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A Kinase-Independent Activity of Cdk9 Modulates Glucocorticoid Receptor-Mediated Gene Induction

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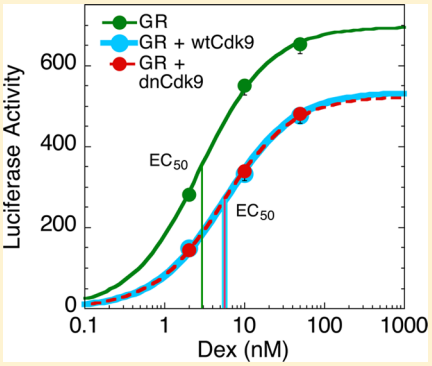
Supporting Information

ABSTRACT: A gene induction competition assay has recently uncovered new inhibitory activities of two transcriptional cofactors, NELF-A and NELF-B, in glucocorticoid-regulated transactivation. NELF-A and -B are also components of the NELF complex, which participates in RNA polymerase II pausing shortly after the initiation of gene transcription. We therefore asked if cofactors (Cdk9 and ELL) best known to affect paused polymerase could reverse the effects of NELF-A and -B. Unexpectedly, Cdk9 and ELL augmented, rather than prevented, the effects of NELF-A and -B. Furthermore, Cdk9 actions are not blocked either by Ckd9 inhibitors (DRB or flavopiridol) or by two Cdk9 mutants defective in kinase activity. The mode and site of action of NELF-A and -B mutants with an altered NELF domain are similarly affected by wild-type and kinase-dead Cdk9. We conclude that Cdk9 is a new modulator of GR action, that Ckd9 and ELL have novel activities in GR-regulated gene expression, that NELF-A and -B can act separately from the NELF complex, and that Cdk9 possesses activities that are independent of Cdk9 kinase activity. Finally, the competition assay has succeeded in ordering the site of action of several cofactors of GR transactivation. Extension of this methodology should be helpful in determining the site and mode of action of numerous additional cofactors and in reducing unwanted side effects.



Steroid hormones, acting through their cognate receptors, are critical regulators of gene expression during development, differentiation, homeostasis, and endocrine therapies for numerous inflammatory diseases and lung development in premature infants.[1−3] Typically, steroids enter the cell by passive diffusion and bind to cognate intracellular receptors to cause activation and an increased residency of the receptor−steroid complex in the nucleus, where the complex binds to DNA at biologically active hormone response elements (HREs) to induce or repress gene transcription. More than 350 cofactors have been described to modify the maximal activity (A_{max}) of steroid-regulated gene activation.[4,5] Many of these cofactors have been found to interact with HRE-bound receptors or other factors at the transcriptional start site (TSS).[6] Of those cofactors that have been examined, most also modulate both the concentration of the agonist steroid required for half-maximal induction, or EC_{50}, and the residual partial agonist activity of antisteroids.[7−10] However, despite the dramatic advances over the past two decades, both the precise molecular action of each DNA-associated cofactor and where it acts in the overall sequence of events remain mostly a matter of speculation.

Recently, our attention has been drawn to factors involved in steps of steroid-modulated gene activation that are downstream of the TSS. One particularly attractive, potential control point for many genes that has emerged over the past few years involves paused RNA polymerase II.[11−16] Paused polymerase is usually located 25−100 bp downstream of the TSS.[11,12,16,17] Pol II is held at this position by the poorly defined actions of several proteins, including the heterotetrameric negative elongation factor (NELF) complex and DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity-inducing factor (DSIF).[12,16,18,19] Release of the paused polymerase is proposed to be a rate-limiting step and is initiated by signals that allow the polymerase-bound mediator complex to recruit "super-elongation complexes" (SECs) containing, among other proteins, eleven-nineteen lysine-rich leukemia (ELL) and positive transcription elongation factor b (P-TEFb).[15,16,20] Our interest in paused polymerase, and associated factors, was heightened by our recent findings that two NELF complex components, NELF-A and NELF-B, each inhibit GR-regulated gene expression by acting at two separate sites in the sequence of steps leading to an increased level of gene expression. One site does not appear to involve the NELF complex.[21] Of the numerous factors that are associated with the release of paused polymerase and could participate in the second site of action of NELF-A and NELF-B, ELL and cyclin-dependent kinase 9 (Cdk9), which is the catalytic subunit of P-TEFb, were of particular interest to us.

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ELL was first reported to be required for full induction of heat shock genes in *Drosophila* and to be involved in the regulation of paused polymerases.[16,22] However, ELL also has other activities, such as transcription elongation and cotranscriptional RNA processing.[23,24] ELL was also reported to display specificity among steroid receptors.[25] Thus, ELL increased the A_{max} of mineralocorticoid receptor (MR) transactivation and reduced the EC_{50} by a factor of ≈10 to cause a left shift in the position of the dose−response curve. In contrast, ELL had no effect on androgen or progesterone receptors but decreased the level of glucocorticoid receptor (GR) transactivation by an undetermined mechanism while binding to GR. Interestingly, the actions of ELL on MR and GR are even more divergent because the L214V mutant of ELL disrupts its activity as a coactivator with MR but not its corepressor properties with GR.[25]

P-TEFb is thought to be the critical factor for the escape of paused polymerase into the productive elongation phase of transcription.[16−19,26] This occurs following the actions of the kinase subunit of P-TEFb, Cdk9, which phosphorylates both the NELF and DSIF complexes, to cause the release of NELF from the paused polymerase, and the Ser-2 residues of the CTD of pol II, to permit transcriptional elongation by pol II. Cdk9 has been reported to have other targets, such as the kinase-independent actions of Cdk9 during repression of B-Myb transactivation activity [27] and in transcriptional elongation once the pol II complex is released from the pause site.[28] Cdk9 has also been found to bind to estrogen receptor α[29] and to phosphorylate androgen receptors.[30,31]

The reports described above suggest that factors well-known to be involved in the status of paused RNA polymerase II may have other, less common activities. A recently developed theory and associated competition assay[9,10,32,33] would be helpful in continuing our characterization of the actions of NELF-A and -B.[21] This competition assay has advantages in that it yields information about both the kinetically defined mechanism of action and the relative order of factor action without the knowledge of the precise biochemical mechanisms involved. Factors that have been previously examined and could be eliminated as direct functional targets of NELF-A and -B are (a) GR, which binds to NELF-B but acts upstream of the NELFs, (b) transcriptional intermediary factor 2 (TIF2), which acts after the NELFs, and (c) CREB-binding protein (CBP), which exerts its effects downstream of the NELFs.[21]

The purpose of this study is to examine whether other potential interacting factors could reverse the effects of the NELFs. Because one site of action of the NELFs could still be via the NELF complex, we considered two factors that are known to be involved in the release of the paused polymerase (ELL and Cdk9), one of which (Cdk9) phosphorylates the NELF complex to cause its dissociation.[16,17,26] Interestingly, we find that both ELL and Cdk9 are competitive decelerators[9] that impede GR transactivation. Unexpectedly, Cdk9 does not hinder the actions of NELF-A and NELF-B but rather cooperates to cause an increased level of inhibition. Even more surprising is that Cdk9 kinase activity is not involved in the ability of Cdk9 to attenuate GR transactivation. These studies thus document new activities of NELFs, ELL, and Cdk9 that impact GR-controlled gene activation in manners that differ from their better known mechanisms of action in the release of paused RNA polymerase II.

EXPERIMENTAL PROCEDURES

Unless otherwise indicated, all cell growth occurred at 37 °C and all other operations were performed at 0 °C.

Chemicals. Dexamethasone (Dex), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and flavopiridol hydrochloride hydrate were purchased from Sigma (St. Louis, MO). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA), and the dual-luciferase reporter assay was from Promega (Madison, WI).

Plasmids. Renilla-TS reporter, rat GR (pSG5-GR), GREtkLUC, pSG5-TIF2, and GAL/GR525C have been previously described.[34] FR-LUC reporter is from Stratagene (La Jolla, CA). Human NELF-A (missing the first 11 residues) and chimeric Flag/NELF-B[35] were from R. Li (University of Texas Health Science Center at San Antonio, San Antonio, TX). Full length wild-type (wt) NELF-B and mutants of wt NELF-A and -B, in which four amino acids of the NELF domain in each protein have been changed to alanine, have been described elsewhere.[21] pCMV-XL6/ELL (K. Gardner, National Cancer Institute, National Institutes of Health), Rc/CMV-dnCkd9 (D167N) (X. Grana, Temple University, Philadelphia, PA), and Flag/K44,48R Cdk9 (AcMtCdk9) (M. Giacca, ICGEB, Trieste, Italy) were generously provided as gifts.

Antibodies and Western Blotting. Anti-GR mouse and rabbit monoclonal antibodies [MA1−510 and PA1−516A, respectively (Affinity BioReagents)], anti-NELF-B rabbit polyclonal antibody [ab48336 (Abcam)], anti-NELF-A rabbit polyclonal antibody and anti-Cdk9 mouse monoclonal antibody [sc-23599 and sc-13130, respectively (Santa Cruz)], anti-β-actin mouse monoclonal antibody [A2228 (Sigma)], and anti-Flag mouse monoclonal antibody [F3165 (Sigma)] are commercially available. The rabbit anti-ELL antibody was generously provided by K. Gardner. Western blots were prepared, probed, and visualized by ECL detection reagents as described by the manufacturer (Amersham Biosciences).

Cell Culture, Transient Transfection, and Reporter Analysis. Monolayer cultures of U2OS, U2OS.rGR, COS-7, CV-1, and 293 cells were grown as described previously.[34,36,37] Briefly, triplicate samples of cells were seeded into 24-well plates at a density of 20000 cells/well and transiently transfected the following day with luciferase reporter and DNA plasmids by using 0.7 μL Lipofectamine (Invitrogen) or Fugene 6 (Roche) per well according to the manufacturer's instructions. The level of total transfected DNA was adjusted to 300 ng/well of a 24-well plate with pBluescriptII SK+ (Stratagene). The molar amount of plasmids expressing different protein constructs was kept constant with added empty plasmid or plasmid expressing human serum albumin.[34] Renilla-TS (10 ng/well of a 24-well plate) was included as an internal control. After transfection (32 h), cells were treated with medium containing appropriate hormone dilutions. The cells were lysed 20 h later and assayed for reporter gene activity using dual luciferase assay reagents according to the manufacturer's instructions (Promega). Luciferase activity was measured by an EG&G Berthold's luminometer (Microlumat LB 96P). The data were normalized to Renilla null luciferase activity and expressed as a percentage of the maximal response with Dex before being plotted ± the standard error of the mean, unless otherwise noted.

Two-Factor Competition Assays. The predictions of the mechanism and site of action of the factors were accomplished using a two-factor competition assay.[9,32,33] This assay is based on a theory of steroid-mediated gene induction that exploits the

property that the dose−response curve for the amount of gene product as a function of the added steroid precisely follows a Michaelis−Menten (MM) function (see the Supporting Information). Mathematically, this is unexpected for the sequence of complex forming reactions that is presumed to exist in gene transcription. However, it has been shown that if the individual reactions in the sequence each possess a MM input−output relationship, then the dose response of an arbitrarily long sequence of events will have a MM dose−response curve.[32] Biochemically, this constraint can be satisfied if the formed complexes are short-lived. A formula for the dose−response curve can then also be explicitly computed. The action of the factors in the sequence can be classified in terms of activators and inhibitors as they are in enzyme kinetics. To avoid confusion with the commonly used terms coactivator and corepressors, we have adopted the terms accelerators and decelerators for enzymatic activators and inhibitors, respectively.[9] It should be noted that the action of an accelerator or decelerator is with respect to only the specific reaction in which it acts and does not pertain to its global effect on the final gene product. The advantage of this classification system is that the predicted mechanism is not context-dependent. In the presence of two factors, a distinct formula (parametric statistical model) can be written down for each combination of factor type and location with respect to each other and a distinguished step, called the concentration-limiting step (CLS). The CLS is the step in the sequence beyond which the concentration of bound factors is negligible with respect to the free concentrations. Given that the experimental measurements are taken after the system has reached dynamic equilibrium, there are no fluxes through the system but the CLS can be considered the equilibrium analogue of a rate-limiting step. The CLS has been found to be the invariant site of action of the reporter gene, acting as an accelerator, and thus is a marker in the reaction scheme of steroid receptors about which all other factors can be organized.[9,21]

The individual parametric models can be compared directly to the data to make predictions about the actions of the factors. This task can be simplified because the formulas for the parametric models all have a fractional linear form and are thus completely specified by the potency (EC_{50}) and maximal induced activity (A_{max}). Hence, instead of direct curve fitting, graphical analyses of the behavior of combinations of EC_{50} and A_{max} will give equivalent predictions. A full description has been published.[9,10,33] A flowchart is given as Figure S1 of the Supporting Information. Briefly, the effect of four concentrations of each of two factors (total of 16 combinations) on the maximal induced activity (A_{max}) and EC_{50} is determined from directly fitting a Michaelis−Menten curve to the average ($n = 3$) value of induced luciferase activity from transiently transfected reporter (GREtkLUC) with EtOH and three subsaturating concentrations of Dex (192 total samples). The curve fitting for the dose−response curves is extremely good for a first-order Hill plot [R^2 for 368 randomly selected curves = 0.997 ± 0.004 (standard deviation)]. Graphs of $1/EC_{50}$ and A_{max}/EC_{50} (and EC_{50}/A_{max} when the plot of A_{max}/EC_{50} is decreasing) versus the concentration of one cofactor are constructed at each of the concentrations of the second factor. In all cases, though, it is critical to make corrections if Western blots show that expression of the transiently transfected protein (at constant levels either of total cellular protein or of an internal standard, such as β-actin) is nonlinear. This is because the interpretation of the graphs is predicated on the x-axis being a linear scale. To determine the linear equivalent of the expressed plasmid, the nonlinear plot of OD versus the nanograms of transfected plasmid is first fit to a Michaelis−Menten plot of

$$A_{max} = m_1 \times \text{plasmid}/(m_2 + \text{plasmid})$$

The functional equivalent of the transfected plasmid that gives a linear OD versus plasmid plot is then obtained from the formula

$$\text{plasmid (linear)} = m_2 \times \text{plasmid}/(m_2 + \text{plasmid})$$

The x-axis value of the amount of plasmid in the various graphs is then this "corrected plasmid" value. These same Western blots are used to determine the relative amount of endogenous factor (in units of nanograms of factor plasmid) when dealing with an accelerator. It is not necessary to quantitate the relative amount of an endogenous factor that displays decelerator activity. If an experiment used n concentrations for each cofactor, then there would be a total of four to six graphs, each with n separate curves. The shape of the curves and how they change with the other cofactor are then compared to Table S1 of the Supporting Information to determine the kinetically defined mechanism of action and site of action, relative to each other and to the CLS. Our Table S1 is an updated version of Table S1 of ref 33. Many of the entries in Table S1 of the Supporting Information require an estimate of the intersection point of a set of linear regression fits to the graphs. For a family of lines of the form $y = a + bx$, an unbiased estimate of the intersection can be obtained from "a versus b plots", which are a linear regression on the graph of a versus b to give a new plot of the form $a = y' + x'b$, where y' is the y-axis value of the intersection point of the family of lines in the original graph and the negative value of x' corresponds to the x-axis value of the intersection point.

Statistical Analysis. Unless otherwise noted, all experiments were performed in triplicate multiple times. KaleidaGraph version 3.5 (Synergy Software, Reading, PA) was used to determine a least-squares best fit of the experimental data to the theoretical dose−response curve, which is given by the equation derived from Michaelis−Menten kinetics of y = [free steroid]/[free steroid + dissociation constant (K_d)] (where the concentration of total steroid is approximately equal to the concentration of free steroid because only a small portion is bound), to yield a single EC_{50} value. The values of n independent experiments were then analyzed for statistical significance by the two-tailed Student's t test using InStat version 2.03 for Macintosh (GraphPad Software, San Diego, CA). The Mann−Whitney test or the Alternate Welch t test is used when the difference between the standard deviations of two populations is statistically significant. The Bayesian Information Criterion was used to determine the better of two types of fits for a particular graph (e.g., linear vs quadratic).

RESULTS

Application of the Competition Assay To Determine the Mechanism and Site of Cofactor Action. The competition assay was selected to determine whether any one factor, assayed in combination with NELF-A or -B, affects the competitive decelerator activity of NELF-A or -B during GR-regulated transactivation of an exogenous reporter (GREtkLUC) in transiently transfected U2OS cells. If the selected factor is found to reverse the actions of the NELF protein by acting at the same site as the NELF protein, we can propose that the activity of the factor in question directly counters the step inhibited by NELF. Conversely, if the factor is found to function before or

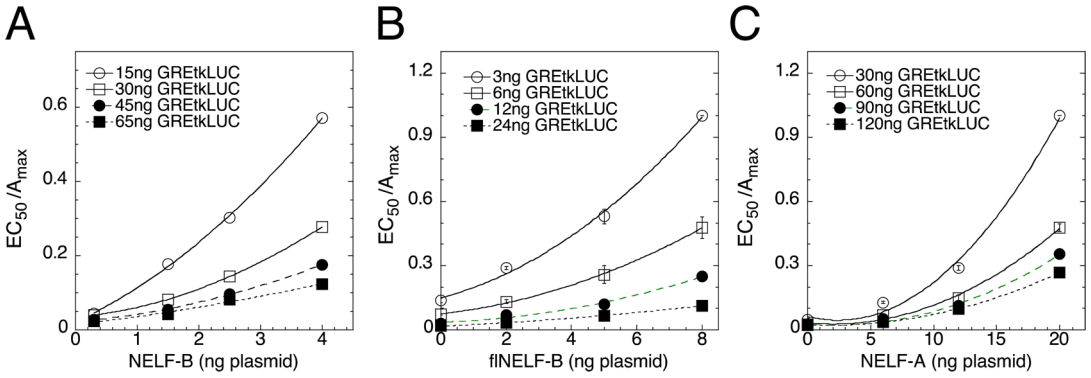

Figure 1. Full length and chimeric NELF-B and full length NELF-A similarly influence GR transactivation by acting at two sites. Graphical analyses of competition assays with four amounts of chimeric NELF-B (A), full length wild-type NELF-B (B), and NELF-A (C) plasmids each with four concentrations of GREtkLUC were conducted in U2OS cells and a constant amount of transfected GR plasmid as described in Experimental Procedures. Plots of EC_{50}/A_{max} vs NELF were best fit with quadratic equations. Similar results were obtained in two additional independent experiments for chimeric NELF-B. Panels B (wild-type NELF-B) and C (NELF-A) are averaged data (±standard error of the mean) of four and six experiments, respectively, after normalization to the LUC activity with the smallest amount of GREtkLUC reporter and the largest amount of NELF plasmid.

after the site of NELF action, then that factor's actions cannot be the direct target of NELF even if the factor is able to reverse the inhibitory activity of NELF.

The competition assay consists of determining the dose–response curves for dexamethasone (Dex) induction of GR-controlled expression of luciferase activity from a GREtkLUC reporter under all 16 possible combinations of four concentrations of each of the two factors being examined (see Figure S1 of the Supporting Information for a flowchart). A series of graphs are constructed, as described in Experimental Procedures, and then matched with the possible graphs in Table S1 (see the Supporting Information). Each graph in Table S1 is associated with one or more mechanistic descriptions. After the mechanistic descriptions from all graphs for each factor have been compared, a single consistent mechanism is identified.[9,10,21]

NELF-A and -B Act at Two Sites in GR-Regulated Transactivation. The NELF complex, composed of the four subunits NELF-A, -B, -C/D, and -E, plays a pivotal role in immobilizing RNA polymerase II shortly after the start of transcription.[11−16,19] NELF-A and -B are particularly interesting because each protein has been found to act as a competitive decelerator of GR transactivation at two sites before or at the CLS.[21] Two forms of NELF-B (full length protein and a chimera lacking the 30 C-terminal amino acids) were initially examined to eliminate the functional relevance of the C-terminal sequence. Subsequently, they were used interchangeably because they had identical properties with two key components (GR and the reporter gene, GREtkLUC) in the mechanistically sensitive competition assay. At least one site of action appears to be independent of polymerase pausing.[21]

The critical feature of two-site action is upward curving plots of EC_{50}/A_{max} versus NELF. Panels A and B of Figure 1 reconfirm the identical behavior of chimeric (Figure 1A) and full length (Figure 1B) NELF-B. Plots of EC_{50}/A_{max} versus NELF-A (Figure 1C) display the same upward curvature. The theory of the competition assay says that a polynomial fit of order "n" to these plots is diagnostic of a competitive decelerator acting at n sites before or at the CLS (abbreviated as = C at n sites ≤ CLS) (see the Supporting Information for a theoretical explanation). This was tested directly for panels B and C of Figure 1, which are averaged plots from four and six normalized experiments, respectively. Bayesian Information Criterion analysis was used to determine the goodness of fit of linear (data not shown) versus quadratic plots for the data depicted in panels B and C of Figure 1. That plot with the lower score is to be preferred. Quadratic fits gave a much lower score than linear fits for both Figure 1B (32.7 vs 113.2) and Figure 1C (104 vs 1863). These results rigorously support the preference of quadratic versus linear fits, which is also indicated by the higher R^2 value of quadratic versus linear fits for all three graphs of Figure 1 (data not shown). Thus, the quadratic fits in Figure 1 are diagnostic of each factor acting as a C at 2 sites ≤ CLS. A competitive decelerator acting at only one site before or at the CLS gives a linear plot with a positive slope in graphs of EC_{50}/A_{max}. Conversely, an accelerator gives a decreasing plot for EC_{50}/A_{max}, and an increasing plot for A_{max}/EC_{50}, versus factor.[9,33] Factors acting at two sites are especially valuable in mechanistic studies because the elimination of just one site of action has to be selective and cannot result from general inactivation of the protein or reaction system.

It should be realized that the actions of NELF-A and -B are additive and thus may be at different steps.[21] Finally, we note that the corrections for nonlinear expression of NELF-B in the previously reported graphs[21] do not cause the upward curvature because it is still evident in panels A and B of Figure 1 with NELF-B plasmid concentrations that give linear protein expression.

Cdk9 Augments NELF Inhibition of GR Transactivation. Cdk9 has been shown to cause the release of paused polymerase, apparently because of the phosphorylation of DSIF, the NELF complex, and pol II.[16−19,26] In view of the activity of both NELF-A and NELF-B at two sites described above, we used the competition assay to ask if exogenous Cdk9 can inhibit or reverse the effects of either NELF protein at either step. Unexpectedly, the competition assay revealed that Cdk9 further reduced A_{max} in the absence or presence of exogenous NELF-A (Figure 2A). This shows that Cdk9 augments, rather than attenuates, NELF-A inhibitory activity. This response is not due to some net decrease in the amount of Cdk9 because Western blots establish that the total amount of Cdk9 protein increases with added Cdk9 plasmid (data not shown). Western blot analysis further revealed both that Cdk9 expression is linear up to 18−20 ng of Cdk9 plasmid (so that no correction is needed for nonlinear expression) and that endogenous Cdk9 in U2OS cells is equal to 9.0 ± 2.6 ng (standard deviation; n = 2) of the Cdk9 plasmid. This last point is especially significant because it establishes that the effects of

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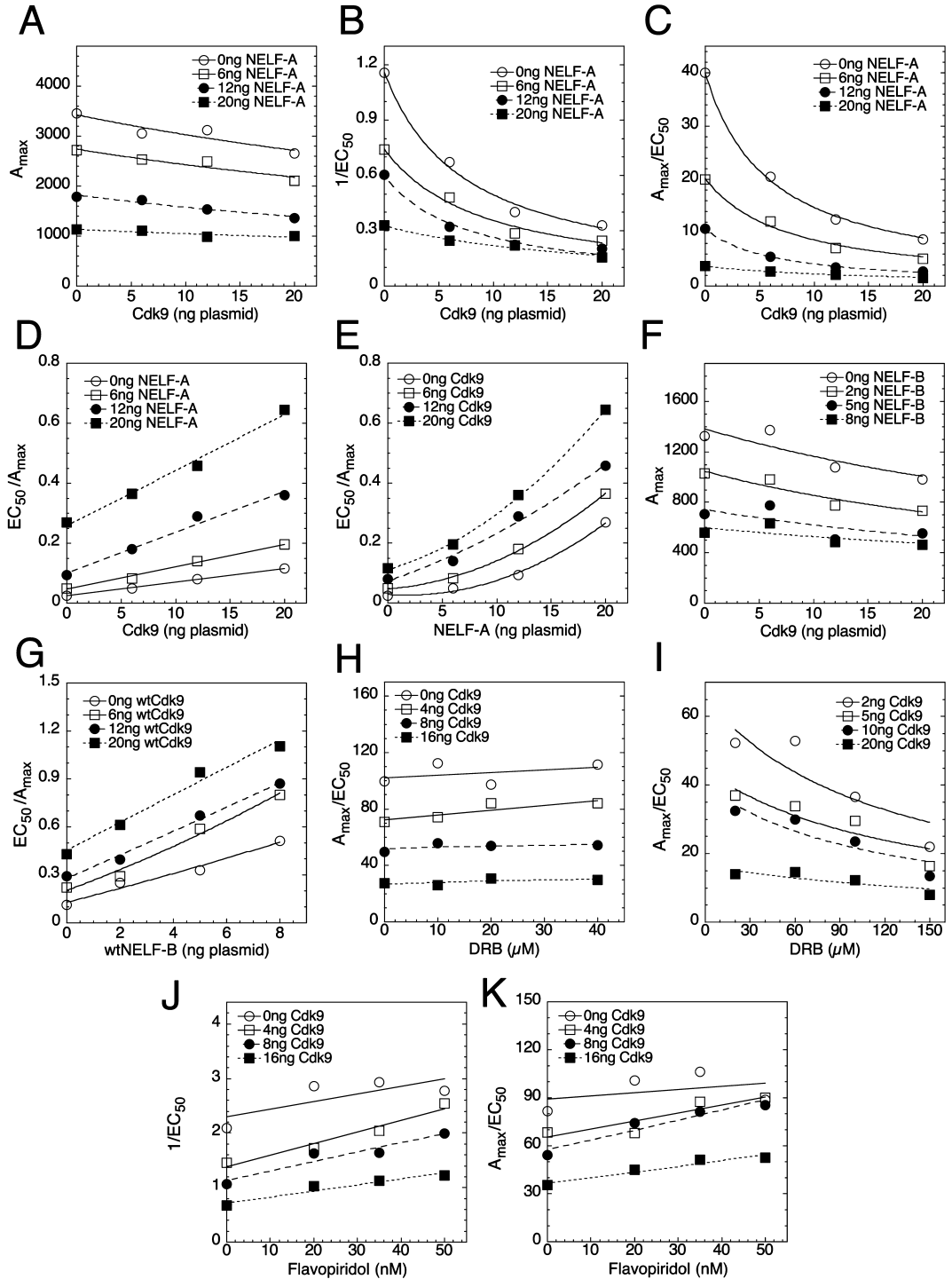

Figure 2. Cdk9 is a decelerator that does not reverse the effects of NELF-A or NELF-B and is not inactivated by chemical inhibitors. (A–G) Cdk9 augments the inhibitory effects of NELF-A and NELF-B. Competition assays of Cdk9 with NELF-A (A–E) or NELF-B (F and G) were performed with the indicated amounts of Cdk9 and NELF plasmids. Graphs of A_{max} vs NELF-A (A) or NELF-B (F) illustrate the additive effects of Cdk9 with each NELF. The other graphs were plotted as in Figure 1. Similar results were obtained in five additional independent experiments. (H–K) Chemical inhibitors of Cdk9 do not block decelerator activity of Cdk9. Assays were performed as described in the legend of Figure 1 with the indicated amounts of Cdk9 and DRB (H and I) or flavopiridol (J and K) and plotted as A_{max}/EC_{50} (H, I, and K) or $1/EC_{50}$ (J). The amount of GREtkLUC reporter was 100 ng in all panels except for F and G, which used 30 ng. Similar results were obtained in two to five additional independent experiments.

added Cdk9 in Figure 2 are seen with relatively small increases in total Cdk9 protein (i.e., ≤122% above endogenous Cdk9)

The graphs of $1/EC_{50}$ versus Cdk9 (Figure 2B) and A_{max}/EC_{50} versus Cdk9 (Figure 2C) display the characteristic nonlinear declining curves, with a decreasing position in the presence of increasing NELF-A, that define Cdk9 as a competitive decelerator. The linear graph of EC_{50}/A_{max} versus Cdk9 (Figure 2D) further specifies Cdk9 actions as a C acting at one site ≤ CLS. At the same time, the quadratic fit for the graph of EC_{50}/A_{max} versus NELF-A, with higher curve positions with increasing amounts of NELF-A (Figure 2E), indicates that NELF-A continues to act as C at two sites ≤ CLS. Similarly, Cdk9 is

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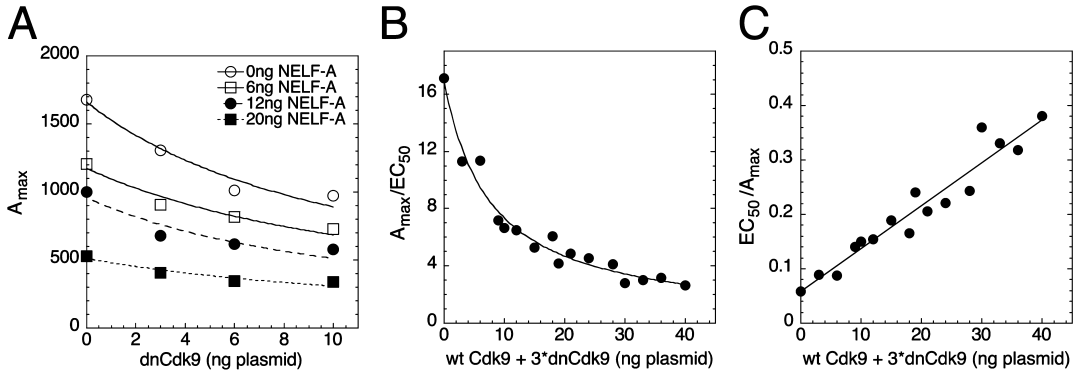

Figure 3. Dominant negative kinase-dead Cdk9 has activity identical to that of wt Cdk9. (A) dnCdk9 augments the inhibitory effects of NELF-A. Competition assays with the indicated amounts of dnCdk9 and NELF-A plasmids were performed and plotted as in Figure 1. Similar results were obtained in four additional independent experiments. (B and C) Competition assays were performed as in Figure 1 with 0, 3, 6, and 10 ng each of wt Cdk9 and dnCdk9 plasmids and a constant amount of GR (0.5 ng) and GREtkLUC (100 ng) plasmids. After adjustment for the 3-fold higher level of protein expression of dnCdk9 compared to that of wt Cdk9, plots of A_{max}/EC_{50} (A) and EC_{50}/A_{max} (B) vs the combined amounts of wt and dnCdk9 were constructed as described in the text. Similar results were obtained in a second independent experiment.

active as C ≤ CLS in competition assays without and with added NELF-B, as shown by the nonlinear decreasing graphs of A_{max} (Figure 2F), $1/EC_{50}$ (data not shown), and A_{max}/EC_{50} (data not shown) and the increasing plots of EC_{50}/A_{max} (data not shown) versus Cdk9. In the presence of added Cdk9, the plots of EC_{50}/A_{max} versus NELF-B have less curvature (Figure 2G). This indicates that Cdk9 has a stronger inhibitory action in the presence of NELF-B, thereby weakening the influence of NELF-B at one of its two sites of action.

One restriction is that if Cdk9 acts as a C at the CLS (abbreviated as = C = CLS), then NELF-A (and NELF-B) must be a C before the CLS (abbreviated as = C < CLS) and vice versa. This is because two C's cannot act at precisely the same step.[33] In either case, however, increased concentrations of Cdk9 do not act as an accelerator to reverse the competitive decelerator effects of NELF-A or NELF-B. Furthermore, Cdk9 exhibits unequal effects in the presence of NELF-A and NELF-B by being a stronger decelerator with added NELF-B.

Classical Inhibitors of Cdk9 Do Not Prevent the Competitive Decelerator Activity of Cdk9. To determine the biochemical mechanism of Cdk9 competitive decelerator action in panels A and F of Figure 2, we asked if Cdk9 action in U2OS cells could be blocked with either of two well-known inhibitors of Cdk9 kinase activity: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)[38] and flavopiridol.[39] Interestingly, DRB concentrations of ≤40 μM have little to no effect on either A_{max}/EC_{50} versus DRB (Figure 2H) or $1/EC_{50}$ versus DRB (data not shown), while higher concentrations (50−150 μM) display the telltale nonlinear decreasing curves in plots of $1/EC_{50}$ (data not shown) and A_{max}/EC_{50} versus DRB (Figure 2I) of a competitive decelerator.[33] Thus, DRB does not prevent, but rather amplifies at high concentrations, the effects of Cdk9 in this system. This may be due to the fact that DRB is known to have different targets in these two concentration ranges: Cdk9 at ∼3 μM DRB and TFIIH at ∼30 μM DRB.[40−42]

Flavopiridol is regarded as a more selective inhibitor of Cdk9 activity with an apparent IC_{50} of <1 μM.[39] In our system, flavopiridol weakly decreases the maximal luciferase activity (data not shown). At the same time, however, our analysis shows that flavopiridol acts as an accelerator (A) of some step after CLS in the concentration range of 1−50 nM, as seen by the linear, positive slope plots of $1/EC_{50}$ and A_{max}/EC_{50} versus flavopiridol[33] (Figure 2J,K). This is an example of the well-documented situation in enzyme kinetics[43,44] in which a factor can decrease A_{max} and still be what we define as an accelerator.[9] Further analysis of the other graphical plots yields the consistent conclusion that flavopiridol is an accelerator after the CLS and Cdk9 is a competitor before flavopiridol. This last conclusion indicates that even though flavopiridol has been shown to bind tightly to, and inactivate, Cdk9,[39] its action in our system occurs after those of Cdk9. Therefore, flavopiridol's ability to reduce the effects of Cdk9 cannot result from inactivating Cdk9 kinase activity and must reflect interactions with either a different function of Cdk9 or some other unidentified target. Interestingly, at concentrations of >40 nM, flavopiridol reverses its effects to act as a competitive decelerator and augment Cdk9 activity (data not shown). Collectively, the results with DRB and flavopiridol suggest that the inhibitory effects of Cdk9 are, unexpectedly, independent of Cdk9 kinase activity.

Mutant Cdk9 Retains Competitive Decelerator Activity. Interpretations based on chemical inhibitors can be misleading because of frequent side effects. A potentially more selective probe of how Cdk9 increases NELF-A and -B activities is a point mutation that prevents Cdk9 kinase activity. HA-tagged D167N Cdk9 is a dominant negative mutant.[45] We confirmed that this mutant (dnCdk9) has negligible kinase activity in a whole cell assay of serine 2 phosphorylation of RNA polymerase II by Cdk9 with or without dnCdk9 (data not shown). Surprisingly, in a competition assay with NELF-A, graphs of A_{max} versus dnCdk9 (Figure 3A) suggest that dnCdk9 is just as active as a competitive decelerator as wt Cdk9 (cf., Figure 2A). Similar results were observed with dnCdk9 in competition assays with NELF-B (data not shown).

A competition assay of dnCdk9 versus wt Cdk9 was then performed to determine whether the dnCdk9 would reduce the competitive decelerator activity of wt Cdk9 in GR induction of the luciferase enzyme from the GREtkLUC reporter. Again, both dnCdk9 and wt Cdk9 appear to have identical activities (data not shown). A stringent test of this conclusion is to see if the activities are additive. Western blot analysis revealed that wt Cdk9 and dnCdk9 are linearly expressed up to at least 20 ng of transfected plasmid and that the expression level of dnCdk9 is ∼3-fold higher than that of wt Cdk9 (data not shown). Therefore, we calculated the total relative amount of expressed Cdk9 as wt Cdk9 + 3 × dnCdk9 and plotted A_{max}/EC_{50} versus the total expressed Cdk9. As shown in Figure 3B, a single well-behaved nonlinear

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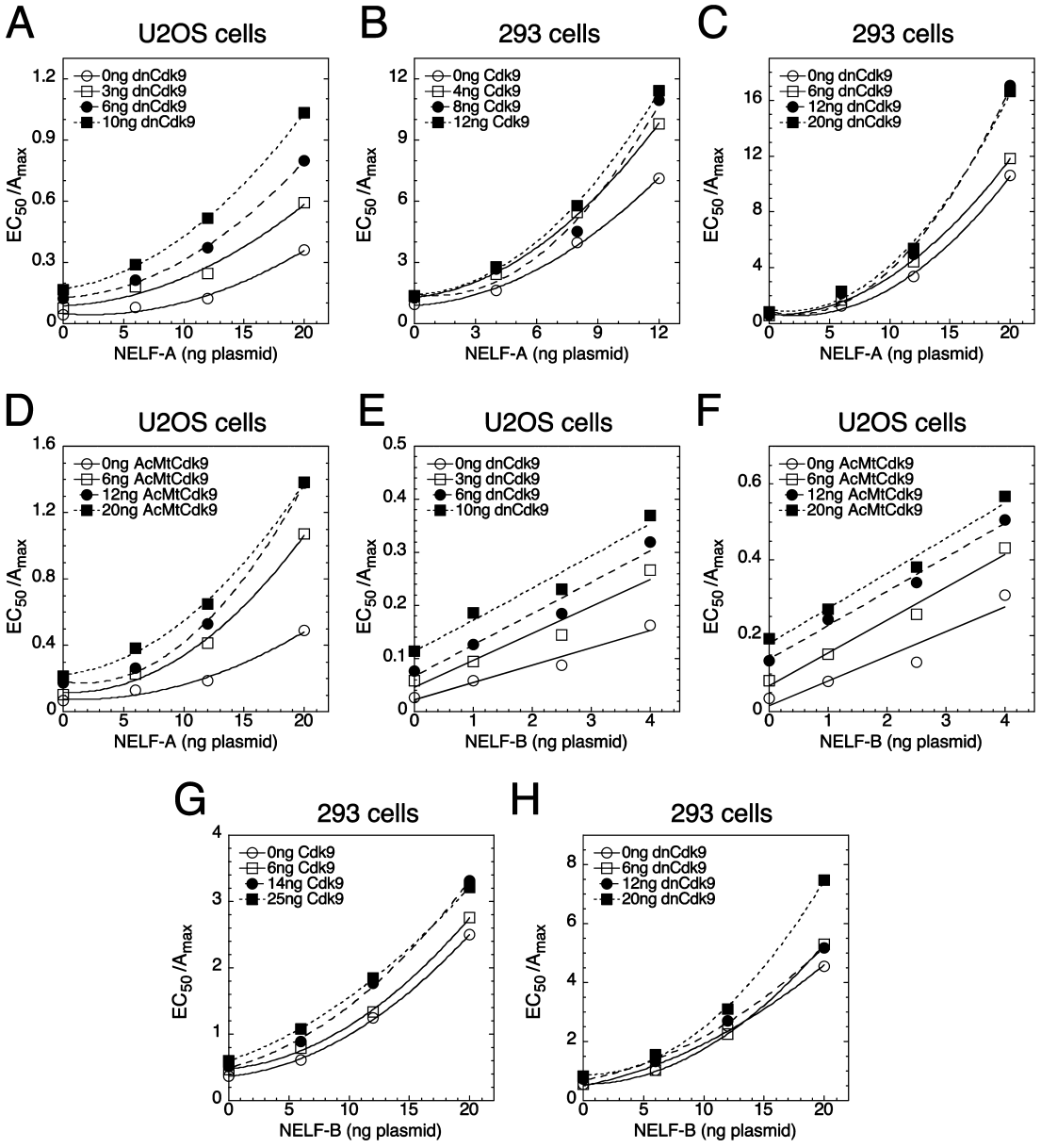

Figure 4. Kinase-defective Cdk9 mutants retain activity of wt Cdk9 with NELF-A and NELF-B in different cells. Competition assays were performed and plotted as in Figure 1 with the indicated amounts of dnCdk9 and NELF-A plasmids in U2OS cells (A) or 293 cells (B and C), with AcMtCdk9 and NELF-A plasmids in U2OS cells (D), with NELF-B in U2OS cells with dnCdk9 (E) or AcMtCdk9 (F), and with NELF-B in 293 cells with wt Cdk9 (G) or dnCdk9 (H). The amount of reporter plasmid was always 100 ng. The amount of GR plasmid used was 0.5 ng in U2OS cells and 5 ng in 293 cells. The range of linear expression of wt Cdk9 (≤40 ng), dnCdk9 (≤20 ng), and NELF-B (≤20 ng) is higher in 293 than in U2OS cells (data not shown). Similar results were obtained in one to four additional independent experiments except for that for NELF-B with AcMtCdk9 in U2OS cells (F), which was performed only once.

decreasing plot is obtained, indicating that the combined wt Cdk9 and dnCdk9 species are acting as a single, uniformly active competitive decelerator.

To determine where the combined Cdk9 species are acting, the data were replotted as EC_{50}/A_{max} versus total expressed Cdk9 (Figure 3C). The resulting linear plot is diagnostic of the combined population of wt Cdk9 and dnCdk9 acting in concert as competitive decelerators before or at the CLS.[33] Similar results were observed in 293 cells (data not shown). Therefore, the ability of wt Cdk9 and dnCdk9 to display identical activities is not limited to U2OS cells. These data support and extend the conclusion described above that the competitive decelerator activity of Cdk9 on GR transactivation is independent of Cdk9 kinase activity.

Mutant Cdk9s Retain the Ability To Augment NELF-A Actions. While Figure 3 indicates that dnCdk9 has wild-type activity with regard to the inhibition of GR transactivation, it was not evident whether dnCdk9 would also have wild-type activity with the NELFs when the other types of competition assay plots are examined. Therefore, we next asked if dnCdk9 could also increase the decelerator activity of NELF-A. Using amounts of dnCdk9 and NELF-A that are in the linear range of expression, the behavior with added dnCdk9 is indistinguishable from that with wt Cdk9 (data not shown). Most importantly, plots of EC_{50}/A_{max} versus NELF-A with increasing dnCdk9 retain the

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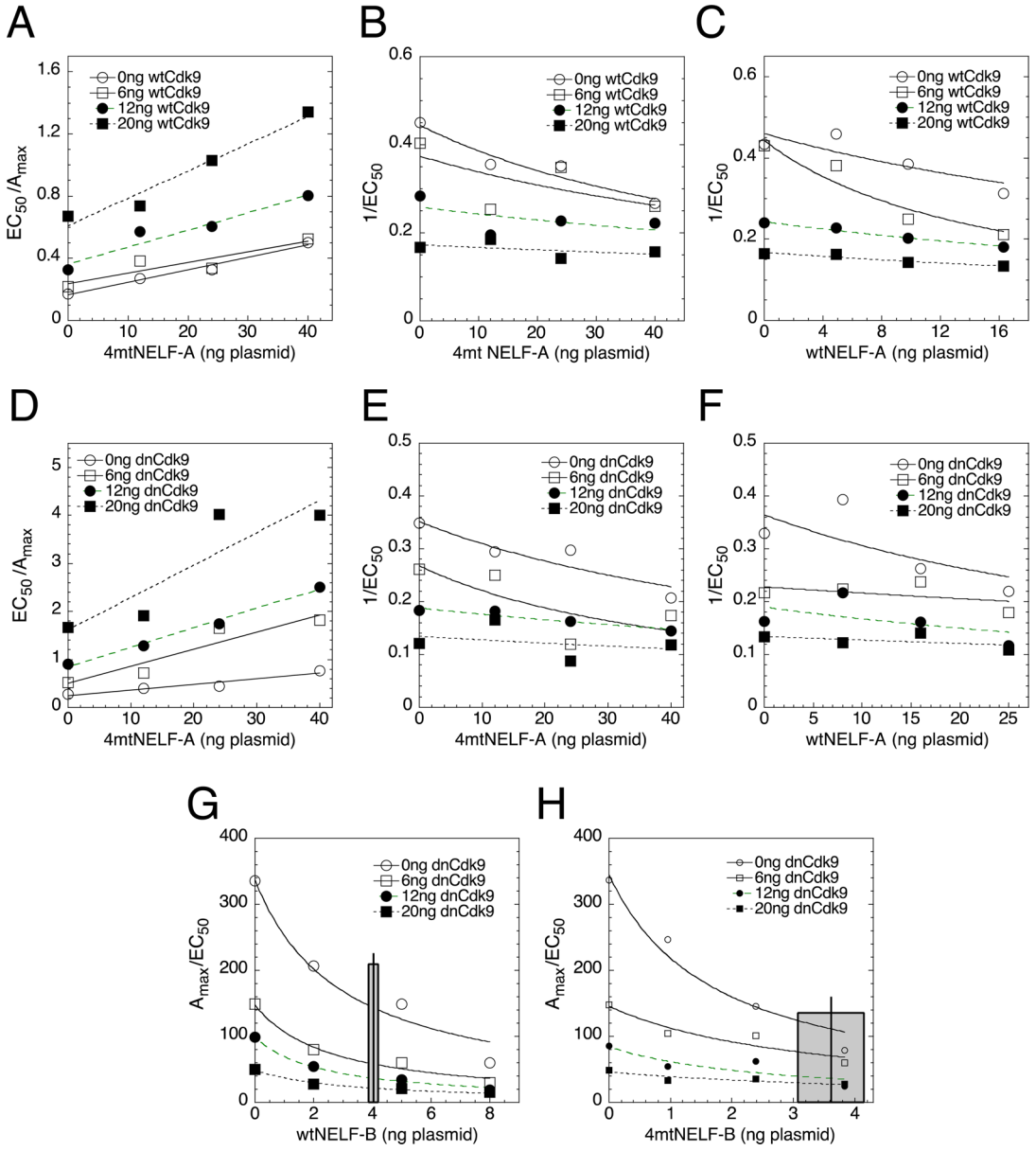

Figure 5. Wild-type and mutant NELF-A and -B activities are not altered by mutations that destroy Ckd9 kinase activity. Competition assays were performed with 0.3 or 0.5 ng of GR plasmid and 30 ng of GREtkLUC, and the results are plotted as in Figure 1 with the indicated amounts of wt Cdk9 and 4mtNELF-A (A and B) or wt NELF-A (C) plasmids or dnCdk9 and 4mtNELF-A (D and E) or wt NELF-A (F) plasmids, in U2OS cells. Similar results were obtained from five to eight independent experiments. (G and H) 4mtNELF-B has reduced potency, relative to that of wt NELF-B, in competition assays with dnCdk9. Competition assays were performed with 0.5 or 5 ng of GR plasmid and 100 ng of GREtkLUC, and A_{max}/EC_{50} vs NELF-B was plotted as in Figure 1 with the indicated amounts of wt Cdk9 and wt NELF-A (G) or 4mtNELF-A (H) plasmids. The gray bar with the extending line represents the standard error of the mean and average amount of plasmid required for half-maximal reduction of A_{max}/EC_{50} from six experiments. To compensate for the 2.09-fold more efficient expression of 4mtNELF-B protein, compared to that of wt NELF-B [as determined by Western blots (not shown)], the *x*-axis values of panel H should be multiplied by 2.09. Similar results were obtained from five additional independent experiments.

same upward curvature with dnCdk9 (Figure 4A) that is seen with wt Cdk9 in Figure 2E. This shows that NELF-A still acts as C at two sites before or at the CLS with dnCdk9, just as is seen with wt Cdk9. Identical experiments were performed in human 293 cells to determine the generality of this response. Panels B and C of Figure 4 show that there is no significant difference in the activities of wt Cdk9 and dnCdk9 with NELF-A in 293 cells. Thus, the ability of NELF-A to act as a C at two sites before or at the CLS in competition assays with Cdk9 and the ability of Cdk9 to act as C before or at the CLS are independent both of the kinase activity of Cdk9 and of the cells used.

To further test this equivalence of wt Cdk9 and dnCdk9, we examined another Cdk9 mutant that cannot be acetylated at the position needed to yield the activated kinase, i.e., K44/48R Cdk9 (or AcMtCdk9).[46] Just as with wt Cdk9 and dnCdk9, the graph of EC_{50}/A_{max} versus NELF-A with increasing AcMtCdk9 displays the same progressively greater upward curvature as with wt Cdk9 (Figure 4D), which is diagnostic of NELF-A again acting as C at two sites ≤ CLS. Thus, two different kinase-deficient mutants of Cdk9 possess the same activities in U2OS cells as wt Cdk9 with NELF-A.

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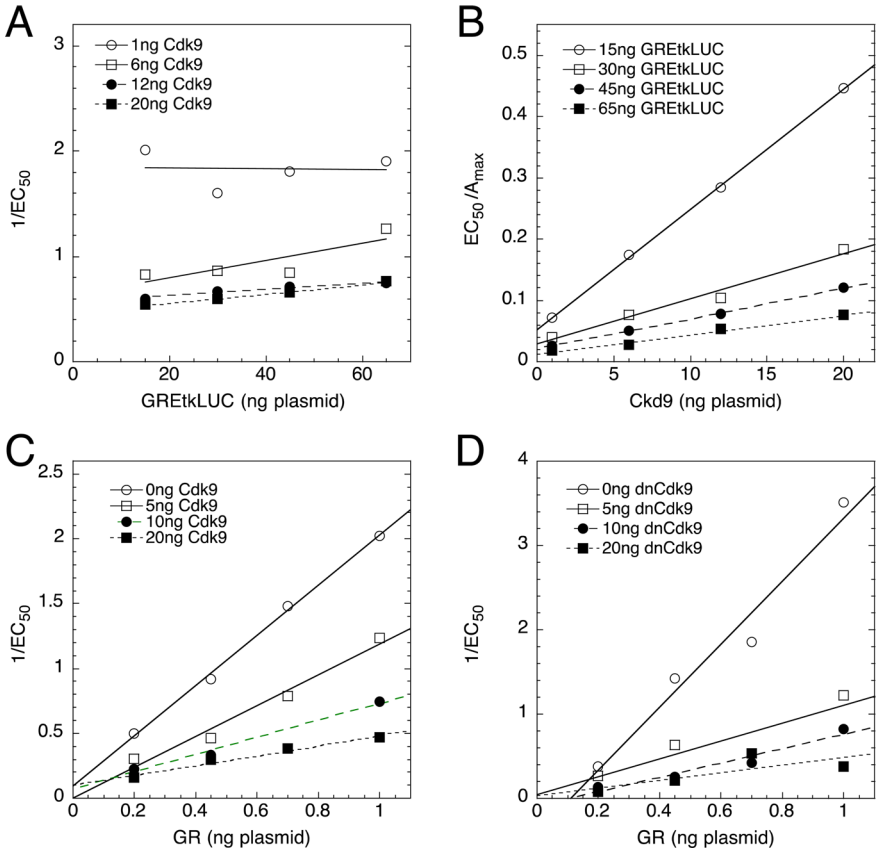

Figure 6. Cdk9 is a competitive decelerator acting after the site of action of GR and before that of the reporter gene. Competition assays with the indicated amounts of (A and B) Cdk9 and GREtkLUC reporter, (C) Cdk9 and GR, and (D) dnCdk9 and GR plasmids were performed in U2OS cells and the results plotted as in Figure 1. Similar results were obtained in five additional (for A and B) and two additional (for C and D) independent experiments.

Mutant Cdk9s Retain the Ability To Augment NELF-B Actions. As seen above in Figure 2G, NELF-B works predominantly at one site as a C ≤ CLS in U2OS cells in the presence of additional wt Cdk9. This contrasts with NELF-A, which still acts as C at two sites ≤ CLS in the absence or presence of exogenous Cdk9 (see Figure 2E). We therefore asked whether the action of NELF-B at the second site ≤ CLS might be restored, or the one site of action eliminated, in the presence of either the dnCdk9 or AcMtCdk9. Interestingly, as determined by the linear plots of EC_{50}/A_{max} versus NELF-B with added Cdk9, NELF-B still appears to function as C at just one site ≤ CLS because of the stronger decelerator activity of both dnCdk9 (Figure 4E) and AcMtCdk9 (Figure 4F) in the presence of NELF-B. These same graphs, and others not shown, identify the actions of dnCdk9 and AcMtCdk9 as C ≤ CLS. Thus, as for NELF-A, the two kinase-defective Cdk9 mutants evoke the same increasingly pronounced responses with NELF-B as seen with wt Cdk9. This is further evidence that the effects of added Cdk9 are independent of Cdk9 kinase activity.

Actions of NELF-B Are Cell Line-Dependent but Still Independent of Cdk9 Kinase Activity. In marked contrast with the nearly linear plots of EC_{50}/A_{max} versus NELF-B with added Cdk9 in U2OS cells (Figure 2G), the same plots for 293 cells are now curved upward (Figure 4G). However, this difference may be simply a matter both of NELF-B protein being expressed ~9-fold more efficiently in 293 cells and of using more NELF-B plasmid in 293 cells, thereby making the curvature more evident. Nevertheless, this upward curvature in plots of EC_{50}/A_{max} versus NELF-B, which is unique for a factor acting as C at two sites ≤ CLS, is retained in dnCdk9 (Figure 4H). Thus, once again, the actions of Cdk9 are independent of its kinase activity.

Mutation of the NELF Domain Has Equal Effects on Activity with wt Cdk9 and dnCdk9. Earlier studies identified a "NELF domain" that is shared by all four subunits of the NELF complex and is required for full competitive decelerator activity of both NELF-A and NELF-B at two sites in competition assays with GR and GREtkLUC.[21] Similarly, the graphs of EC_{50}/A_{max} that are quadratic and upward curving for wt NELF-A (Figure 2E) change to what is generally best fit by linear plots for the 4mtNELF-A mutant, in which four residues in the NELF domain of NELF-A have been changed to alanine (Figure 5A). The linear fits of EC_{50}/A_{max} mean that the actions of 4mtNELF-A with varying wt Cdk9 are predominantly that of a C acting at one site before or at the CLS. Thus, as with varying levels of GR and GREtkLUC,[21] the mutations of the NELF domain have also eliminated most of the ability of NELF-A to act at one of the two sites before or at the CLS in the presence of added wt Cdk9. The nonlinear decreasing graphs of $1/EC_{50}$ versus both 4mtNELF-A (Figure 5B) and wt NELF-A (Figure 5C) approach a flat plot with large amounts of NELF-A. The underlying equations of the competition assay specifically ascribe this particular graphical behavior to a defined relative ordering of two competitive decelerators that are both initially characterized as acting before or at the CLS. More specifically, it means that the single site of action for 4mtNELF-A and the two sites of action with wt NELF-A are still after wt Cdk9.

The activities of 4mtNELF-A and wt NELF-A with the dnCdk9 mutant are very similar to those with wt Cdk9. The

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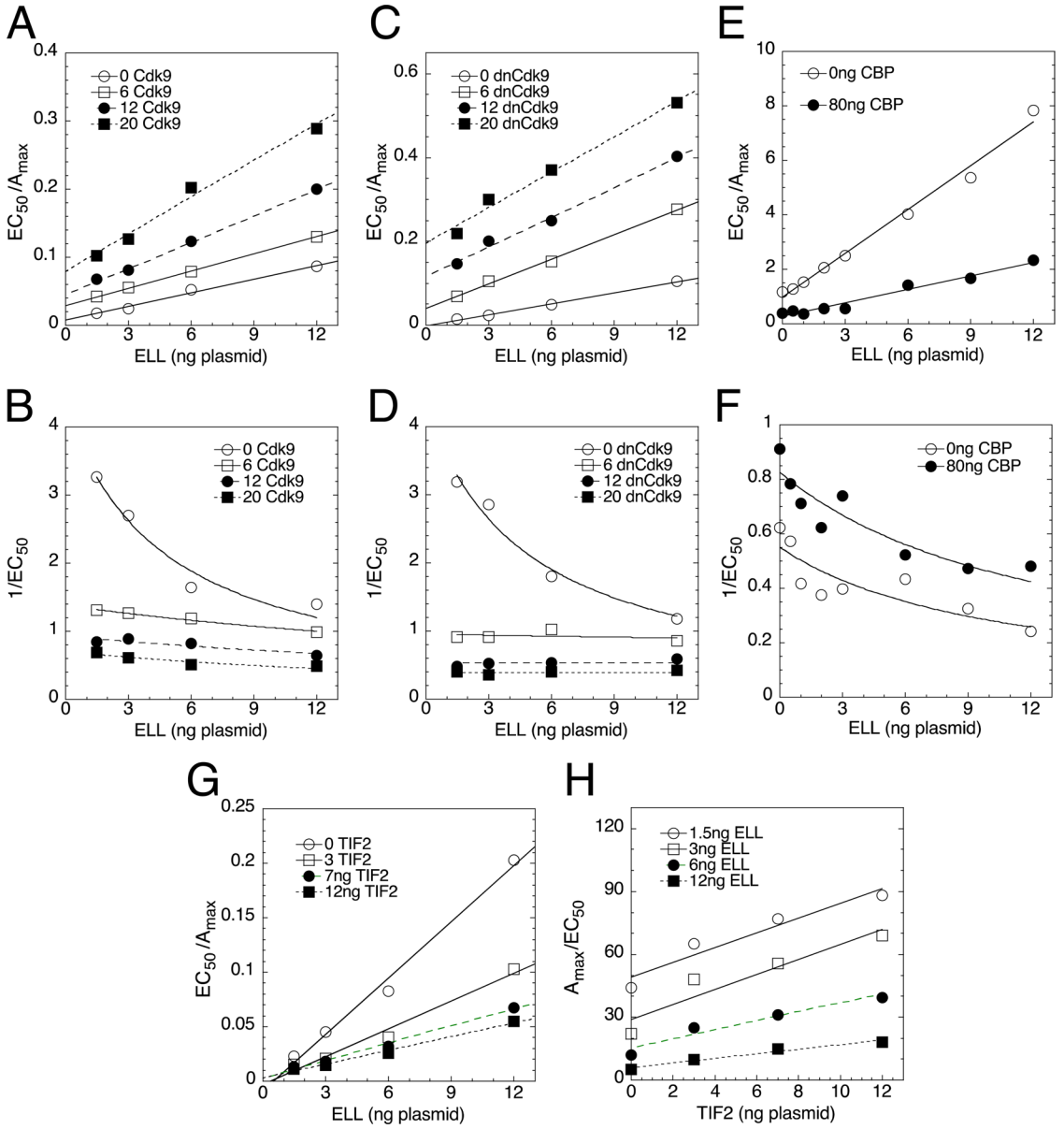

Figure 7. Competitive decelerator activity of ELL that is augmented by Cdk9 in a manner independent of Cdk9 kinase activity and reversed by accelerators. Competition assays with 0.5 or 1 ng of GR plasmid, 100 ng of GREtkLUC, and the indicated amounts of (A and B) Cdk9 and ELL, (C and D) dnCdk9 and ELL, (E and F) CBP and ELL, and (G and H) TIF2 and ELL plasmids were performed in U2OS cells and the results plotted as in Figure 1. Wild-type Cdk9, dnCdk9, and ELL are linearly expressed with up to 20 ng of transfected plasmid; CBP is linearly expressed with up to 80 ng of plasmid, so no corrections are needed. TIF2 expression is corrected as described in the text. Similar results were obtained in five (A and B), two (C and D), one (E and F), and three (G and H) additional independent experiments.

graph of EC_{50}/A_{max} versus NELF-A again changes from a quadratic plot with wt NELF-A (Figure 4A) to a linear graph with 4mtNELF-A (Figure 5D), indicating that 4mtNELF-A also functions with added dnCdk9 at only one site as a C before or at the CLS. The graphs of $1/EC_{50}$ versus both 4mtNELF-A (Figure 5E) and wt NELF-A (Figure 5F), like those of panels B and C of Figure 5 with wt Cdk9, become flatter with increasing dnCdk9, which places dnCdk9 action before both 4mtNELF-A and wt NELF-A. We therefore conclude that the loss of Cdk9 kinase activity in dnCdk9 does not alter the actions of either wt NELF-A or 4mtNELF-A from what they are with wt Cdk9.

We next compared the actions of wt NELF-B and 4mtNELF-B with wt Cdk9 vs dnCdk9. No difference was observed between wt NELF-B and 4mtNELF-B with wt Cdk9. As with wt NELF-B (Figure 2G), the graphs of EC_{50}/A_{max} versus 4mtNELF-B with increasing wt Cdk9 are fit well by linear plots (data not shown) that define a C acting at one site before or at the CLS. The increasingly flat plots of $1/EC_{50}$ versus NELF-B again indicate that Cdk9 is a C acting before NELF-B (data not shown). However, graphs of A_{max}/EC_{50} (Figure 5G,H) illustrate that wt NELF-B and 4mtNELF-B do display quantitatively different abilities to reduce A_{max}/EC_{50}. After correction has been made for the 2.1-fold more efficient expression of 4mtNELF-B (data not shown), in which case 4 ng of 4mtNELF-B in Figure 5H corresponds to 8.4 ng of wt NELF-B plasmid, 4mtNELF-B is seen to be 1.9 ± 0.5-fold (standard error of the mean; $n = 23$; $P = 0.0003$) less potent than wt NELF-B with different concentrations of dnCdk9. Thus, again with mutants of NELF-A and -B, the kinetically defined mechanism and site of action are relatively insensitive to whether Ckd9 possesses kinase activity. These

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results argue strongly that Cdk9 has some new, yet undefined, kinase-independent action in GR-regulated gene induction.

Cdk9 Acts after GR and before or at the Site of Reporter Action. In an effort to define further the novel actions of Cdk9 described above, we performed a competition assay with wt Cdk9 and the reporter GREtkLUC in U2OS cells. In all of the combinations of factors that we have examined to date, GREtkLUC always acts as an accelerator (A) = CLS.[9] Similarly, in competition with Cdk9, the average slope (0.00192 ± 0.00428, standard deviation; $n = 24$) in graphs of $1/EC_{50}$ versus GREtkLUC (Figure 6A), with curve position decreasing with added Cdk9, indicates that GREtkLUC again acts as A = CLS while Cdk9 = C. The linear plots of EC_{50}/A_{max} versus Cdk9, with decreasing position for increasing GREtkLUC (Figure 6B), are diagnostic of Cdk9 = C at one site $\leq$ CLS. These graphs, together with the others in the analysis of the data,[33] uniquely determine that GREtkLUC = A = CLS and Cdk9 = C $\leq$ CLS.

To further restrict the site of action of Cdk9, we allowed Cdk9 to compete with GR, which is known to act before the CLS.[9,21] The defining graph here is $1/EC_{50}$ versus GR. It shows an intersection point that is slightly more negative than that without added GR, which corresponds to no GR when one subtracts the small amount of endogenous GR in U2OS cells (Figure 6C). This behavior, in conjunction with the other graphs (data not shown), places the site of Cdk9 action as being after GR.[33] Similar results and conclusions were obtained for GR versus dnCdk9 (Figure 6D). We therefore conclude that wt Cdk9 and dnCdk9 both act as C $\leq$ CLS and after GR.

Cdk9 Acts before ELL. ELL, like Cdk9, is a component of the super-elongation complexes, which is associated with the release of paused polymerases.[15,16,20] This suggested that ELL might antagonize the inhibitory actions of Cdk9 in our assay, even though, in different systems (RC.SV3 and Cos1 cells), ELL was reported to decrease the level of GR transactivation by an undetermined mechanism.[25] We therefore tested this hypothesis by allowing ELL to compete with Cdk9. The graphs of EC_{50}/A_{max} versus ELL (Figure 7A) are linear with increasing position as more Cdk9 is transfected. The plots of EC_{50}/A_{max} versus Cdk9 and EC_{50}/A_{max} versus ELL are almost identical (data not shown), from which we can conclude that both ELL and Cdk9 are C at one site $\leq$ CLS. The graphs of $1/EC_{50}$, however, are different, with that versus ELL being especially informative (Figure 7B and data not shown). As mentioned above, the mathematics describing nonlinear decreasing curves that become flat, but continue to shift to a lower position, at higher levels of Cdk9 uniquely describes Cdk9 acting before ELL, with both manifesting their actions $\leq$ CLS. The identical behavior is observed in competition assays of ELL with dnCdk9 (Figure 7C,D). Thus, both the mode and position of action of Cdk9 relative to ELL are preserved regardless of whether Cdk9 kinase activity is retained.

ELL Acts before CBP and TIF2. The mechanism of ELL action was further probed in competition assays with two accelerators: CBP[9] and TIF2.[33] The competition assays of ELL versus CBP involved eight concentrations of ELL and two concentrations of CBP, instead of the usual 4×4 setup, because of the relatively small changes in EC_{50} with added ELL under these conditions. Two plots were particularly revealing. The plot of EC_{50}/A_{max} versus ELL (Figure 7E) displays the linear plots with decreasing slope in the presence of added CBP that is diagnostic of ELL being a competitive decelerator before or at the CLS with CBP being an accelerator. The plot of $1/EC_{50}$ versus ELL (Figure 7F) shows decreasing curves that increase in position with more CBP and for which the shape of the curve does not become significantly flatter with no added CBP. In conjunction with the results depicted in Figure 7E, the interpretation of Figure 7F is limited to ELL being a competitive decelerator before or at the CLS while CBP is an accelerator after the CLS. All of the other graphs (not shown) are compatible with this conclusion.

We next examined the competition of ELL with the accelerator TIF2.[10,21,32,33] A critical graph is EC_{50}/A_{max} versus ELL (Figure 7G), which shows the linear plots with decreasing slopes that are diagnostic of ELL acting as C $\leq$ CLS and TIF2 = A. The behavior of ELL is confirmed, and that of TIF2 specified, by the linear plot of A_{max}/EC_{50} versus TIF2 (Figure 7H). The average intersection point of all of the lines is $x = -20.8 \pm 7.3$ ng of TIF2 plasmid and $y = -10.7 \pm 6.6$ (standard deviation; $n = 4$). This point is very close to $y = 0$, in view of the range of y values for the various points, and much more negative than $x = -1.17$, which corresponds to the point at which the level of endogenous TIF2 [determined from Western blots to be equivalent to 1.17 ± 0.31 ng (standard deviation; $n = 3$) of TIF2 plasmid] would be equal to zero. These characteristics of Figure 7H define TIF2 = A > CLS and ELL = C $\leq$ CLS. The other graphs (data not shown) are entirely consistent with this interpretation.

DISCUSSION

This report describes the use of a recently developed competition assay[9,10,21,33] to uncover novel activities in GR-regulated gene induction of two cofactors known to participate in the early stages of gene transcription: Cdk9 and ELL. Cdk9 is widely considered to release pol II from the paused state, caused in part by the NELF and DSIF complexes, by phosphorylating the CTD of RNA pol II and the NELF and DSIF complexes.[16,26] ELL is a constituent of the super-elongation complexes that assist in the release of paused polymerases.[15,16,20] Thus, additional Cdk9 and ELL were predicted to facilitate GR transactivation and antagonize the effects of NELF. Unexpectedly, both Cdk9 and ELL are found to be competitive decelerators of GR transactivation activity and do not interfere with the inhibitory activity of NELF-A and NELF-B. Exhaustive analysis of the kinetically defined activity, and site of action, of ELL and especially Cdk9 yields the internally consistent results that both factors are competitive decelerators acting at one site, with Cdk9 acting before ELL, which acts before or at the CLS. Cdk9 actions in these assays appear to be atypical because they are independent of Cdk9 kinase activity, as shown by equivalent effects by two Cdk9 mutants (dnCdk9 and AcMtCdk9) with little or no kinase activity and the inability to be directly antagonized by two classical inhibitors of Cdk9 kinase activity: DRB and flavopiridol. Low concentrations (≤ 40 μM) of DRB that are reported to inactivate Cdk9 kinase activity[40−42] were essentially inactive. Flavopiridol inactivates Cdk9 kinase activity by forming an essentially irreversible complex with Cdk9.[39] Flavopiridol can reverse the effects of Cdk9 in our system but does so downstream of Cdk9, in which case it cannot be exerting the observed effects via a covalent complex with Cdk9. Therefore, it is not known if the ability of flavopiridol to counter the effects of Cdk9 reflects the different activity of Cdk9 in our system, which flavopiridol is preventing at a downstream site, or an activity of flavopiridol that is unrelated to Cdk9. Finally, the same responses to wt and mutant Cdk9s are seen in two different cell lines in the presence of a variety of other factors. We therefore conclude that these new activities of Cdk9 are not restricted to a unique cell line or combination of cofactors.

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The analysis of competition assays of Cdk9, ELL, and several other factors establishes the following ordered sequence of where each factor acts during the overall reaction sequence: GR < Cdk9 < ELL, NELF-A, and NELF-B ≤ GREtkLUC < TIF2 and CBP. This ability to order the site of factor action is unique and provides the beginnings of a previously unavailable logical framework for manipulating GR transaction outcomes under physiologically relevant conditions. Particularly attractive in the clinical setting is the prospect that modifying steps far downstream of GR and reporter action will have fewer side effects. This is because there would be fewer branch points from a far downstream step, and a reduced number of subsequent steps, that could further influence the final outcome. Such a reduction of side effects is the holy grail of steroid endocrinologists[47] and would greatly increase the number of clinical applications of glucocorticoid therapies.

Complete validation of the conclusions described above, and our approach in general, will require knowledge of all of the steps in steroid-regulated gene expression followed by careful examination of how a specific factor alters the rate or frequency of the affected step. While such confirmation is theoretically possible, the required knowledge of each reaction step does not currently exist. In the meantime, the internally consistent mechanistic conclusions that are accumulating for various factors under different experimental conditions[9,10,21,32,33,48] provide a less rigorous cross-check on the legitimacy of the competition assay and its underlying theory.

We have recently presented evidence that NELF-A and NELF-B each act at two sites to repress GR transactivation. We concluded that at least one action of each protein is independent of the NELF complex and depends upon the integrity of a "NELF domain" for full activity at both sites.[21] However, the second site of action of NELF-A and/or -B could proceed via the NELF complex. In this case, we would expect Cdk9 to inhibit some of the activity of one or both NELF proteins. Instead, we find that Cdk9 increases the inhibitory activity of both NELF-A and -B (Figure 2A,F). This argues that neither site of action of NELF-A or -B involves the NELF complex. Because Cdk9 and ELL augment these new, NELF complex-independent activities of NELF-A and -B, we conclude that the effects of Cdk9 and ELL presented here are also independent of the NELF complex. In fact, polymerase pausing has not been observed under the conditions used here for a reporter that is without chromatin structure and introns[49−51] and does not require synchronized expression during development and differentiation.[52] Thus, these conditions would facilitate the observation of new activities with Cdk9 and ELL.

The activities that we see result from relatively low levels of overexpression that do not appear to saturate the system. The amounts of Cdk9 plasmid used typically cause a no more than 2.2-fold increase in total cellular Cdk9 protein after 48 h with wt Cdk9 plasmid and no more than 3-fold with dnCdk9 plasmid (data not shown). The average fold increase for the other factors was only 8.5 ± 4.5 (standard deviation; $n = 6$ factors). This does not include NELF-B because the commercially available antibody did not recognize the endogenous protein. We cannot eliminate the possibility that endogenous factor levels are already maximal and any added factor results in squelching. For example, HEXIM1 both forms a complex with 7SK small nuclear RNA and P-TEFb and binds by itself to GR to inhibit GR transactivation.[53] Elevated levels of Cdk9, and maybe even dnCdk9, could sequester free HEXIM1, but this should cause an increase in the level of GR transactivation, in contrast to the observed decrease in panels A and F of Figure 2 and panel A of Figure 3. Furthermore, the fact that well-behaved plots for each factor are obtained over the entire range of concentrations, including very low levels of transfected factor plasmid, is consistent with the added factors simply increasing the strength of the response of the endogenous factor. Cdk9, as part of P-TEFb, is known to bind ligand-bound estrogen receptor α and is required for estrogen receptor α-dependent transcriptional elongation of the Myb proto-oncogene.[29] This behavior is diametrically opposed to what we see for the effect of Cdk9 on GR transactivation. Ckd9 has also been observed to enhance androgen receptor transactivation by phosphorylating the receptor protein.[30,31] A similar mechanism cannot be operative in our experiments with GR because Cdk9 is inhibitory and two kinase-defective Cdk9 mutants are still active. Thus, the activities of Cdk9 with GR appear to be unique and are clearly independent of the kinase activity associated with most of the actions of Cdk9.

Cdk9 is best known for its kinase activity. However, other activities of Cdk9 have been reported. Cdk9 represses B-Myb transactivation activity in a manner that is independent of Cdk9 kinase activity. This response appeared to be due to a direct interaction of Cdk9 alone with the carboxyl terminus of the B-Myb protein. The authors speculated that similar binding could negatively regulate other transcription factors.[27] If such a mechanism is operative in our system, it could explain why the Cdk9 inhibitors DRB and flavopiridol do not have the expected effects. Alternatively, the inactivity of DRB and flavopiridol may be attributed to the intronless reporter gene, GREtkLUC, used in our study. CDK9 inhibitors drastically affect the elongation of transcription of the β-actin gene but have little effect on the transcription of short intronless genes, such as the U2 snRNA genes.[54] Finally, although Cdk9 is present in the P-TEFb complex that travels with elongating pol II, its CTD kinase activity is no longer required once the pol II complex is released from the pause site.[28] It will be interesting to see whether future studies identify the new activity of Cdk9 of our study as being the same or different from any of the previously reported mechanisms.

Reports that ELL, in addition to its role in the super-elongation complex, has different effects on various steroid receptors argue against a common role in gene transcription. ELL induces an increase in mineralocorticoid basal activity in a manner that requires the N-terminal AF-1b domain, but not AF-1a or ligand binding domain, of mineralocorticoid receptors. This response is receptor specific, though, because ELL has no effect on androgen receptor or progesterone receptor activity and decreased the level of GR transactivation while binding to GR.[25] Also, the N-terminus of ELL is thought to have inhibitor activity for pol II.[55] Therefore, excess ELL could form premature complexes with pol II prior to entering the preinitiation complex, which could disrupt assembly.

It is important to realize that the results from the competition assays do not include any information about the binding site or biochemical activity of the factor. Only the site of biological activity is identified, and this need not be the same as the binding site. One alternative would be that the factor initiates a sequence of "side" reactions that produces a "product" that participates in the main transcription sequence. This is allowable in the theory behind the competition assay as long as the dose response between the factor and product of the side reaction has a Hill plot coefficient n equal to 1. For example, cofactors that cause acetylation or methylation of nucleosomal histones are thought to be influential in gene transcription. However, it is highly

probable that the act of acetylation or methylation is not the "action", as defined by our model,[33] of these cofactors that alters gene transactivation. Instead, the ability of the modified histones to interact with or recruit other proteins is thought to be the first of several additional biochemical steps (not yet defined), of which one is where the cofactor actually "expresses" its activity in a currently unknown manner. In this respect, the site of cofactor action will be several steps downstream from the site of cofactor binding, and the cofactor may act in either a "*cis*" or "*trans*" mode.

It should be noted that the positioning of GR, TIF2, and CBP relative to GREtkLUC, which acts at the CLS, is the same as seen previously under different conditions.[9,10,33] This observation thus again raises the useful mechanistic hypothesis that the sites of action of numerous cofactors in GR-regulated gene induction may be constant under a variety of conditions.[9,10,33]

In summary, our competition assay has unveiled new activities of Cdk9 and ELL. This is possible because of two features of the assay. First, it involves transiently transfected reporter genes, the responses of which are easier to interpret because of their usually more robust and reproducible responses. While the utility of exogenous reporters has been questioned because of the lack of chromosomal structure,[49] this does not appear to a major issue for GR-regulated gene induction. From 80 to 95% of endogenous GR-regulated genes already have an open chromatin conformation,[56−58] and the kinetically defined actions of several transcription factors are found to be the same in GR induction of exogenous and endogenous genes.[10,21,33] Second, virtually all studies of cofactor action look only at the total activity, or A_{max}. When one includes the EC_{50}, dramatically more and usually novel information becomes available.[32,33,59] We suspect that incorporating both A_{max} and EC_{50} into studies of steroid hormone action, not to mention gene transcription in general, will reveal a wealth of new, physiologically relevant information such as that included in this study.

■ ASSOCIATED CONTENT

Supporting Information

A brief summary of the theory behind the competition assay and its application, a flowchart of the steps of the competition assay, and an update of the original table[33] that is used to analyze the various graphs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

A, accelerator; C, competitive decelerator; CLS, concentrating limiting step; CBP, CREB-binding protein; Cdk9, cyclin-dependent kinase 9; Dex, dexamethasone; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GR, glucocorticoid receptor; DSIF, DRB sensitivity-inducing factor; HRE, hormone response elements; ELL, eleven-nineteen lysine-rich leukemia; MR, mineralocorticoid receptor; NELF, negative elongation factor; P-TEFb, positive transcription elongation factor b; SECs, super-elongation complexes; TIF2, transcriptional intermediary factor 2; TSS, transcriptional start site.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on March 11, 2014. A change has been made to the caption of Figure 5 and the corrected version was reposted on March 13, 2014.