[Frontiers in Bioscience 6, d1073-1082, September 1, 2001]

NEW INSIGHT IN CDK9 FUNCTION: FROM TAT TO MYOD

Cristiano Simone, [1,2] and Antonio Giordano[1]

[1]Dept. of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107 USA, [2]Dept. of Internal Medicine and Public Medicine, Division of Medical Genetics, University of Bari, Bari 70124 Italy

TABLE OF CONTENTS

1. ABSTRACT

Cdk9 is a serine-threonine cdc2-related kinase and its activity is not cell cycle-regulated. Cdk9 function depends on its kinase activity and also on its regulatory units: the T-family cyclins and cyclin K. Recently, several studies confirmed the role of cdk9 in different cellular processes such as signal transduction, basal transcription, HIV-Tat- and MyoD-mediated transcription and differentiation.

All the referred data strongly support the concept of a multifunctional protein kinase with specific cytoplasmic and nuclear functions.

2. INTRODUCTION

The cell cycle in mammalian cells consists of four phases, G_1, S, G_2 and M, which govern cell fate, allowing the formation of two fully functional daughter cells.

The first evidence of the molecular basis underlying cell division arose more than 20 years ago, from studies involving both budding and fission yeast (for review article see 1). After discovering cdc2 (cell division cycle 2) in Schizosaccaromyces pombe and CDC28 in Saccharomyces cerevisiae, in 1987 two different research groups characterized the human homologue of these yeast regulatory proteins, which was collectively referred as to cdc2 (2, 3). Since that time, a multitude of cdc2-related kinases have been cloned based on sequence and functional similarity, and functionally characterized as having distinct roles in cell cycle control. Cdks, cyclin-dependent kinases, are the catalytic subunits of complexes whose regulatory subunits are the cyclins. They are a family of proteins named for their cyclic expression and degradation and they play an important role in regulating cell division. Cyclins are synthesized immediately before they are used and their levels fall abruptly after their action because of degradation through ubiquitination (4). Interaction between the cyclins and the cdks occurs at specific stages of the cell cycle, and their activities are required for progression through the cell cycle. Unlike the cyclins, the protein levels of the cdks do not oscillate throughout the cell cycle, allowing the cdks to be present in an inactive form until the formation of complexes with the cyclins (4).

The current model explaining cell cycle involves cyclin-cdk complexes that, by phosphorylating different substrates, govern not only cell division, but also several cellular pathways such as signal transduction, differentiation and apoptosis. One of the best examples to present a new dimension of the concept of cyclin/cdk complexes is cdk9 and its related T cyclins (T1, T2a and T2b) and cyclin K. Cyclin-dependent kinase 9 (cdk9) is a cdc2-related serine/threonine kinase previously named PITALRE, which was isolated using degenerate oligonucleotide primers derived from conserved regions found in cdc2 and cdk2 (5). The deduced amino acid sequence is 47% identical to that of CHED kinase involved in cell cycle control in hematopoietic cells (6) and it is 42% identical with the Saccharomyces cerevisiae SGV1 gene product, a kinase involved in the pherormone response pathway (5). The cdk9 gene is widely

expressed in human and murine tissues with higher levels found in terminally differentiated cells and, in muscle cells, its promoter activity parallels the protein levels (7, 8, 9). The regulatory units of cdk9 are the T-family cyclins (T1, T2a and T2b) and cyclin K (10). Cyclin T1 and cyclin T2 mRNAs are widely expressed in adult human tissues with higher levels in the muscle, spleen, thymus, testis, ovary and peripheral blood lymphocytes. In HeLa nuclear extracts, roughly 80% of cdk9 is complexed with cyclin T1 and 10% with cyclin T2a and cyclin T2b (11, 12). Cyclin K is ubiquitously expressed in adult mouse and human tissue, but is most abundant in developing germ cells of adult testes and ovaries (13). The elevated levels of cdk9 and its regulatory subunits (cyclins T1 and T2) in terminally differentiated cells, together with the fact that cdk9/cyclinT complexes are not cell cycle-regulated, distinguish cdk9 from the other cdks (1, 4). Moreover, unlike the other cdks, which regulate cell cycle progression and phosphorylate histone H1, cdk9 fails to phosphorylate H1. Notably, cdk9 phosphorylates *in vitro* the C-terminus of pRb, the protein product of the Retinoblastoma gene, showing a phosphorylation pattern involving only Ser residues (5, 7, Simone *et al*., submitted). Other *in vitro* substrates of cdk9 include casein and MBP (Myelin Basic Protein) (5). As cyclin T levels are not cell cycle-regulated, cdk9 kinase activity does not change during the different phases of the cell cycle. The catalytic activity of the cdk9 immunocomplex results only from cdk9 and it is abolished using a kinase inactive mutant that behaves as a *dominant negative* form (cdk9dn) (4). This consists of a point mutation within the ATP binding domain that changes the Asp to Asn at residue 167. In addition to these data, colony formation assay performed using several cell lines demonstrated that cdk9dn does not reduce cell growth compared to a mock control, if cdc2dn arrests cells in G_2 (Simone *et al*., unpublished results).

All of these characteristics of cdk9/cyclin function confirm the new concept of a multifunctional cyclin-dependent kinase. This is not the first cdk/cyclin complex to not be involved in the cell cycle; in fact, cdk7/cyclin H, forming TFIIH, cdk8/cyclin C, involved in transcription, and cdk5/p35, active during neural differentiation, confirm the idea of a multifunctional kinase.

3. CDK9-INTERACTING PROTEINS

Immunoprecipitation experiments using anti-cdk9 antibodies have shown that cdk9 co-immunoprecipitates with several proteins. Some of them are now well-known and their identification has given us the opportunity to better understand the function of cdk9 immunocomplexes.

In our lab in 1998, MacLachlan *et al*. used a yeast two hybrid system to identify TRAF2 (a cytoplasmic tumor necrosis factor signal transducer) as a cdk9-interacting protein, suggesting a role for cdk9 in the TNF signal transduction pathway. Cdk9 interacts with a conserved domain in the TRAF-C region of TRAF2, a motif that is known to bind other kinases responsible for TRAF-mediated signaling (14) (Fig 1A). This finding is relevant because it represents a new link between cdk9 and HIV infection due to the involvement of NFkB in both the TNF pathway and HIV genome-activated transcription. The relevance of this interaction is further supported by data on TRAF2 knock-out mice which are defective in the ability to protect skeletal muscle cells against apoptosis. Finally, that report implicated a role of cdk9 in the cytoplasm. In the first studies, cdk9 was characterized as a nuclear protein (5), but Western blotting of nuclear and cytoplasmic extract in human and rodent cells confirmed that the kinase is present in both subcellular compartments (Simone *et al*., unpublished results).

Two years later, we demonstrated cdk9-B-Myb binding by identifying a mechanism in which cdk9 could inhibit B-Myb autoregulation (15) (Fig 1B). This is interesting because it further suggests that cdk9 could be involved in the signaling pathways that promote differentiation and inhibit cell cycle progression and DNA synthesis.

More recently, O'Keeffe et al. described other cdk9-associated proteins through affinity purification of the immunocomplex in human cells. They demonstrated that cdk9 could be present in the cell in at least three major complexes in addition to free cdk9 and that it requires the interaction with the kinase-specific chaperone pathway. In light of this observation, it seems that the pathway involving the sequential actions of Hsp70 and Hsp90/Cdc37 may be used for cdk9 folding and cdk9/cyclin T1 assembly. In an initial step, newly translated cdk9 may be bound by Hsp70, which acts to stabilize cdk9 and to assist the correct folding of the nascent polypeptide. Once cdk9 has reached a certain folded state, Hsp70 may mediate its transfer to Hsp90/Cdc37. This kinase-specific chaperone complex probably establishes and maintains the cdk9 molecule in a state that can readily form a complex with cyclin T1 during the final stage of the assembly process. When many cdk9 proteins are moving along this pathway to eventually become part of the mature cdk9/cycT1 complex, a major portion of cdk9, either translated without the protection of Hsp70 or dissociated from the chaperones along the pathway, is rapidly degraded in the cell. Finally, among the three major cdk9 complexes, only the cdk9/cyclin T1 dimer contains all the activities attributed to P-TEFb. The two chaperone-cdk9 complexes are important precursors that are essential for the generation of the mature and active cdk9/cyclin T1 P-TEFb complex (16) (Fig 1C).

Finally, we characterized the physical interaction between cdk9, cyclin T2 and pRb, and found that the C-terminus of pRb (835-928) directly interacts with the region surrounding the kinase domain of cdk9 (129-195) and with both isoforms of cyclin T2 (a and b), suggesting that the region of interaction involves the common sequence 1-642 aa. In addition, cdk9 complexes phosphorylate the C-terminus of pRb (793-928) *in vitro* and our data suggest that the phosphorylated region spans the amino acids 793-834 (Simone *et al*., submitted; Fig 1D). This evidence is intriguing because it confirms the division of the pRb C-terminus into two subdomains. In fact, several groups have reported that the pRb C-terminus (793-928) can be separated into two subdomains with specificity for cdk/cyclin complexes. The first region (793-829) contains

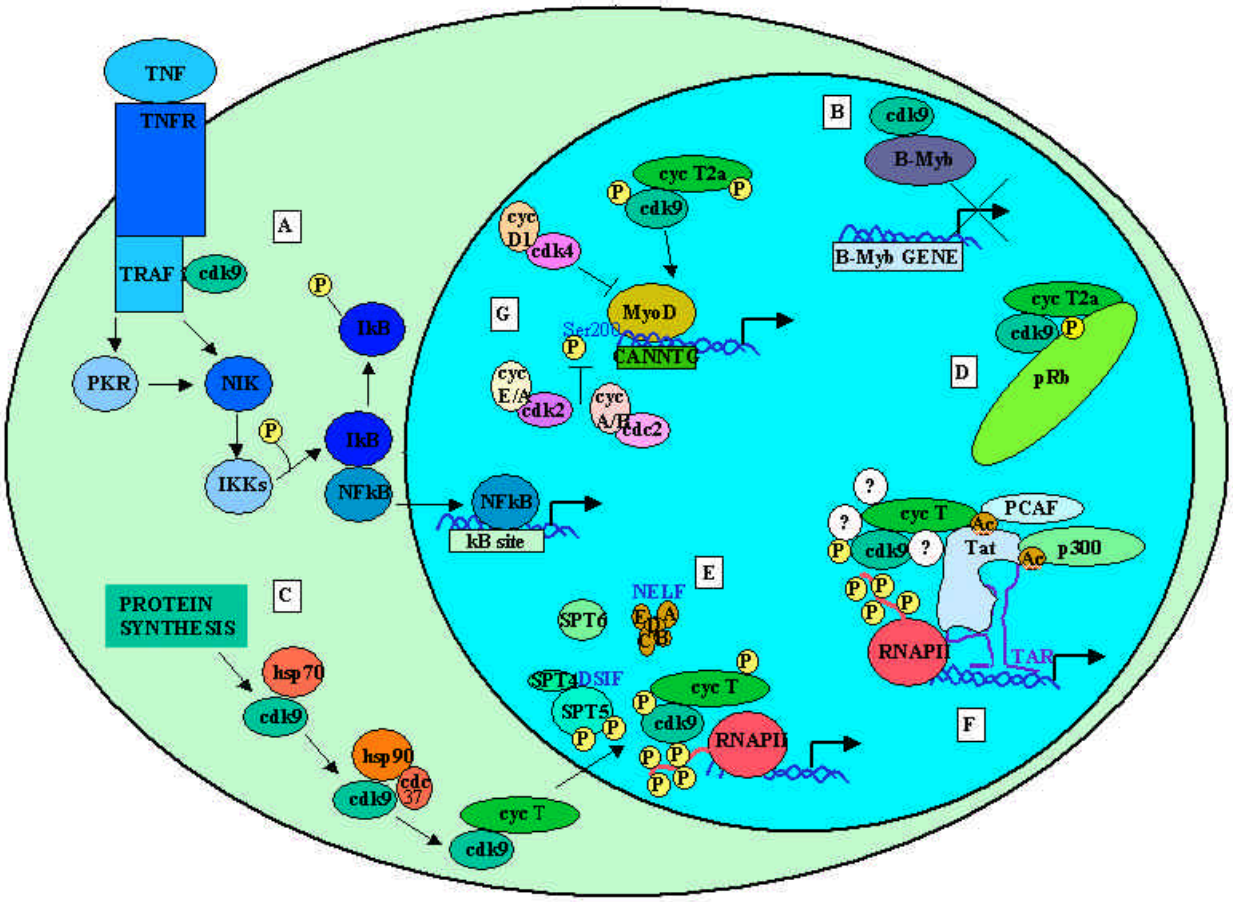

Figure 1. Schematic model of cellular pathways involving cdk9 (A) Cdk9 physically interacts with TRAF2 in differentiated muscle cells, (B) Cdk9-B-Myb interaction down-regulates B-Myb gene expression, (C) The kinase-specific chaperone pathway is required to produce stable cdk9/cyclin complexes, (D) Cdk9/cycT2 complex interacts and phosphorylates the C-terminal domain of pRb, (E) Cdk9/cycT is involved in transcriptional elongation phosphorylating the CTD of RNA pol II and the regulator Spt5, (F) HIV Tat-mediated transcription requires several cellular factors including cdk9/cycT1, (G) Cdk9/cycT2a activates MyoD-dependent transcription

the cdk phosphorylation sites and the second (829-928) is the recognition/binding site for cdk/cyclin complexes (17, 18, 19). During the cell cycle, pRb phosphorylation mediated by cdk/cyclin complexes is required for the cell to progress through the different phases. Several pieces of evidence could help explain the meaning of cdk9-mediated phosphorylation of pRb *in vivo*. First, pRb has been identified to functionally interact with factors which influence RNA polymerase II-dependent gene transcription (20, 21). This important effect of pRb on RNA pol II transcription could represent a functional link with PTEF-b, and cdk9 phosphorylation of pRb could be a signal involved in transcriptional control. The second point of interest is the involvement of pRb during cellular differentiation and in terminally differentiated cells, which we will discuss hereafter.

4. CDK9 AND TRANSCRIPTION

It is now widely accepted that a large number of periodically expressed genes are regulated at the transcriptional level during specific stages of the cell cycle by the concerted action of appropriate transcription repressors and/or activators (22).

Recent data indicate a role for cdk complexes in regulating transcription by functional and physical interaction with components of the basal transcription apparatus (23). Three different cdks (cdk7, cdk8 and cdk9) appear to have a role in basal transcription. Indeed, all three cdks have been reported as being capable of mediating catalytic hyperphosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II (RNA pol II). A number of studies have underlined the importance of CTD phosphorylation in transcription (22). The CTD of RNA pol II molecules present in the pre-initiation complexes (PIC) is unphosphorylated (RNA pol IIa), whereas phosphorylated CTD is a hallmark of active elongating polymerase (RNA pol IIb) complexes (24). It has also been proposed that CTD phosphorylation is critical for the release of the PIC complex from the promoter by distruption of contacts between the CTD and transcriptional mediator and/or other basal factors, such as TBP, which is absent in the elongation complex (22).

Transcription in eukaryotic cells involves three major steps: initiation, elongation and termination. It has been thought that regulation occurs primarily at the level of

the initiation step. It is now recognized that transcription elongation is a critical step for regulation of gene expression (25, 26, 27). Shortly after initiation, RNA pol II faces a barrier of negative transcription elongation factors (N-TEF) and enters abortive elongation. The action of positive transcription elongation factors (P-TEF) lowers the barrier of N-TEF and allows the transition into productive elongation producing longer mRNA transcripts (28). Two N-TEFs have recently been identified and characterized: DSIF (DRB-sensitivity-inducting factor) and NELF (negative elongation factor). DSIF, composed of Spt5 (p160) and Spt4 (p14), and NELF, composed of five polypeptides (NELF-A to –E), functionally cooperate to strongly repress RNA pol II elongation (28). The factors that positively regulate the elongation step form an essential multiprotein complex named P-TEFb. P-TEFb was originally identified in Drosophila and mammals and it is composed of two subunits: the catalytic subunit cdk9 and the regulatory subunit cyclin T1 (29) (Fig 1E).

In Drosophila cells, it has been demonstrated that the direct recruitment of a Gal4-binding domain P-TEFb hybrid to an hsp70 promoter is sufficient to activate transcription. Mutational studies have shown that this stimulation depends on cdk9 kinase activity and on cdk9 interaction with cyclin T. These results, coupled with the frequent colocalization of p-TEFb and the hypophosphorylated form of RNA pol II found at promoter-pause sites, support a model in which P-TEFb acts to stimulate promoter-paused RNA pol II to enter into productive elongation (30). It has also been demonstrated that P-TEFb regulates transcriptional elongation through cdk9 phosphorylation of Spt5, one of the two subunits of DSIF (31, 32, 28). It is a highly conserved protein that, with Spt4 and Spt6, can regulate transcriptional elongation in both a positive and a negative manner, and it has two important domains that are involved in transcriptional control. The Spt5 domains that bind Spt4 and RNA pol II in addition to the CTR1 domain, a C-terminal repeat element, are critical for mediating DRB (ATP analogue 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole) inhibition and Tat activation. P-TEFb phosphorylates the CTR1 domain of SPT5, suggesting that P-TEFb phosphorylation of both RNA pol II and Spt5 may be critical for the regulation of transcriptional elongation (31, 32, 28).

In mammalian cells, targeted recruitment of cdk9/cyclin T1 to specific promoters stimulates transcription *in vivo* (33). Transcriptional enhancement is dependent on active cdk9, as the catalytically inactive form has no transcriptional effect. It has been demonstrated that, unlike conventional activators, DNA-bound cdk9 does not activate enhancerless TATA-promoters unless TBP is overexpressed, suggesting that cdk9 acts *in vivo* at a step subsequent to DNA-bound TFIID recruitment. Finally, it has been determined that cdk9-mediated transcriptional activation is mediated by preferentially stimulating productive transcription elongation (33). In addition it has been found that cdk9/cyclin T1-mediated transcription requires CTD-containing RNA pol II, suggesting that the CTD is the major target of the cdk9/cyclin T complex *in vivo*. Unlike cdk7 and cdk8, the other two cdks that are able to phosphorylate the CTD *in vitro*, only the cdk9 complex activates gene expression in a catalytic-dependent manner. Finally, unlike cyclin T1 and cyclin T2, the targeted recruitment of cyclin K to promoter DNA does not stimulate transcription *in vivo* (34). Collectively, these data strongly indicate that the P-TEFb kinase subunits-cdk9/cyclin T complex is specifically involved in transcription and the CTD domain of RNA pol II is the major functional target of this complex *in vivo*. In addition, PTEF-b has been demonstrated to localize throughout the non-nucleolar nucleoplasm, especially in the nuclear speckles, subnuclear structures that are associated with increased concentrations of factors involved in transcription and pre-mRNA splicing (35).

How cellular promoters recruit specific kinase complexes to RNA pol II remains to be investigated. The ability of cdk9/cyclin T to function in a catalytic-dependent manner when bound to the upstream promoter sequences raises the possibility that promoter-specific activation of transcription elongation might occur through interaction between cdk9/cyclin T and a dedicated DNA-bound activator. Recent studies suggest that cdk9/cyclin T complex may be recruited to specific promoters by cellular transcription factors that bind to the promoter sequences of genes regulated at the level of transcriptional elongation. This is the case of HIV-Tat-mediated and MyoD-mediated transcription, two aspects of cdk9 function that we analyze in the next two sections.

5. CDK9 AND HIV INFECTION

The transcriptional activator protein (Tat) of HIV-1 enhances synthesis of viral RNA recognizing TAR, a hairpin loop structure near the 5' end of the viral RNA, and stimulating RNA pol II processivity, thus causing an increase in the fraction of transcripts that extend all the way to the 3' end of the provirus (36). The link between Tat and transcriptional activation has been identified in a complex which exerts a kinase activity on the CTD of RNA pol II. This complex is composed at least of cdk9 and cyclin T1. Cdk9 was identified independently as a kinase that binds the activation domain of Tat *in vitro* and phosphorylates RNA pol II (37). Cyclin T was identified as a protein that binds to a Tat affinity resin (11). The important role of cdk9/cyclin T was demonstrated by its immunodepletion from transcription extracts, which was found to inhibit activation by Tat as well as basal transcription (29, 38, 11). The interaction between Tat and cdk9/cyclin T is mediated by cyclin T-Tat physical binding. Cyclin T-Tat complex binds TAR with higher affinity than Tat alone, thereby identifying in cyclin T the host-cell factor that allows HIV infection of human cells. Cyclin T1 fulfills the criteria for the species-specific host-cell factor: the human gene maps on chromosome 12, which was identified as the human locus for Tat cofactor, and its

over-expression in HIV infection nonpermissive rodent cells is sufficient to permit Tat activation (39, 40, 36). One study on human-rodent hybrid complexes showed that stable complexes containing human cyclin T1 and human or rodent cdk9 supported Tat transcription, while rodent cyclin T1 and human cdk9 failed to interact with Tat and to mediate Tat transactivation (41) (Fig 1F). Interestingly, it seems that only human cyclin T1 cooperates with Tat; in fact, Tat transactivation in rodent cells occurs in the presence of hcyclin T1 but not in the presence of hcyclin T2. Overexpression of hcyclin T2 inhibits Tat function in both rodent and human cells (42). The difference in Tat transactivation between the two T cyclins confirms the idea of different roles for cdk9 kinase activity in several cellular processes depending on its interaction with cyclins. Cdk9/cyclinT1-Tat complex is regulated by positive and negative signals that start from the complex itself and from other cofactors. The acetyltransferases p300 and PCAF directly acetylate Tat on two different functional domains. p300 acetylates Lys 50 in the TAR RNA binding domain and PCAF acetylates Lys 28 in the activation domain of Tat. Acetylation at Lys 28 by PCAF enhances Tat binding to cdk9/cyclin T1, while acetylation by p300 at Lys 50 promotes the dissociation of Tat from TAR RNA that occurs during early transcriptional elongation. These data suggest that acetylation of Tat regulates two functionally critical steps in cdk9/cyclin T1-Tat-mediated transcription (43). Another mechanism that regulates the activity of the complex depends on the ability of Tat to modify the substrate specificity of cdk9/cyclin T1. In the absence of Tat, cdk9 and cdk7 phosphorylate the CTD on two serines, Ser 2 and Ser 5, respectively. In the presence of Tat, the substrate specificity of cdk9 is altered, such that the kinase phosphorylates both Ser 2 and Ser 5 of CTD RNA pol II instead of phosphorylating only Ser 2. The CTD phosphorylation state indicates a biphasic modification pattern and the second phase is Tat- and TAR-dependent and is sustained by cdk9 only (44) (Fig 1F). In addition, cdk9/cyclin T1 complex is intrinsically incapable of forming a stable complex with Tat and TAR due to two built-in autoinhibitory mechanisms in P-TEFb. Both mechanisms exert little effect on the cdk9/cyclin T1-Tat interaction, but prevent the P-TEFb-Tat complex from binding to TAR RNA. The first autoinhibition arises from the unphosphorylated state of cdk9, which establishes a P-TEFb conformation that is unfavorable for TAR recognition. Autophosphorylation of cdk9 overcomes this inhibition by inducing conformational changes in P-TEFb, thereby exposing a region in cyclin T1 for possible TAR binding. An intramolecular interaction between the N- and C-terminal regions of cyclin T1 sterically blocks the P-TEFb-TAR interaction and constitutes the second autoinhibitory mechanism. This inhibition is relieved by the binding of the C-terminal region of cyclin T1 to the transcription elongation factor Tat-SF1 and perhaps other cellular factors. Upon release from the intramolecular interaction, the C-terminal region also interacts with RNA pol II and is required for HIV-1 transcription, suggesting its role in bridging the cdk9/cyclin T1-Tat-TAR complex and the basal elongation apparatus (45, 46) (Fig 1F). Finally, Sune and colleagues characterized the protein components of kinase activity present in HeLa cell nuclear extracts. They found the presence of cyclin T1, cdk9, Tat-SF1 and at least three unidentified proteins (47) (Fig 1F). These data indicate that the transcriptional regulation of HIV infection is still unclear and that more studies are awaited to further define the molecular mechanism underlying cdk9/cyclin T1-Tat interaction and functional regulation.

6. CDK9 AND CELLULAR DIFFERENTIATION

The difference between cdk9 and the cdks involved in the regulation of the cell cycle might also reflect a different function during terminal differentiation. In addition, the role of cyclin T2 a and b isoforms in regulating cdk9 activity is still unknown. We have investigated cdk9 function in a paradigmatic differentiation model: a skeletal muscle differentiation system. In this context, the induction of myocyte differentiation upon serum withdrawal is accompanied by the down-regulation of cyclins D1, E and A and the up-regulation of several cdk inhibitors, which lock the cyclin-cdk complexes in an inactive form. This allows exit from the cell cycle and the expression of muscle-specific genes (48). Transcription of genes that characterize the phenotypic and biochemical identity of differentiated muscle cells is activated by myogenic basic helix-loop-helix (bHLH) transcription factors. These are the myogenic regulatory factors (MRFs) MyoD, Myf5, myogenin and MRF4. They operate by heterodimerizing with the E2A gene products (E12 and E47) via the HLH domain and by binding to certain recognition sites (E-box -> CANNTG) in the regulatory regions of muscle-specific genes (49, 50, 51). Overexpression of cdk/cyclins has been reported to inhibit the activity of MyoD and prevent myogenic differentiation by different modalities. Overproduction of cyclins E and A together with their associated kinase, cdk2, impinge on MyoD function by virtue of their ability to phosphorylate pRb (52, 53), an essential cofactor for MyoD-dependent transcription (54, 55). In addition, cdk2 and cdc2 can inactivate MyoD function by phosphorylating Ser 200, thereby triggering MyoD degradation (56, 57). Cyclin D1 overexpression inhibits myogenic differentiation by promoting the nuclear accumulation of cdk4, which in turn binds the C-terminus of MyoD and prevents MyoD DNA binding and activation of myogenic transcription (58, 59). Our studies have shown that overexpression of cdk9/cyclin T2a, unlike other cdk/cyclin complexes, enhances MyoD function and promotes myogenic differentiation, while inhibition of cdk9 kinase activity by a dominant negative form prevents the activation of the myogenic program. The N-terminal region of cdk9 and full-length cyclin T2a directly interact with the bHLH region of MyoD, allowing the formation of a complex that stimulates transcription of specific muscle genes (Simone *et al.*, manuscript in preparation) (Fig 1G).

Cdk9 involvement in the differentiation program is supported by recent studies. First of all, cdk9 is involved in transcriptional elongation of many, but not all, genes in Drosophila cells (30) and, in human cells, controls histocompatibility class II (MHC II) genes (60), but not CD96, CD25 or IL-2 genes (61), suggesting a role of cdk9 complexes in specific transcription of targeted promoters. In addition, P-TEFb is activated differentially in lymphocytes and monocytes. In fact, cdk9/cycT1 activation in peripheral blood lymphocytes (PBLs) involves an increase in mRNA and protein levels of both proteins (62); conversely, differentiation of promonocytic cells involves only cycT1 protein levels through posttranscriptional modifications (63). In monocytes/macrophages, the overexpression of cdk9dn blocks the differentiation program rendering U937 cells sensitive to apoptosis (61). The involvement of the kinase activity of cdk9 in monocyte differentiation reflects its involvement in the myogenic program. In mouse myoblasts, the overexpression of cdk9dn inhibits muscle gene transcription and promotes cell death (Simone *et al.*, manuscript in preparation).

How cdk9/cyclin T2a enhances MyoD trancriptional activity is still the subject of study, but the cdk9/cyclin T1-Tat complex could give us several research inputs. Data regarding the involvement of the HAT proteins, essential cofactors of MyoD-mediated transcription, in Tat-mediated transcription; the role of cdk9 in the transcriptional elongation complex; the physical interaction between MyoD and cyclin T2a; and cdk9 phosphorylation of MyoD *in vitro* support the Tat-like model in which cdk9 recruitment on myogenic promoters is mediated by MyoD and allows transcriptional elongation. The intricate regulation of Tat complex could suggest that cdk9/cyclin T2a-MyoD complex has several positive and negative regulating signals and one of them could be the cdk9 phosphorylation of MyoD.

Several pieces of evidence have implicated pRb as an essential cofactor during muscle differentiation. Muscle cells derived from pRb-/- mice fail to irreversibly exit the cell cycle (64), express reduced levels of late differentiation markers and display impaired fusion into multinucleated myotubes (55). Several hypotheses have been proposed to justify pRb involvement in differentiation, including a physical binding with MyoD (54) and the inhibition of the anti-myogenic activity of E2F (65, 66, 67). More recently, pRb has been shown to promote functional synergism between MyoD and MEF2 protein, a coactivator of specific gene transcription (68). However, the molecular mechanisms through which pRb promotes myogenic transcription remain unclear. We found that cdk9/cycT2 binds and phosphorylates the C-terminus of pRb (Simone *et al.*, submitted). Immunoprecipitated cdk9 complexes from C2C12 mouse myoblasts induced to differentiate have an increasing kinase activity on p56/pRb with a peak of phosphorylation at 96 h of the myogenic program (8). During muscle differentiation, pRb is present in the active hypophosphorylated form, especially due to down-regulation of cyclins A, E and D1 and the up-regulation of cdk inhibitors (69). It is possible that cdk9/cycT2 kinase activity is involved in the basal phosphorylation of the retinoblastoma protein and that pRb and cdk9/cycT2 cooperate to support MyoD-mediated myogenic transcription.

In addition, the MyoD region of interaction with cdk9 is the bHLH domain. This is a conserved region in the bHLH family of transcription factors and several of them are involved in transcriptional regulation of the differentiation program. It will be interesting to study the action of cdk9/cycT2a on bHLH transcription factors in other models of differentiation.

7. CONCLUSION

All the data discussed in this review have opened several fields of research in basic molecular biology, tumorigenesis, HIV infection disease and differentiation, but the one concept emphasized herein is the idea of a gene codifying for a kinase that exerts several functions in different subcellular compartments, the idea of a multifunctional kinase.

8. ACKNOWLEDGMENT

We thank Marie Basso for her editorial assistance and Dr. Lorenzo Puri for his comments and helpful discussion during the preparation of the manuscript. We also thank Drs. Ginevra Guanti, Nicoletta Resta and Alessandro Stella for critically reading this manuscript. Finally, we thank Dionisio Simone and Margherita D'Aprile for their encouragement and support. This work was funded by grants from the National Institute of Health (NIH) to A.G.

9. REFERENCES

1. MacLachlan, T. K., N. Sang & A. Giordano: Cyclins, cyclin-dependent kinases and cdk inhibitors: implication in cell cycle control and cancer. *Crit Rev Eukaryot Gene Expr* 5, 127-156 (1995)

2. Lee, M. G. & P. Nurse: Complementation used to clone a human homolog of fission yeast cell cycle control gene cdc2. *Nature* 327, 31-35 (1987)

3. Draetta, G., L. Brizuela, J. Patashkin & D. Beach: Identification of p34 and p13, human homologs of cell cycle regulators of fission yeast encoded by cdc2 and suc1. *Cell* 50, 319-325 (1987)

4. De Falco, G. & A. Giordano: CDK9 (PITALRE): a multifunctional cdc2-related kinase. *J Cell Physiol* 177, 501-506 (1998)

5. Grana, X., A. De Luca, N. Sang, Y. Fu, P.P. Claudio, J. Rosenblatt, D. O. Morgan & A. Giordano:

PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein *in vitro*. *Proc Natl Acad Sci* 91, 3834-3838 (1994)

6. Lapidot-Lifson, Y., D. Patinkin, C.A. Prody, G. Dhrlich, S. Seidman, A.R. Ben, F. Bensele, F. Eckstein, H. Zakut & H. Soreq: Cloning and antisense oligodeoxynucleotides inhibition of a human homolog of cdc2 required in hematopoiesis. *Proc Natl Acad Sci USA* 89, 579-583 (1992)

7. De Luca, A., V. Esposito, A. Baldi, P.P. Claudio, Y. Fu, M. Caputi, M. Pisano, F. Baldi & Giordano: A. Cdc2-related kinase PITALRE phosphorylates pRb exclusively on serine and is widely expressed in human tissues. *J Cell Physiol* 172, 265-273 (1997)

8. Bagella, L., T. K. MacLachlan, R .J. Buono, M.M. Pisano, A. Giordano & A. De Luca: A. Cloning of murine CDK9/PITALRE and its tissue-specific expression in development. *J Cell Physiol* 177, 206-213 (1998)

9. Bagella, L., P. Stiegler, A. De Luca, L.D. Siracusa & A. Giordano: Genomic organization, promoter analysis, and chromosomal mapping of the mouse gene encoding Cdk9. *J Cell Biochem* 78, 170-8 (2000)

10. Fu, T.J., J. Peng, D.H. Price & O. Flores: Cyclin K functions as a cdk9 regulatory subunit and participates in RNA Polymerase II transcription. *J Biol Chem* 274, 34527-34530 (1999)

11. Wei, P., M.E. Gender, S.M. Fang, W.H. Fisher & K.A. Jones: 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 (1998)

12. Peng, J., Y. Zhu, J.T. Milton & D.H. Price: Identification of multiple cyclin subunits of human P-TEFb. *Genes & Dev* 12, 755-762 (1998)

13. Edwards, M.C., C. Wong & S.J. Elledge: Human cyclin K, a novel RNA polymerase II-associated cyclin possessing both carboxy-terminal domain kinase and Cdk-activating kinase activity. *Mol Cell Biol* 18, 4291-4300 (1998)

14. MacLachlan, T.K., N. Sang, A. De Luca, P.L. Puri, M. Levrero & A. Giordano: Binding of CDK9 to TRAF2. *J Cell Biochem* 71, 467-478 (1998)

15. De Falco, G., L. Bagella, P.P. Claudio, A. De Luca, Y. Fu, B. Calabretta, A. Sala & A. Giordano: A. Physical interaction between cdk9 and B-Myb results in suppression of B-Myb gene autoregulation. *Oncogene* 19, 373-379 (2000)

16. O'Keeffe, B., Y. Fong, D. Chen, S. Zhou & Q. Zhou: Requirement for a kinase-specific chaperone pathway in the production of a cdk9/cyclin T1 heterodimer responsible for P-TEFb-mediated Tat stimulation of HIV-1 transcription. *J Biol Chem* 275, 279-287 (1999)

17. Pan, W., T. Sun, R.H. Hoess & R.H. Grafstrom: Defining the minimal portion of the retinoblastoma protein that serves as an efficient substrate for cdk4 kinase/cyclin D1 complex. *Carcinogenesis* 19, 765-769 (1998)

18. Adams P.D., X. Li, W.R. Sellers, K.B. Baker, X. Leng, J.W. Harper, Y. Taya & W.G. Kaelin: Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. *Mol Cell Biol* 19, 1068-1080 (1999)

19. Pan, W., S. Cox, R.H. Hoess & RH. Grafstrom: A cyclin D1/cyclin-dependent kinase 4 binding site within the C domain of the retinoblastoma protein. *Cancer Res* 61, 2885-2891 (2001)

20. De Luca, P., B. Majello & L. Lania: Retinoblastoma protein tethered to promoter DNA represses TBP-mediated transcription. *J Cell Biochem* 70, 281-287 (1998)

21. Fanciulli, M., T. Bruno, M. Di Padova, R. De Angelis, S. Iezzi, C. Iacobini, A. Floridi & C. Passananti: Identification of a novel partner of RNA polymerase II subunit 11, Che-1, which interacts with and affects the growth suppression function of Rb. *FASEB J* 14, 904-912 (2000)

22. Lania, L., B. Majello & G. Napolitano: Transcriptional control by cell-cycle regulators: a review. *J Cell Physiol* 179, 134-141 (1999)

23. Dynlacht, B.D.: Regulation of transcription by proteins that control the cell cycle. *Nature* 389, 149-152 (1997)

24. Dahmus, M.E.: Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J Biol Chem* 271, 19009-19012 (1996)

25. Bentley, D.L.: Regulation of transcriptional elongation by RNA pol II. *Curr Opin Genet Dev* 5, 210-216 (1995)

26. Li, X.Y. & M.R. Green: Transcriptional elongation and cancer. Tumorigenesis. *Curr Biol* 6, 943-944 (1996)

27. von Hippel, P.H.: An integrated model of the transcription complex in elongation, termination, and editing. *Science* 281, 660-665 (1998)

28. Ping, Y.H. & T.M. Rana: DSIF and NELF interact with RNA polymerase II elongation complex and HIV-1 Tat stimulates P-TEFb-mediated phosphorylation of RNA polymerase II and DSIF

during transcription elongation. *J Biol Chem* 276, 12951-12958 (2001)

29. Zhu, Y., T. Pelery, J. Peng, Y. Ramanathan, N. Marshall, T. Marshall, B. Amendt, M. Mathews & D. Price: Transcription elongation factor P-TEFb is required for HIV-1 Tat transactivation *in vitro*. *Genes & Dev* 11, 2622-2632 (1997)

30. Lis, J.T., P. Mason, J. Peng, D.H. Price & J. Werner: P-TEFb kinase recruitment and function at heat shock loci. *Genes & Dev* 14, 792- 803 (2000)

31. Ivanov, D., Y.T. Kwak, J. Guo & R.B. Gaynor: Domains in the SPT5 protein that modulate its transcriptional regulatory properties. *Mol Cell Biol* 20, 2970-2983 (2000)

32. Andrulis, E.D., E. Guzman, P. Dòring, J. Werner & J.T. Lis: High-resolution localization of Drosophila SPT5 and SPT6 at heat shock gene *in vivo*: roles in promoter proximal pausing and transcriptional elongation. *Genes Dev* 14, 2635-2649 (2000).

33. Majello, B., G. Napolitano, A. Giordano & L. Lania: Transcriptional regulation by targeted recruitment of cyclin dependent cdk9 kinase *in vivo*. *Oncogene* 18, 4598-605 (1999)

34. Napolitano, G., B. Majello, P. Licciardo, A. Giordano & L. Lania: Trancriptional activity of positive transcription elongation factor b kinase *in vivo* requires the C-terminal domain of RNA polymerase II. *Gene* 254, 139-145 (2000)

35. Herrmann, C.H. & M.A. Mancini: The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. *J Cell Sci* 114, 1491-1503 (2001)

36. Yankulov, K. & D. Bentley: Transcriptional control: Tat cofactors and transcriptional elongation. *Curr Biol* 8, R447-R449 (1998)

37. Yang, X.Z., M.O. Gold, D.N. Tang, D.E. Lewis, E. Aguilar-Cordova, A.P. Rice & C.H. Hermann: TAK, an HIV Tat-associated kinase, is a member of the cyclin dependent family of protein kinase and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. *Proc Natl Acad Sci USA* 94, 12331-12336 (1997)

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

39. Hart, C., J. Galphin, M. Westhafer & G. Schochetman: TAR loop-dependent human immunodeficiency virus trans activation requires factors encoded on human chromosome 12. *J Virol* 67, 5020-5024 (1993)

40. Alonso, A., T. Cujec & B. Petrlin: Effects of human chromosome 12 on interaction between Tat and TAR of human immunodeficiency virus type 1. *J Virol* 66, 6505-6513 (1994)

41. Chen, D., Y. Fong, Y. & Q. Zhou: Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-transcription. *Proc Natl Acad Sci USA* 96, 2728-33 (1999)

42. Napolitano, G., P. Licciardo, P. Gallo, B. Majello, A. Giordano & L. Lania: The cdk9-associated cyclins T1 and T2 exert opposite effects on HIV-1 Tat activity. *AIDS* 13, 1453-1459 (1999)

43. Kiernan, R.E., C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K.T. Jeang, M. Benkirane & C. Van Lint: HIV-1 Tat transcriptional activity is regulated by acetylation. *EMBO J* 18, 6106-6118 (1999)

44. Zhou, M., M.A. Halansky, M.F. Radonovich, F. Kashanchi, J. Peng, D.H. Price & J.N. Brady: Tat modifies the activity of cdk9 to phosphorylate serine 5 of the RNA plymerase II carboxy-terminal domain during human immunodeficiency virus type 1 transcription. *Mol Cell Biol* 20, 5077-5086 (2000)

45. Fong, Y.W. & Q. Zhuo: Relief of two built-in autoinhibitory mechanisms in P-TEFb is required for assembly of a multicomponent transcription elongation complex at the human immunodeficiency virus type 1 promoter. *Mol Cell Biol* 20, 5897-907 (2000)

46. Garber, M.E., T.P. Mayall, E.M. Suess, J. Meisenhelder, N.E. Thompson & K.A. Jones: CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol Cell Biol* 20, 6958-69 (2000)

47. Sune, C., A.C. Goldstrohm, J. Peng, D.H. Price & M.A. Garcia-Blanco: An *in vitro* transcription system tha recapitulates Equine infectious anemia virus Tat-mediated inhibition of human immunodeficiency virus type 1 Tat activity demonstrates a role for positive transcription elongation factor b and associated proteins in the mechanism of Tat activation. *Virology* 274, 356-366 (2000)

48. Walsh, K. & H. Perlman: Cell cycle exit upon myogenic differentiation. *Curr Opin Genet Dev* 7, 597-602 (1997)

49. Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, Y.N. Jan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka & A.B. Lassar: Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* 58, 537-544 (1989)

50. Lassar, A.B., R.L. Davis, W.E. Wright, T. Kadesch, C. Murre, A. Voronova, D. Baltimore & H. Weintraub: Functional activity of myogenic HLH proteins requires hetero- oligomerization with E12/E47-like proteins *in vivo*. *Cell* 66, 305-315 (1991)

51. Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T.K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, and *et al*.: The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* 251, 761-766 (1991)

52. Skapek, S.X., J. Rhee, P.S. Kim, B.G. Novitch & A.B. Lassar: Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. *Mol Cell Biol* 16, 7043-7053 (1996)

53. Guo, K. & K. Walsh: Inhibition of myogenesis by multiple cyclin-Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. *J Biol Chem* 272, 791-797 (1997)

54. Gu, W., J.W. Schneider, G. Condorelli, S. Kaushal, S. Mahadavi & B. Nadal-Ginard: Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell* 72, 309-324 (1993)

55. Novitch, B.G., G.J. Mulligan, T. Jacks & A.B. Lassar: Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J Cell Biol* 135, 441-456 (1996)

56. Song, A., Q. Wang, M. Goebl & M. Harrington: M. Phosphorylation of nuclear MyoD is required for its rapid degradation. *Mol Cell Biol* 18, 4994-4999 (1998)

57. Kitzmann, M., M. Vandromme, V. Schaeffer, G. Carnac, J.C. Labbe, N. Lamb & A. Fernandez: cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. *Mol Cell Biol* 19, 3167-3176 (1999)

58. Zhang, J.M., Q. Wei, X. Zhao & B.M. Paterson: Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4. *EMBO J* 18, 926-933 (1999a)

59. Zhang, J.M., X. Zhao, Q. Wei & B.M. Paterson: Direct inhibition of G (1) cdk kinase activity by MyoD promotes myoblast cell cycle withdrawal and terminal differentiation. *EMBO J* 18, 6983-6993 (1999b)

60. Kanazawa, S., T. Okamoto & B.M. Peterlin: Tat competes with CIITA for the binding to P-TEFb and blocks the expression of MHC class II genes in HIV infection. *Immunity* 12, 61-70 (2000)

61. Foskett, S.M., R. Ghose, D.N. Tang, D.E. Lewis & A.P. Rice: Antiapoptotic function of Cdk9 (TAK/P-TEFb) in U937 promonocytic cells. *J Virol* 75, 1220-1228 (2001)

62. Garriga, J., J. Peng, M. Parreno, D.H. Price, E.E. Henderson & X. Grana: Upregulation of cyclin T1/CDK9 complexes during T cell activation. *Oncogene* 17, 3093-3102 (1998)

63. Herrmann, C.H., R.G. Carroll, P. Wei, K.A. Jones & A.P. Rice: Tat-associated kinase, TAK, activity is regulated by distinct mechanisms in peripheral blood lymphocytes and promonocytic cell lines. *J Virol* 72, 9881-9888 (1998)

64. Schneider, J., W. Gu, L. Zhu, V. Mahdavi & B. Nadal-Ginard: Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. *Science* 264, 1467-1471 (1994)

65. Shin, E., A. Shin, C. Paulding, B. Schaffhausen & A. Yee: Multiple change in E2F function and regulation occur upon muscle differentiation. *Mol Cell Biol* 15, 2252-2262 (1995)

66. Corbeil, H., P. Whyte & P. Branton: Characterization of transcription factor E2F complexes during muscle and neuronal differentiation. *Oncogene* 11, 909-920 (1995)

67. Puri, P.L., C. Balsano, V. Burgio, P. Chirillo, G. Natoli, L. Ricci, E. Mattei, A. Graessmann & M. Levrero: MyoD prevents cyclinA/cdk2 containing E2F complexes formation in terminally differentiated myocytes. *Oncogene* 14, 1171-1184 (1997)

68. Novitch, B.G., D.B. Spicer, P.S. Kim, W.L. Cheung, W.L. & A.B. Lassar: pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. *Curr Biol* 9, 449-459 (1999)

69. Puri, P.L. & V. Sartorelli: Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-transcriptional modifications. *J Cell Physiol* 185, 155-73 (2000)

Key Words: Cdk9, PITALRE, Cyclins, Cyclin-dependent Kinases, Transcriptional Elongation, MyoD, Muscle Differentiation, Tat, HIV**,** Review

Send correspondence to: Antonio Giordano, M.D., Ph.D., Dept. Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Rm. 226, Philadelphia, PA 19107, Tel.: 215-503-0781, Fax: 215-923-9626, E-mail: agiordan@lac.jci.tju.edu