activation induced by CKS-17

Based on these results suggesting that cAMP may be involved in the initial decline of MAP kinase activation, we next determined whether elevated intracellular cAMP suppresses CKS-17-induced MAP kinase phosphorylation using C1 or G7 cells. These cells were pretreated with forskolin or dibutyryl-cAMP (db-cAMP), a membrane-permeable cAMP analog, and then incubated with CKS-17 for 2, 10, 60, and 120 min. Fig. 4, A and B, shows that both forskolin and db-cAMP dramatically reduced ERK1/2 phosphorylation induced by CKS-17 using C1 or G7 cells. Thus, our experiments using mutant THP-1 cells had suggested that cAMP was responsible for the initial decline of MAP kinase activation by CKS-17. Experiments were then designed to further investigate this hypothesis. Fig. 5, A and B, shows that the elevated intracellular cAMP also suppressed kinase activity of MAP kinase or MEK using THP-1 cells.

Discussion

In this report, we have added another novel modality for the action of a highly conserved retroviral peptide on the immune system. The most important issue of this study is that CKS-17, known for its immunosuppressive characteristics, for its regulation of cytokines, and for its induction of cAMP, activates MAP kinase, a crucial signaling molecule that is activated by a variety of extracellular stimuli through its receptor (15). In these experiments we used THP-1 cells in which CKS-17 induces a dramatic increase of...
intracellular cAMP levels and mutant THP-1 cells that are unable to increase cAMP levels when treated with CKS-17. Using THP-1 cells we show that CKS-17 induces an initial phosphorylation and kinase activity of ERK1/2 or MEK followed by a rapid decline of activation. With THP-1 mutant cells this rapid decline of activation is abolished, suggesting that cAMP is responsible for the inhibition of activation of MEK and ERK1/2 by CKS-17 observed in THP-1 cells. Pretreatment of both THP-1 and mutant cells with cAMP analog or forskolin inhibited MEK and ERK1/2 activation induced by CKS-17.

It is noteworthy that the activation of MAP kinase signaling pathway enhances HIV-1 replication and infection (16), suggesting that activation of MAP kinase is beneficial to viral infection. In our new evidence, it is possible that viruses or the highly conserved domain of retroviral transmembrane proteins may interact with a putative molecule, activate the MAP kinase cascade, and contribute to the immunopathogenesis associated with enhancement of retroviral infections, viral infectivity, and replication. Further studies, which are beyond the scope of these experiments, are necessary to determine this hypothesis.

From an immunological point of view it is interesting to note that using a murine system activation of the Ras/MAP kinase pathway has been shown to up-regulate Th2 cell differentiation and down-regulate Th1 cell differentiation (23). Furthermore, enhanced activation of ERK results in inhibition of macrophage IL-12 production (17). Previously we and others have reported that CKS-17 primarily down-regulates Th1-type immune response, including inhibition of IL-12 production (reviewed in Ref. 8). As indicated earlier, in our current studies we show that the activation of MAP kinase by CKS-17 is inhibited by pretreatment of a cAMP analog or forskolin. Using mutant THP-1 cells isolated in our laboratory, which unlike the original THP-1 cells do not induce cAMP upon activation by CKS-17, we have demonstrated clearly that the rapid and significant transient down-regulation of MAP kinase phosphorylation is abolished in these cells, thus supporting our hypothesis that elevated cAMP levels are responsible for the brief decline in the process of MEK and ERK1/2 activation by CKS-17. Our finding is also in agreement with the observations from other laboratories using many types of cells, where an increase in intracellular cAMP levels is associated with down-regulation of the Ras/Raf-1/MEK/ERK pathway (24–30). Also, our provocative observations showing that increased levels of cAMP down-regulate MAP kinase challenge us to understand the cross-talk between CKS-17-induced MAP kinase and cAMP.

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References