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ARTICLES

Binding of CDK9 to TRAF2

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Abstract CDK9 has been recently shown to have increased kinase activity in differentiated cells in culture and a differentiated tissue-specific expression in the developing mouse. In order to identify factors that contribute to CDK9's differentiation-specific function, we screened a mouse embryonic library in the yeast two-hybrid system and found a tumor necrosis factor signal transducer, TRAF2, to be an interacting protein. CDK9 interacts with a conserved domain in the TRAF-C region of TRAF2, a motif that is known to bind other kinases involved in TRAF-mediated signaling. Endogenous interaction between the two proteins appears to be specific to differentiated tissue. TRAF2-mediated signaling may incorporate additional kinases to signal cell survival in myotubes, a cell type that is severely affected in TRAF2 knockout mice. J. Cell. Biochem. 71:467–478, 1998.

Key words: cell cycle; kinase; signal transduction; differentiation

cdc2-related kinases have historically been described as direct effectors on cell cycle phase control. The G1 phase in particular possesses at least three of these that associate with cyclically expressed activating proteins called cyclins, leading to the terminology of kinases as cyclin-dependent kinases (CDKs). As a result of this activation, kinase activity of such CDKs as CDK2 and CDK4 varies with cell cycle phase and forces the cell to progress further toward division [MacLachlan et al., 1995]. However, in recent years, several cdc2 relatives have been discovered that diverge from this functional paradigm of cell cycle control, but that have proven no less important to certain critical cellular functions. One cdc2 relative anomaly, CDK5, is in fact involved in keeping cells out of the cell cycle. Kinase activity and expression of CDK5 are localized primarily to neural tissue that is permanently withdrawn from the cell cycle, and is also necessary for normal myogenesis in *Xenopus* [Tsai et al.,1993; Philpott et al., 1997]. Another kinase, CDK7, is involved in phosphorylating the C-terminal domain of RNA polymerase II in a noncell cycle-dependent manner.

The CDK9 kinase also falls into this class of proteins. Cloned by PCR using primers representing conserved domains of all cdc2 relatives, CDK9 (originally termed PITALRE due to its homology to cdc2 in the PSTAIRE region) was identified as a 45-kDa protein that possesses noncell cycle-regulated kinase activity against the pRb protein [Grana et al., 1994]. CDK9 phosphorylates substrates exclusively on serine residues, and is dependent on its associated proteins for optimal kinase activity [Garriga et al., 1996; De Luca et al., 1997]. The CDK9 gene is located on chromosome 9p34.1, a region found altered in myeloproliferative disorders and deleted in 40% of bladder cancers [Bullrich et al., 1995]. An essential cellular function of this cdc2 relative has been elusive until recently, with the discovery of CDK9 involvement in transcrip-

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tion elongation. CDK9 has been shown to bind to the HIV Tat protein, resulting in enhancement of transcription elongation of the HIV genome, and CDK9 has also been found to be the kinase component of the *Drosophila* P-TEFb elongation complex [Mancebo et al., 1997; Zhu et al., 1997]. Both Tat and P-TEFb complexes are able to phosphorylate the C-terminal domain (CTD) of RNA polymerase II which is one of, if not the critical step, in enhancing RNA Pol II's processivity along the DNA template. Kinase activity of CDK9 appears to be essential for this function, as inactivating mutants of CDK9 are able to inhibit Tat-dependent transactivation of an HIV long terminal repeat (LTR)-luciferase construct in Jurkat cells.

CDK9 kinase activity is increased upon terminal differentiation of skeletal muscle and neural cells in vitro, and is expressed in terminally differentiated tissues of the developing mouse such as the dorsal root ganglia and skeletal muscle fibers [Bagella et al., 1998]. CDK9 CTD kinase activity is also induced upon stimulation of T cells and differentiation of monocytes to macrophages [Yang et al., 1997]. Although a GST-Tat protein can strongly precipitate the CDK9 protein from human cells, binding between the two proteins does not appear to be direct. Identification of a cyclin-binding partner for CDK9, called cyclin T, provides the link between the two proteins [Wei et al., 1998]. Cyclin T stabilizes an otherwise weak interaction between Tat and TAR, an RNA component of the HIV genome also essential for efficient transcription elongation. It is has been shown that CDK9 is directed to the appropriate region of the HIV genome by Tat and cyclin T, where it phosphorylates the C-terminal domain of RNA polymerase II (CTD). Activation of CDK9 is also accomplished by this and two additional cyclins, cyclin T2a and T2b [Peng et al., 1998].

Another factor involved in activating transcription of the HIV genome is the NF-κB transcription factor complex [Baltimore, 1995]. NF-κB is retained in the cytoplasm by an associated anchoring protein, IκB, until the appropriate stimulus causes this inhibitor to be phosphorylated and degraded [Baeuerle and Baltimore, 1996]. Intense study has been performed on the regulation of NF-κB activity over the last decade, with many of the proteins contributing to its activation being discovered in the last few years. One of these proteins, TRAF2, is associated with the intracellular domain of the tumor necrosis factor receptor [Rothe et al., 1994, 1995]. Once the receptor is activated by ligand binding, a signaling cascade begins that intimately involves the TRAF2 protein and results in, among other things, the activation of the IκB kinase (IKK) complex [Woronicz et al., 1997; Regnier et al., 1997; Zandi et al., 1997]. It is thought that one reason why tumor necrosis factor (TNF) cannot induce apoptosis in some cell lines is due to TRAF2-dependent activation of NF-κB, the activation of which is antiapoptotic in nature [Van Antwerp et al., 1996; Beg and Baltimore, 1996]. TRAF2 is also associated with TNF receptor 2, a receptor of TNF that predominantly evokes antiapoptotic and proliferative signals [Tartaglia et al., 1991, 1993; Rothe et al., 1994]. Therefore, TRAF2 is apparently essential for cellular survival in the presence of circulating TNF. A number of TRAF2 binding proteins have been identified since its cloning, all playing some role in regulating its ability to transduce the NF-κB activating signal from a cell surface receptor [Cheng and Baltimore, 1996; Song et al., 1996; Lee et al., 1997b]. Mice lacking the TRAF2 gene are deficient in the ability to protect thymocytes and skeletal muscle cells against apoptosis [Yeh et al., 1997; Lee et al., 1997a]. Although much work has been performed in human tumor cell lines and cells of hematopoietic origin to decipher the roles TRAF2 plays in TNF-mediated signal transduction, involvement of TRAF2 in other tissues such as skeletal muscle has not yet been investigated.

Here we describe CDK9's interaction with TRAF2 in vivo upon induction of myogenic differentiation. CDK9 interacts with TRAF2 through the WKI (Tryptophan-Lysine-Isoleocine) motif present in the C-terminus of TRAF2, a motif essential for NF-κB activation mediated by TRAF2. These results suggest an additional kinase acting through TRAF2 cascades and a new scenario in which TRAF2 may function.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

A mouse embryonic cDNA library subcloned into a vector expressing the library as a fusion protein to the VP16 transcriptional activation domain [Hollenberg et al., 1995] was cotransformed into the Y190 yeast strain [Harper et al., 1993] with a vector encoding the open reading frame of CDK9 fused to the GAL4 DNA binding domain. Screening for interacting pro-

teins was performed on synthetic medium lacking tryptophan, leucine, and histidine and incorporating 3-aminotriazole. Positive clones were tested for β-galactosidase activity by freezing in liquid nitrogen and subsequent incubation in Z buffer (16.1 g/l of $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/l of $NaH_2PO_4 \cdot H_2O$, 0.75 g/l of KCl, 0.246 g/l of $MgSO_4 \cdot 7H_2O$, 2.7 ml/l BME (β-mercapto-ethanol), and 0.334 g/l X-gal, pH 7.0).

Glutathione-S-Transferase Fusion and In Vitro Translated Protein Production

DH5α bacteria transformed with pGEX2T-CDK9 or TRAF2 (Pharmacia, Piscataway, NJ) were grown to mid-log phase and subsequently incubated at room temperature for 4 h in the presence of 0.25 mM IPTG. Bacteria were pelleted, resuspended in NENT (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40), and disrupted in a Fisher (Malvern, PA) sonic dismembranator. Supernatant was collected and incubated with glutathione sepharose (Pharmacia), rocking overnight at 4°C. Beads were pelleted, washed, and stored at 40°C in a 50/50 beads/NENT slurry. Proteins were in vitro translated from pcDNA3 vectors (1 μg) using 1U T7 RNA polymerase (Promega, Madison, WI) in the presence of ^{35}S-methionine (10 μCi/ml), amino acid mixture minus methionine (1 mM), and rabbit reticulocyte lysate to a total volume of 25 μl. In vitro binding assays were performed by mixing 5 μg of GST fusion protein to 1 μl of in vitro translated protein and bringing this up to a volume of 300 μl with NENT. Mixtures were rocked for 1 h at 4°C, and beads were pelleted, washed, resuspended in Laemmli sample buffer, and run on a 10% polyacrylamide gel.

Cell Culture and Transfection

C2C12 mouse myoblasts and 293 human embryonic kidney cells were maintained in DMEM supplemented with 2 mM glutamine, 100 μg/ml penicillin/streptomycin, and 10% fetal bovine serum. Differentiation of C2C12 cells was induced after 2 days in growth medium by switching to DMEM (Dulbecco's Modified Eagle's Medium) containing 2% heat-inactivated horse serum for the final 4 days of culture. Unless otherwise noted, growth or differentiation media for all cell types were changed every other day. Two hundred and ninety-three cells were transfected by the calcium phosphate precipitation method, as previously described [Li et al., 1995]. C2C12 cells were transfected with Superfect reagent (Qiagen, Valencia, CA) as per the manufacturer's instructions. Recombinant human TNFα was obtained from Sigma.

Western Blot and Immunoprecipitation

Cell lysis, immunoprecipitations, Western blotting, and affinity purification of anti-CDK9 serum were performed as previously described [Bagella et al., 1998]. Affinity-purified anti-C-terminal TRAF2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Kinase Assays

GST fusion proteins were prepared as described above. Two hundred micrograms of C2C12 cell lysate were precleared with GST protein and subsequently incubated with GST alone, GST-CDK9, or GST-TRAF2 conjugated to sepharose beads. Precipitated beads were washed in lysis buffer and divided into two fractions. The first fraction, in conjunction with beads not incubated with cell extract, was washed in kinase buffer (20 mM HEPES, pH 7.4, and 10 mM MgAcetate) and incubated with 20 μl of reaction buffer (50 mM HEPES, pH 7.4, 25 mM MgAcetate, 50 μM ATP, 2.5 mM DTT (Dithiothreitol), 5 μCi γ^{32}P-ATP, and 1 μg substrate (purified GST-Rb(328–916) protein) at 30°C for 20 min. Thirty microliters of 2 × Laemmli sample buffer were added to stop the reaction, and 30 μl of the reaction were loaded to a 10% polyacrylamide gel.

Immunofluorescence

C2C12 cells were plated sparsely on glass coverslips, placed in growth medium for 24 h, and then shifted to differentiation medium for 96 h. Cells were fixed in fixation buffer (100 mM PIPES, pH 6.8, 5 mM EGTA, 2 mM $MgCl_2$, 3.7% formaldehyde, 1% methanol, and 0.2% Triton X-100), followed by dehydration in ice-cold ethanol. Rehydration was performed with phosphate-buffered saline (PBS), and cells were blocked for nonspecific antibody binding sites by initial incubation with 10% normal goat serum in 5% bovine serum albumin (BSA) in PBS. Primary antibody was incubated with the slides in 0.5% BSA/PBS followed by washes in PBS, and secondary anti-mouse Flourescein-conjugated antibody was incubated with the cells at a concentration of 1:400 followed by washes. UV-fluorescing cells were visualized by

use of a Zeiss Axiophot microscope (Thornwood, NY) equipped with the appropriate filters.

Luciferase Assays

Cells transfected with the appropriate vectors were lysed in reporter lysis buffer (Promega) and run through one freeze-thaw cycle, and cell debris was pelleted. Cell lysates were tested for β-Gal activity to normalize transfection efficiencies by incubating 10 µl of lysate with β-Gal assay buffer (10 mM $MgCl_2$, 4.5 mM BME, 1 mg/ml ONPG, and 0.1 M Na_2HPO_4); cell lysates were then incubated for 30 min at 37°C, brought up to a volume of 700 µl with 1 M $NaCO_3$, and measured for absorbance at 420 nm. Assay for luciferase was performed by incubation of 20 µl of lysate with 100 µl of luciferase assay reagent (Promega), and fluorescence was measured using a luminometer (Berthold Lumat LB9501, Bumdoura, Australia).

RESULTS

Isolation and Characterization of TRAF2 as a Binding Protein of CDK9

In order to identify factors that bind CDK9 during differentiation, we screened a mouse embryonic library derived from days 9–12 of murine gestation, with CDK9 as a bait protein. After subcloning the full coding region of CDK9 into the two-hybrid system bait vector pGBT9, $\sim 1 \times 10^6$ independent clones were screened from the mouse embryonic cDNA library. After 1 week of growth at 30°C, 75 His^+ colonies were obtained, of which 15 were also strongly $LacZ^+$ on the β-galactosidase filter assay [Harper et al., 1993]. Restriction mapping placed these double positives into three groups. Sequence analysis identified group one as the cytoplasmic protein CLIP-115 and group two as an Expressed Sequence Tag, both neural-specific genes that will be described elsewhere. The third group, named PIT4, contained an average 900-bp insert that was 100% homologous to murine TRAF2. The full-length clone of TRAF2 was subcloned into the pGADGH activation domain vector to check for the specificity of interaction with CDK9 in the two-hybrid system. Full-length TRAF2 and PIT4 were able to activate transcription of the LacZ reporter in the presence of CDK9, but not by itself or with a GAL4-DBD/lamin fusion (Fig. 1A). We then extended these results to a separate in vitro binding method to confirm the specificity of interaction between the proteins. pcDNA3-CDK9, -TRAF1, and -TRAF2 constructs were in vitro-translated (ivt) in the presence of ^{35}S-methionine to obtain labeled protein. The proteins were then precipitated with a variety of GST fusion proteins. GST-TRAF2 was able to precipitate ivt CDK9, and GST-CDK9 was able to

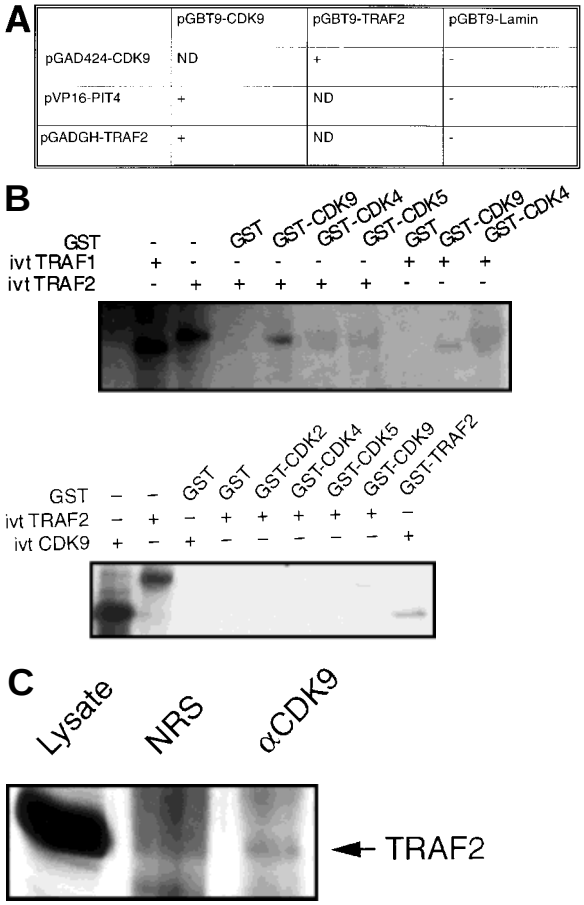

Fig. 1. Interaction of CDK9 and TRAF2. **A:** Summary of yeast two-hybrid interaction. Y190 yeast was transformed with the indicated plasmids and grown at 30°C for 3 days. Individual colonies were picked and streaked on 3-mm paper. Yeast cells were broken by immersing the colonies in liquid nitrogen. Activation of the β-Gal reporter was visualized by subsequently incubating the broken yeast cells in a solution containing X-Gal. pGBT9 is a GAL4-DNA binding domain fusion vectors, and pGAD424, pGADGH, and pVP16 are either GAL4- or VP16-activation domain vectors. +, positive production of β-galatosidase when trasfected with the indiated vectors. ND, not done. **B:** Five micrograms of the indicated GST fusion proteins coupled to beads were incubated with 1 µl of the indicated in vitro-translated protein. Beads were precepitated, washed, and resuspended in Laemmli sample buffer. Associated proteins were run on a 10% polyacrylamide gel. **C:** 293 cells were transfected with overexpression vectors encoding CDK9 and TRAF2 (5 µg of each). Lysates from these cells were immunoprecipitated with polyclonal anti-CDK9 and were Western blotted with anti-TRAF2.

precipitate ivt TRAF2 (Fig. 1B). A weak interaction between GST-CDK9 and TRAF1 and no interaction between GST-cdk 2, 4, or 5 was found; therefore, the binding between CDK9 and TRAF2 appears to be specific. To determine if both proteins interacted in vivo, 293 cells were transfected with expression vectors coding for the full-length CDK9 and TRAF2 proteins. Cell lysates were immunoprecipitated with polyclonal anti-CDK9 and subsequently Western blotted with anti-TRAF2, revealing a weak in vivo interaction (Fig. 1C).

Region of TRAF2 Binding to CDK9

The TRAF2 protein contains distinct domains that are responsible for protein binding and/or signal transduction. Other protein kinases have been found to bind to the TRAF2 domain, while transduction of TRAF2-mediated signaling is dependent on the N-terminal RING and Zn finger domains. To identify the region of TRAF2 with which CDK9 interacts, mutants of TRAF2 that lack specific domains (Fig. 2A) were in vitro-translated, and GST-CDK9 was used to precipitate these mutants in an in vitro binding assay. CDK9 bound strongly to $TRAF2_{1–358}$, but did not bind to $TRAF2_{1–355}$ (Fig. 2B). This implicates a three amino-acid motif present in the TRAF domain (C-terminus) of the protein. It has been reported [Takeuchi et al., 1996] and replicated here (Fig. 2C) that this motif (WKI) is essential for the ability

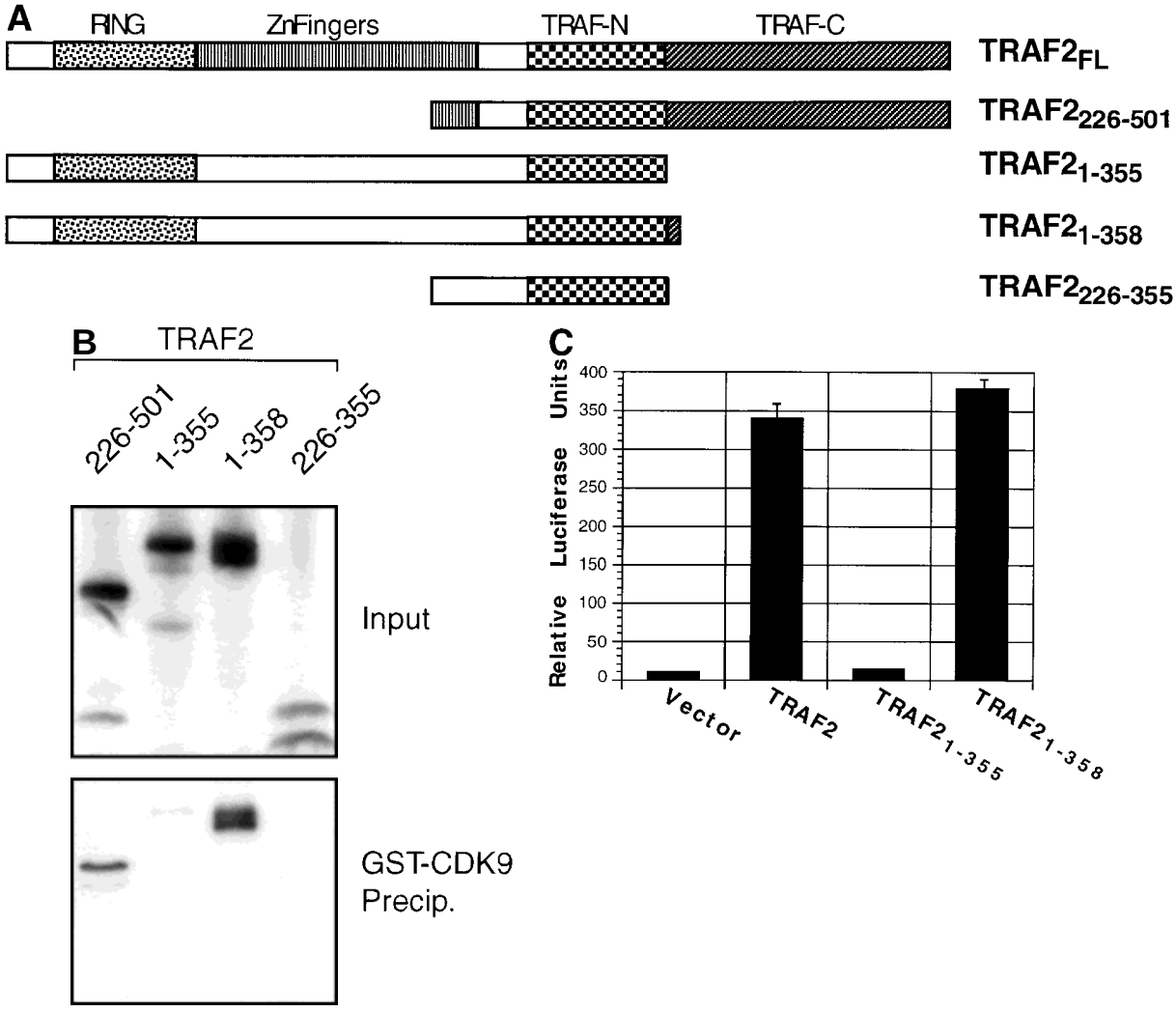

Fig 2. CDK9 interacts with the C-terminus of TRAF2. Deletion mutants of TRAF2 **(A)** were in vitro-translated and precipitated with GST-CDK9 **(B)**. **C:** Wild-type and mutants of TRAF2 were also transfected into C2C12 cells (1 μg) with an NF-κB-controlled luciferase construct, NF-κB-Luc (0.5 μg), and a β-Gal expression vector, and were measured for luciferase activity. Luciferase measurements were normalized for transfection efficiency by measurement of β-Gal activity.

of overexpressed TRAF2 to induce NF-κB activity [Takeuchi et al., 1996]. This motif is also necessary for interaction with the NIK and RIP kinases essential for transduction of TRAF2-mediated TNF signaling [Song et al., 1997]. WKI is present in all TRAF family members, including TRAF1. However, as shown in Figure 2, CDK9 does not bind TRAF1 as stably as TRAF2, suggesting that regardless of the well-conserved nature of this motif, CDK9 is only able to interact with WKI in the context of TRAF2.

Active CDK9 Kinase Complexes Contain TRAF2

To determine if TRAF2 was present in active CDK9 kinase complexes, we sought to determine if a recombinant monomeric GST-CDK9 protein, upon incubation with a cell lysate, possesses kinase activity and if TRAF2 is in this complex. An active CDK9 kinase complex was formed upon incubation with a C2C12 cell extract, while CDK9 alone was not able to phosphorylate pRb (Fig. 3A). TRAF2 is also able to precipitate pRb kinase activity. The second half of the extract-incubated beads was run on a 10% SDS-polyacrylamide gel. A Western blot from this gel identified TRAF2 as being present in the activated CDK9 kinase complex as well as CDK9 being precipitated by GST-TRAF2 (Fig. 3B). These results show that CDK9 stably binds TRAF2 in a cell lysate, but also implicate TRAF2 as a regulatory subunit of CDK9 kinase activity. In CDK9 immunoprecipitates, recombinant TRAF2 does not change CDK9 kinase activity, and neither does overexpression of TRAF2 or TNF treatment of cells prior to lysing (data not shown). These data taken together suggest that TRAF2 is not able to augment CDK9 kinase activity on its own, but may act to solidify the complex or direct CDK9 to substrates.

CDK9 Alters Subcellular Localization During C2C12 Differentiation

CDK9 has been described as a nuclear protein in asynchronously growing HeLa and Saos-2 cells [Grana et al., 1994; De Luca et al., 1997]. This localization poses a problem with regard to its potential interaction with TRAF2. Since CDK9 activity is altered in differentiated systems, we determined if CDK9 subcellular location changes, using muscle differentiation as a model. C2C12 cells were plated onto microscope slides and induced to differentiate. While asynchronously growing myoblasts contained

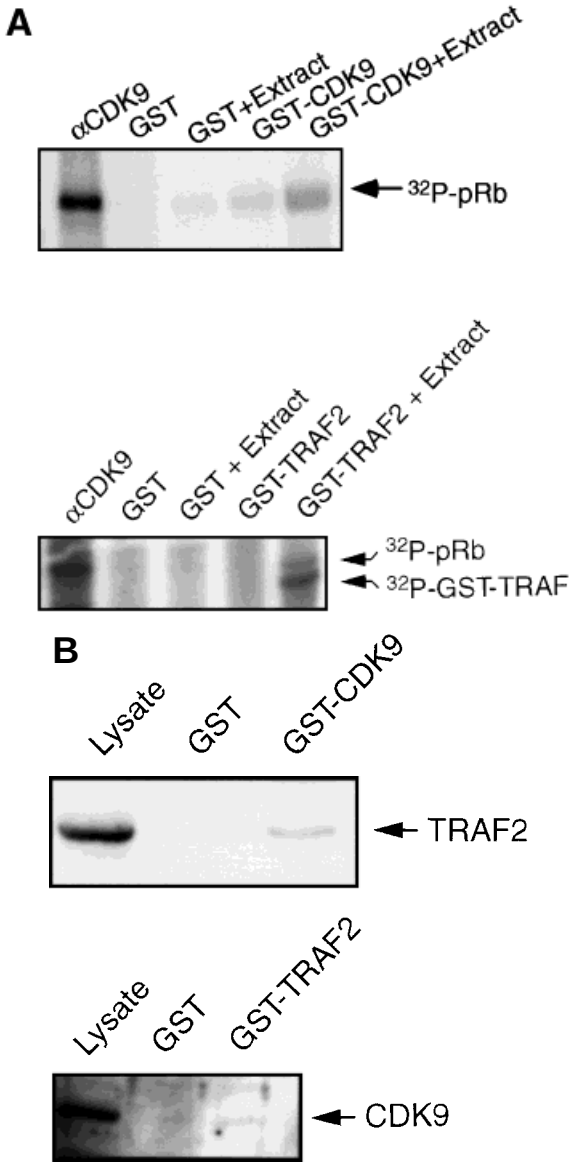

Fig. 3. Presence of TRAF2 in active CDK9 kinase complexes. Glutathione beads coupled to GST, GST-CDK9, or GST-TRAF2 were incubated with a C2C12 cell lysate, washed, and split into two fractions. One half, along with beads that had not been incubated with cell extract, was tested for kinase activity against a recombinant Rb protein **(A)**, and the second half was resuspended in Laemmli sample buffer, run on a gel, and Western blotted for the presence of TRAF2 or CDK9 **(B)**.

CDK9 protein only in the nucleus (Fig. 4A), C2C12 cells incubated in differentiation medium for 96 h contained CDK9 in the cytoplasm as well as in the nucleus (Fig. 4B,C). Similar results were obtained when C2C12 cells were transfected with a vector encoding a fusion protein of green fluorescent protein and CDK9 (Fig. 4D,E,G). This localization change was accompanied by expression of myosin heavy chain,

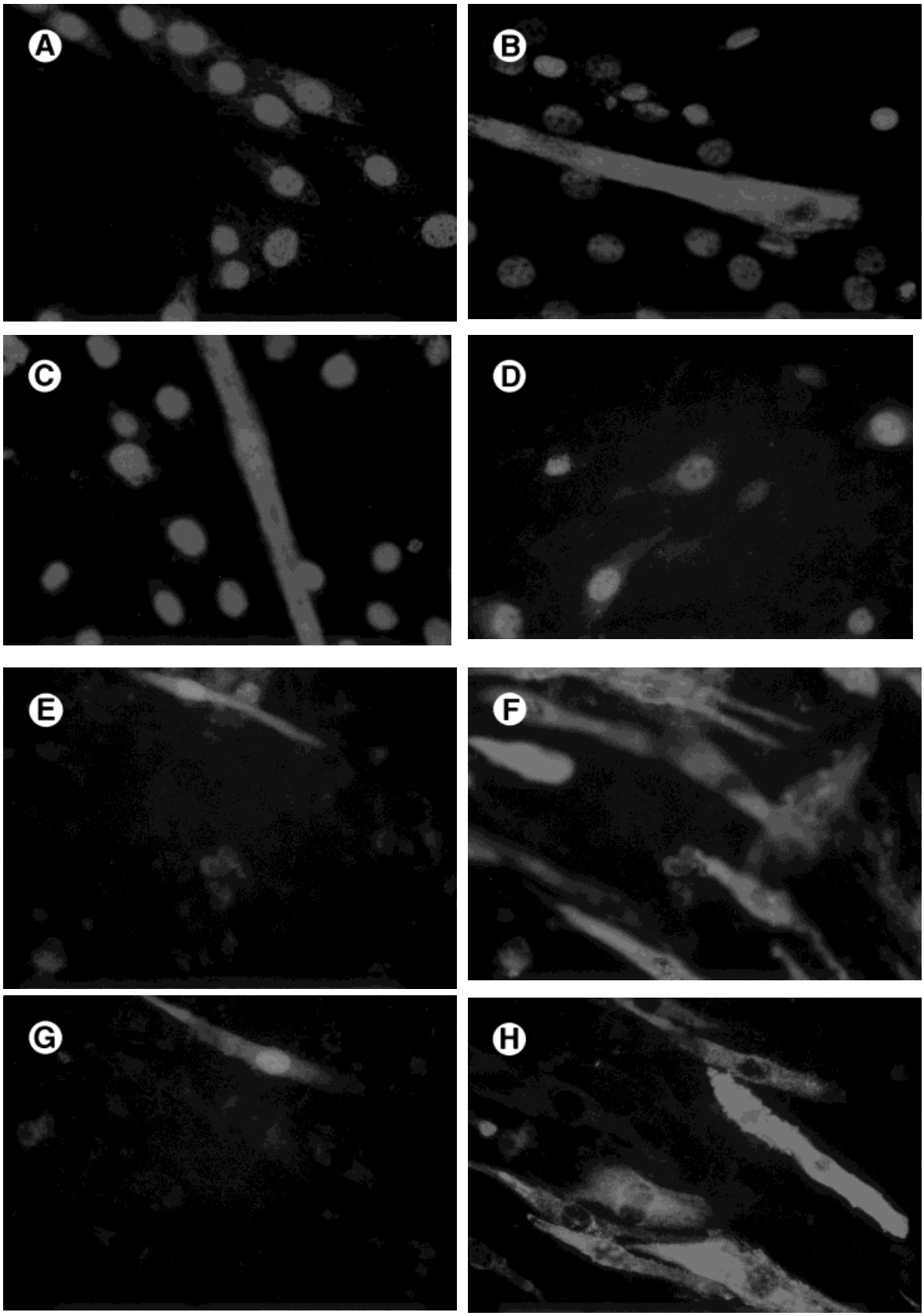

Fig. 4. Localization of CDK9 to the cytoplasm in myotubes. C2C12 cells were grown on glass coverslips and were stained with affinity-purified anti-CDK9 antibody before **(A)** or 96 h after induction of differentiation **(B, C)**. Note the punctate nuclear staining in undifferentiated cells compared to whole-cell staining once differentiation has occurred. C2C12 cells were also transfected with a vector encoding a fusion protein of green fluorescent protein and CDK9, and were fixed for microscopy and visualized before **(D)** or after induction of differentiation **(E, G)**, and were also stained for myosin heavy chain protein induction **(F, H)**. Note that the movement of the fusion protein to the cytoplasm correlates with muscle specific markers (E with F, and G with H).

a marker for muscle differentiation (Fig 4F,H). Significantly less CDK9 was found in the cytoplasm of GFP-CDK9 expressing cells. Since CDK9 possesses an intact nuclear localization sequence, this may be an effect of overexpressing CDK9 from a constitutive promoter. CDK9 equilibrates between the nucleus and the cytoplasm when expressed from its native promoter, as shown in Figure 4B and C.

Endogenous CDK9 and TRAF2 Interact in Differentiated Myocytes

The finding of CDK9 in the cytoplasm in myotubes opened up the possibility for a true in vivo interaction between TRAF2 and CDK9. To determine if this is indeed the case, C2C12 cells were induced to differentiate and cells were harvested every 24 h for 4 days. Lysates from each time point were immunoprecipitated with affinity-purified anti-CDK9 antibody and were subsequently Western blotted with anti-TRAF2 antibody. While an interaction between the two proteins was not detectable during asynchronous growth or early differentiation, a strong interaction was seen with the endogenously expressed proteins when myotubes began to form at 72 h (Fig. 5A). A low-stringency wash identified increasing interaction of CDK9 and TRAF2 that peaked at 72 h of differentiation (Fig. 5B).

Since this and previously published data suggest that this interaction and/or CDK9 kinase activity may be necessary for normal myogenesis to occur, we transfected mutants of CDK9

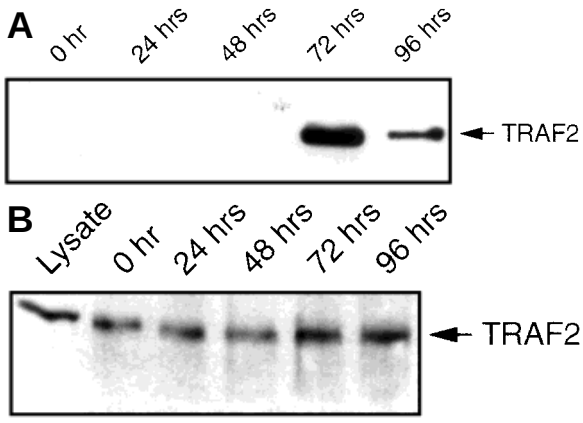

Fig. 5. Interaction of endogenous CDK9 and TRAF2 in myotubes. C2C12 cells were induced to differentiate and were harvested at 24-hour intervals. Lysates from each point were immunoprecipitated with affinity-purified anti-CDK9 and were washed under stringent **(A)** or nonstringent **(B)** conditions. Precipitates were then Western blotted for TRAF2.

and TRAF2 into C2C12 cells to see if this might abrogate myotube formation. A kinase-inactive mutant of CDK9 [Garriga et al., 1996] and the $TRAF2_{1-355}$ mutant that does not interact with CDK9 were found to be expressed in cells that coexpressed myosin heavy chain (data not shown), as determined by double immunofluorescence. Therefore, neither CDK9-TRAF2 interaction nor the kinase activity of CDK9 is required for the progression of differentiation in these cells. However, both of these functions may be necessary for the maintenance of the differentiated phenotype.

CDK9 Effects on TNF-Induced NF-κB Activity in Myotubes

Given the recent data describing TRAF2-interacting kinases, such as NIK [Malinin et al., 1997], that regulate NF-κB activity induced by TNF, we hypothesized that CDK9 kinase activity might be necessary for TRAF2-mediated NF-κB induction. C2C12 cells were used as a model to determine if a kinase-inactive mutant of CDK9, a mutant carrying an Asp → Asn amino-acid change at position 154 resulting in phosphotransfer incapability, blocks NF-κB induction. Cells were transfected with a luciferase construct under the control of NF-κB with the wild-type or kinase-inactive mutant of CDK9 or the pcDNA3 vector alone. Twenty-four hours after transfection, the myoblasts were induced to differentiate into myotubes and were then treated with TNFα. Cells transfected with a kinase-inactive mutant of CDK9 exhibited a decrease in NF-κB activity upon treatment with TNFα compared to cells transfected with the vector alone (Fig. 6). However, the wild-type form of CDK9 equally decreased NF-κB activity. Since CDK9 contains a nuclear localization sequence, it is possible that inappropriate nuclear accumulation may occur when CDK9 is overexpressed (as seen in Fig. 4E and G), interfering with its cytoplasmic function. To address this issue, we constructed a C-terminal deletional mutant of CDK9 that lacks the NLS (CDK9ΔC). Luciferase assays utilizing this form of CDK9 did not show the inhibition of the previous two CDK9 constructs and displayed a mild synergism with TNFα in activation of NF-κB.

DISCUSSION

TRAF2 interaction with kinases has been increasingly documented with the further un-

derstanding of TNF-mediated signal transduction. RIP, a serine/threonine kinase that also interacts with TNFR1 and TRADD proteins, associates with TRAF2. When associated in a complex with TRADD and TNFR1, RIP mediates stimulation of both NF-κB activation and apoptosis. The NF-κB-inducing kinase (NIK) also has been found to associate with TRAF2 through two-hybrid screening. TRAF2's ability to activate NF-κB is absolutely dependent on NIK kinase activity. Indeed, NIK is part of the IKK complex that directly phosphorylates IκB and allows nuclear entry of NF-κB.

We report here that TRAF2 also interacts with CDK9. CDK9, as well as the aforementioned kinases, interacts with a conserved WKI amino acid motif present in the TRAF domain of TRAF2, a domain essential for both kinase interaction and NF-κB activation. Contrary to this point, most data involving mutants of TRAF2 have identified the N-terminal RING finger motif as the indispensable domain for activation of NF-κB, regardless of the fact that all the essential proteins involved in this cascade bind TRAF2 in the C-terminus. One hypothesis raised is that while the RING finger does not directly associate with these other factors, it may modulate or direct the activity of the bound proteins (Song et al., 1997). Therefore, mutants of TRAF2 lacking the RING finger may bind the kinases and other proteins but will not be able to activate them or recruit them to their substrates.

We investigated the possibility of TRAF2 functioning as an activity-modifying subunit of CDK9. Bacterially produced CDK9 protein alone possesses no kinase activity. Only when incubated with a cell extract will the kinase associate with factors that positively affect its kinase activity towards the Retinoblastoma protein. Indeed, TRAF2 is present in this complex, and GST-TRAF2 is able to precipitate CDK9- and pRb-directed kinase activity. However, we found that addition of TRAF2 protein to CDK9 immunoprecipitates or overexpression of TRAF2 in cells does not affect the intensity of CDK9 kinase activity. Therefore, it is possible that TRAF2 does not function as a regulatory subunit for CDK9, but acts as a scaffolding or adaptor protein. A similar scenario has been presented for the CDK7 kinase, which exists as a trimeric complex consisting of CDK7, cyclin H, and MAT1 (DeVault et al., 1995). MAT1, like TRAF2, is also a RING finger protein. Although is not involved in modulating CDK7 kinase activity directly, MAT1 stabilizes the CDK7 complex and directs it to substrates (Yankulov et al., 1997). Perhaps the same is the case for TRAF2-incorporating CDK9 complexes.

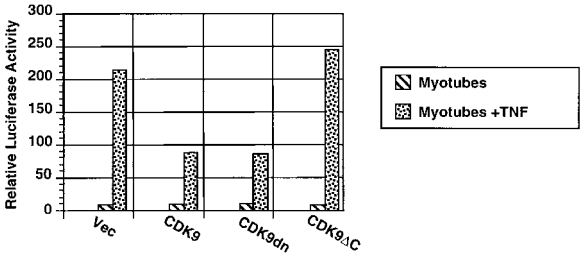

Fig. 6. Effects of CDK9 mutants on NF-κB activation in myocytes and myotubes. C2C12 cells were transfected with β-Gal (0.5 μg), NF-κB Luc (0.5 μg), and the indicated CDK9 expression constructs (1 μg). Cells were treated (myoblasts + TNF) or untreated (myoblasts) with TNFα (20 ng/ml) for 8 h or were allowed to differentiate for 4 days (myotubes, and myotubes + TNF) and were then assayed for luciferase activity. Readings were normalized for transfection efficiency by measurement of β-Gal activity.

We also found that CDK9 undergoes a partial subcellular localization change during myogenesis, an event that has been shown to accompany high levels of CDK9 kinase activity. It is in this locale that it is able to interact with endogenous TRAF2 in vivo, an exclusively cytoplasmic protein. Such localization for cdc2-related kinases is not unprecedented. In fact, one of the kinases that has shared functional homology with CDK9, CDK5, is also localized to the cytoplasm of differentiated neurons. CDK5, like CDK9, increases in kinase activity during myogenesis and neurogenesis, and is thought to phosphorylate cytoplasmic proteins in the terminally differentiated neuron such as the neurofilament and Tau proteins. Additional CDK9-associated proteins identified in the screen that isolated TRAF2 were neural-specific in nature and exclusively cytoplasmic. However, our data on CDK9 suggest that its kinase activity and association with TRAF2 are not essential for differentiation to occur. Maintenance of the differentiated state or survival of the differentiated cell may be the function of CDK9 and/or CDK9-TRAF2 binding. Also supporting a possible cytoplasmic role of CDK9 is the presence of a putative nuclear export signal in the C-terminus of the protein. The leucine- and isoleucine-rich amino-acid sequence LDLIDKLLVL, located at amino acids 289–299 of the kinase, is conserved with the recently

identified Nuclear Export Signals (NES) of the aryl hydrocarbon receptor and of MAP kinase kinase [Ikuta et al., 1998; Fukuda et al., 1997]. It is unusual, however, to find the protein in both the nuclear and cytoplasmic compartments. Experiments are ongoing to further characterize this anomaly as well as to determine if the putative NES truly acts as an export signal.

We show here that overexpression of a kinase-inactive mutant of CDK9 results in a decrease of TRAF2 and TNF-induced NF-κB activity. Kinase-inactive CDK9 and other mutant cdc2 relatives appear to be incapable of nuclear entry (MacLachlan, unpublished observations). Therefore, this form of CDK9 normally resides in the cytoplasm, where it interacts with TRAF2 and decreases NF-κB activation. In order to verify that this decrease is due to the loss of kinase activity, a wild-type CDK9 must lack this inhibition or synergize with TNFα in the induction of NF-κB. However, the full-length wild-type protein, when transfected into C2C12 cells, inhibits NF-κB induction equally as well as the kinase-inactive mutant. We hypothesize that this effect is due to aberrant nuclear localization resulting from overexpression. In differentiated myotubes, we have seen endogenous CDK9 distributed equally between the cytoplasm and nucleus; however, in GFP-CDK9-overexpressing cells, the majority of the protein is transported to the nucleus. It is possible that the overabundance of nuclear CDK9 function may interfere with cytoplasmic function, leading to an inability to view cytoplasmic effects when overexpressed. To alleviate this concern we expressed a C-terminal deletional mutant of CDK9, resulting in loss of the Nuclear Localization Signal (NLS). This form of CDK9 does not possess any inhibitory activity against NF-kB activation upon TNF stimulation, and in fact, mildly synergizes with it. This difference may reflect the possibility that CDK9 cytoplasmic and nuclear functions may be opposing or at least antagonizing.

Finally, with respect to recent data confirming CDK9 involvement in transcriptional elongation, we propose that CDK9 possesses dual functionality. Tat-binding and P-TEFb-incorporating CDK9 clearly is important for efficient transcription elongation, a function that takes place entirely in the nucleus. Immunodepletion experiments performed using antibodies directed against the three T-type cyclin subunits of CDK9 that support this process appear to precipitate almost all CDK9 present in these extracts. However, the extracts used were HeLa nuclear extracts, discounting any cytoplasmic constituent of CDK9. Additionally, ammonium sulfate precipitation fractions from whole-cell extracts has identified both CTD phosphorylating and nonphosphorylating populations of CDK9. Cofractionating with the non-CTD phosphorylating fraction is TRAF2 (Pe'ery and Mathews, personal communication). In fact, GST-TRAF2 precipitations of CDK9, while able to phosphorylate pRb (Fig. 3), were not able to phosphorylate CTD (MacLachlan, unpublished data). We believe that this is preliminary evidence for the prospect of two distinct functional counterparts of CDK9 kinase.

In conclusion, we have identified an interaction between CDK9 and TRAF that takes place in vivo in differentiated myotubes. Given the differentiated feature of this phenomenon, we can begin to understand the previously described differentiation-specific qualities of CDK9 as well as to decipher the role of TRAF2 in nondividing cells.

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REFERENCES

Baeuerle PA, Baltimore D. 1996. NF-kappa B: ten years after. Cell 87:13–20.

Bagella L, MacLachlan TK, Buono RJ, Pisano MM, Giordano A, De Luca A. 1998. Cloning of murine PITALRE and its tissue specific expression. J Cell Physiol, in press.

Baltimore D. 1995. The enigma of HIV infection. Cell 82: 175–176.

Beg AA, Baltimore D. 1996. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274:782–784.

Bullrich F, MacLachlan TK, Sang N, Druck T, Veronese ML, Allen SL, Chiorazzi N, Koff A, Heubner K, Croce CM, Giordano A. 1995. Chromosomal mapping of members of the cdc2 family of protein kinases, cdk3, cdk6, PISSLRE, and PITALRE, and a cdk inhibitor, p27Kip1, to regions involved in human cancer. Cancer Res 55:1199–1205.

Cheng G, Baltimore D. 1996. TANK, a co-inducer with TRAF2 of TNF- and CD 40L-mediated NF-kappaB activation. Genes Dev. 10:963–973.

De Luca A, Esposito V, Baldi A, Claudio PP, Fu Y, Caputi M, Pisano MM, Baldi F, Giordano A. 1997. CDC2-related kinase PITALRE phosphorylates pRb exclusively on serine and is widely expressed in human tissues. J Cell Physiol 172:265–273.

Devault A, Martinez AM, Fesquet D, Labbe JC, Morin N, Tassan JP, Nigg EA, Cavadore JC, Doree M. 1995. MAT1 ("menage a trois"): a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and *Xenopus* CAK. EMBO J 14:5027–5036.

Fisher RP, Jin P, Chamberlin HM, Morgan DO. 1995. Alternative mechanisms of CAK assembly require an assembly factor or an activating kinase. Cell 83:47–57.

Fukuda M, Gotoh I, Adachi M, Gotoh Y, Nishida E. 1997. A novel regulatory mechanism in the mitogen-activated protein (MAP) kinase cascade. Role of nuclear export signal of MAP kinase kinase. J Biol Chem 272:32642–32648.

Garriga J, Mayol X, Grana X. 1996. The CDC2-related kinase PITALRE is the catalytic subunit of active multimeric protein complexes. Biochem J 319:293–298.

Grana X, De Luca A, Sang N, Fu Y, Claudio PP, Rosenblatt J, Morgan DO, Giordano A. 1994. PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein in vitro. Proc Natl Acad Sci USA 91:3834–3838.

Harper JW, Adami GR, Wei N, Keyomarski K, Elledge SJ. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805–816.

Hollenberg SM, Sternglanz R, Cheng PF, Weintraub H. 1995. Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. Mol Cell Biol 15:3813–3822.

Ikuta T, Eguchi H, Tachibana T, Yoneda Y, Kawajiri K. 1998. Nuclear localization and export signals of the human aryl hydrocarbon receptor. J Biol Chem 273:2895–2904.

Lee SY, Reichlin A, Santana A, Sokol KA, Nussenzweig MC, Choi Y. 1997a. TRAF2 is essential for JNK but not NF-kappa-B activation and regulates lymphocyte proliferation and survival. Immunity 7:703–713.

Lee SY, Choi Y. 1997b. TRAF-interacting protein (TRIP): a novel component of the tumor necrosis factor receptor (TNFR)- and CD30-TRAF signaling complexes that inhibits TRAF2-mediated NF-kappaB activation. J Exp Med 185:1275–1285.

Li S, MacLachlan TK, De Luca A, Claudio PP, Condorelli G, Giordano A. 1995. The cdc-2-related kinase, PISSLRE, is essential for cell growth and acts in G2 phase of the cell cycle. Cancer Res 55:3992–3995.

MacLachlan TK, Sang N, Giordano A. 1995. Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit Rev Euk Gene Exp 5:127–156.

Malinin NL, Boldin MP, Kovalenko AV, Wallach D. 1997. MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 385:540–544.

Mancebo HS, Lee G, Flygare J, Tomassini J, Luu P, Zhu Y, Peng J, Blau C, Hazuda D, Price D, Flores O. 1997. P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. Genes Dev 11:2633–2644.

Peng J, Marshall NF, Price DH (1998): Identification of a cyclin subunit required for the function of Drosophila P-TEF. J Biol Chem 273:13855–13860.

Philpott A, Porro EB, Kirschner MW, Tsai LH. 1997. The role of cyclin-dependent kinase 5 and a novel regulatory subunit in regulating muscle differentiation and patterning. Genes Dev 11:1409–1421.

Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. 1997. Identification and characterization of an IkappaB kinase. Cell 90:373–383.

Rothe M, Wong SC, Henzel WJ, Goeddel DV. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 78:681–692.

Rothe M, Sarma V, Dixit VM, Goeddel DV. 1995. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science 269:1424–1427.

Song HY, Rothe M, Goeddel DV. 1996. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation. Proc Natl Acad Sci USA 93:6721–6725.

Song HY, Regnier CH, Kirschning CJ, Goeddel DV, Rothe M. 1997. Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. Proc Natl Acad Sci USA 94:9792–9796.

Takeuchi M, Rothe M, Goeddel DV. 1996. Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. J Biol Chem 271:19935–19942.

Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA, Goeddel DV. 1991. Two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc Natl Acad Sci USA 88:9292–9296.

Tartaglia LA, Goeddel DV, Reynolds C, Fogari S, Weber RF, Fendy BM, Palladino MA. 1993. Stimulation of human T cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J Immunol 151:4637–4641.

Tsai LH, Takahashi T, Caviness VS Jr, Harlow E. 1993. Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development 119:1029–1040.

Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 274:787–789.

Wei P, Garber ME, Fang SM, Fischer WH, Jones KA (1998): A novel CDK9-associated C-type cyclin interacts directly with HIV-1 TAT and mediates its high-affinity, loop-specific, binding to tar RNA. Cell 92:451–462.

Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel DV. 1997. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. Science 278:866–869.

Yang X, Gold MO, Tang DN, Lewis DE, Aguilar-Cordova E, Rice AP, Herrmann CH. 1997. TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. Proc Natl Acad Sci USA 94:12331–12336.

Yankulov KY, Bentley DL. 1997. Regulation of CDK7 substrate specificity by MAT1 and TFIIH. EMBO J 16:1638–1646.

Yeh WC, Shahinian A, Speiser D, Kraunus J, Billia F, Wakeham A, Delapompa JL, Ferrick D, Hum B, Iscove N, Ohashi P, Rothe M, Goeddel DV, Mak TW. 1997. Early lethality, functional NF-kappa-B activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 7:715–725.

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 91:243–252.

Zhu Y, Pe'ery T, Peng J, Ramanathan Y, Marshall N, Marshall T, Amendt B, Mathews MB, Price DH. 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. Genes Dev 11:2622–2632.