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Mutations within a conserved protein kinase A recognition sequence confer temperature-sensitive and partially defective activities onto mouse c-Rel

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Abstract

We have created two mutants of mouse transcription factor c-Rel (c-G29E and c-R266H) that are analogous to mutants previously shown to have temperature-sensitive (ts) functions for the homologous *Drosophila* protein Dorsal and the retroviral oncoprotein v-Rel. In vitro, c-R266H shows both a ts and a concentration-dependent ability to bind DNA, suggesting that the lesion affects the ability of c-Rel to form homodimers. In contrast, the ability of mouse c-G29E to bind DNA in vitro is not ts. c-Rel mutant c-R266H also shows a ts ability to activate transcription from a κB-site reporter plasmid, whereas c-G29E activates transcription well above control levels at both 33 and 39 °C. Insertion of two amino acids (Pro–Trp) between amino acids 266 and 267 in mouse c-Rel (mutant c-SPW) also creates a c-Rel protein with distinct properties: mutant c-SPW is partially defective in that it cannot form DNA-binding homodimers but can form DNA-binding heterodimers with p50. Interestingly, the mutations in c-Rel that affect homodimer formation (c-R266H and c-SPW) fall within a consensus protein kinase A recognition sequence but are not predicted to lie in the dimer interface. Conditional and partially defective mutants such as those described herein may be useful for identifying physiological responses and genes regulated by specific Rel/NF-κB family members.

Keywords: NF-κB; Rel; c-Rel; RelA; Transcription factor; Site-directed mutagenesis; Temperature-sensitive mutant

The Rel/NF-κB proteins comprise an evolutionarily conserved family of transcription factors that regulate genes involved in many cellular and physiological pathways, including cell growth, differentiation, immune and inflammatory responses, and apoptosis [1]. In mammalian cells, this family has five members: p50/p105, p52/p100, c-Rel, RelA (p65), and RelB. These factors form homodimers or heterodimers and these dimers have distinct affinities for DNA target sites, known as κB sites (reviewed in [2]). There are also three Rel family members in *Drosophila* (Dif, Dorsal, and Relish) and one avian retroviral oncoprotein (v-Rel). Rel/NF-κB proteins share a conserved region in their N-terminal halves called the Rel homology (RH) domain, which contains sequences essential for DNA binding, dimerization, inhibitor (IκB) binding, and nuclear localization. In addition, most Rel/NF-κB proteins have a conserved protein kinase A recognition sequence (Arg–Arg–Pro–Ser) towards the C-terminal end of the RH domain.

Various extracellular signals can activate Rel/NF-κB and several hundred genes are influenced by these transcription factors (reviewed in [2]; see also http://www.nf-kb.org). The preferential affinities of different Rel/NF-κB dimers for diverse regulatory sites and the diversity provided by the various combinations of dimers have made it difficult to assess the contribution of individual Rel/NF-κB complexes to the control of specific cellular processes.

Isoda et al. [3] described two point mutations that conferred a temperature-sensitive (ts) biological activity onto the *Drosophila* Dorsal protein. That is, *Drosophila*

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embryos expressing these Dorsal mutants display a normal phenotype at the permissive temperature of 18 °C, but the embryos lack ventral structures (i.e., are "dorsalized") at the non-permissive temperature of 22 °C, due to the inactivation of Dorsal at the higher temperature. These mutations are located within the RH domain of Dorsal: dl^2 has a mutation at amino acid (aa) 68 from glycine (G) to glutamate (E) and dl^{PZ} has a mutation at aa 310 from arginine (R) to histidine (H). The analogous mutations also confer ts transforming and DNA-binding functions onto the retroviral oncoprotein v-Rel: that is, the analogous v-Rel mutants can both bind DNA in vivo and transform chicken spleen cells at 36.5 °C but not at 41.5 °C [4]. The precise effects of these mutations on Dorsal and v-Rel structures are not known. However, the N-terminal G-to-E mutation is located adjacent to residues that participate in DNA binding [5,6], and the R-to-H mutation is within the protein kinase A recognition sequence (Arg–**Arg**–Pro–Ser) and near sequences that are important for dimer formation [7]. Formation of dimer complexes is a necessary step for Rel/NF-κB proteins to bind DNA.

With the long-term goal of developing genetic systems to study the control of Rel/NF-κB signaling, we have created analogous mutations in mouse c-Rel (c-G29E and c-R266H) and mouse RelA (RelA–G40E and RelA–R274H), and have characterized their in vitro and in vivo activities.

Materials and methods

Plasmids and site-directed mutagenesis. Mouse c-Rel mutants c-G29E and c-R266H and mouse RelA mutants G40E and R274H were created by single-stranded site-directed mutagenesis in M13 phage as described previously [4,8]. The oligonucleotides used to make c-Rel mutants c-G29E and c-R266H were 5′GCTACCTGCAGATCT CTCTTCACAC3′ and 5′CTGGTCAGAAGGATGCCGCAGCTG C3′, respectively. After site-directed mutagenesis in an M13 vector, an *Eco*RI–*Pst*I fragment from M13-c-G29E was subcloned into pGEM4-c-Rel and a *Pst*I–*Nde*I fragment from M13-c-R266H was subcloned into pGEM4-c-Rel, in both cases to replace wild-type c-*rel* sequences. The oligonucleotides used to make mutants RelA–G40E and RelA–R274H were 5′CGCTATAAATGCGAGGAACGATCGGCGGG C3′ and 5′GCAGCTACGGCATCCTTCCGATCGCGAGC3′, respectively. After mutagenesis in an M13 vector, *Sac*II/T4–*Xba*I RelA mutants (G40E and R274H) were subcloned into *Hinc*II–*Xba*I cut pGEM3. To create c-Rel mutant c-SPW, a palindromic oligonucleotide containing Pro and Trp codons (5′CCATGG3′) was annealed and then subcloned at a unique *Stu*I site of wild-type c-*rel*. All mutants were confirmed by DNA sequencing.

To express c-Rel proteins in mammalian cells, *Eco*RI–*Hin*dIII c-*rel* fragments were subcloned into pcDNA3.1-. For electrophoretic mobility shift assays (EMSAs), in vitro expression plasmids encoding truncated versions of c-Rel and RelA containing the entire N-terminal RH domain but lacking the C-terminal activation region were created. pGEM4-based c-Rel plasmids (encoding full-length wild-type or mutant proteins) were digested with *Nde*I and religated to remove the C-terminal activation domain and to create expression vectors for c-Rel proteins with aa 1–293. To create expression vectors for truncated RelA proteins, *Hin*dIII–*Bsp*HI/Klenow fragments from full-length RelA, which contain codons 1–313, were subcloned into pGEM3 that had been digested with *Hin*dIII–*Hinc*II. pGEM expression vectors for v-Rel proteins (aa 1–331) have been described previously [9].

In vitro translations and electrophoretic mobility shift assays. Proteins were translated in vitro, normalized by running aliquots on SDS–polyacrylamide gels, and analyzed for DNA binding in EMSAs as described previously with a radiolabeled probe containing a κB site from the chicken c-*rel* promoter (5′TCGAGAGGTCGGGAAA TTCCCCCCCG3′) [9,10]. For EMSAs, c-Rel, v-Rel, and RelA proteins deleted of the C-terminal transactivation region were used (see above).

Reporter gene assays. The κB-site reporter gene assays were performed as described previously [11]. Briefly, A293 cells were co-transfected with 1 μg of c-Rel expression vectors (in pcDNA3.1), 0.5 μg of 3× κB-site (5′TGGGGATTCCCCA3′) luciferase reporter plasmid, and 0.5 μg of pGK-β-gal reporter using SuperFect Reagent (Qiagen) according to manufacturer's protocol. Transfected cells were incubated at 37 °C and 5% CO_2 for 48 h. Cells were lysed using the Reporter Lysis Buffer (Promega) and luciferase and β-gal activities were determined on aliquots of the lysates. β-Galactosidase activity was used to normalize for transfection efficiency and to determine relative luciferase activity.

Results

Mouse c-Rel mutant c-R266H shows a temperature-sensitive ability to bind DNA in vitro, whereas the ability of c-Rel mutant c-G29E to bind DNA is not thermolabile

The glycine and arginine residues that were changed to glutamate and histidine, respectively, to confer ts activity onto Dorsal and v-Rel [3,4] are conserved in mouse c-Rel and RelA (Fig. 1). To determine whether the same mutations could conditionally affect the activity of a mammalian c-Rel protein, the analogous mutations were introduced into mouse c-*rel* to create mutant c-Rel proteins c-G29E and c-R266H. Wild-type mouse c-Rel, c-G29E, and c-R266H were translated in vitro and their abilities to bind DNA at three temperatures (30, 37, and 40–42 °C) were determined in EMSAs using a probe corresponding to a κB site that is found in the chicken c-*rel* and human β-interferon promoters [10,12]. Wild-type c-Rel and c-G29E bound DNA at all three temperatures (Fig. 2A), whereas c-R266H bound DNA efficiently at 30 °C but not at 37 or 41 °C (Fig. 2B).

Previous results from our laboratory [9] reported that v-Rel mutants v-G37E and v-R273H were both ts for DNA binding when translated in vitro. Because our DNA-binding results with mouse c-Rel mutant c-G29E were different from the published results with v-Rel mutant v-G37E, we reanalyzed the DNA-binding ability of in vitro-synthesized v-Rel mutants v-G37E and v-R273H. Consistent with previous results, v-R273H was indeed ts for DNA binding; in contrast, v-G37E behaved similar to wild-type v-Rel in terms of DNA binding (Fig. 3). Further investigations revealed that the in vitro DNA-binding assays using v-G37E (reported in Fig. 8b in [9]) were actually performed with mutant v-R273H, due to a plasmid mislabeling. (However, all in

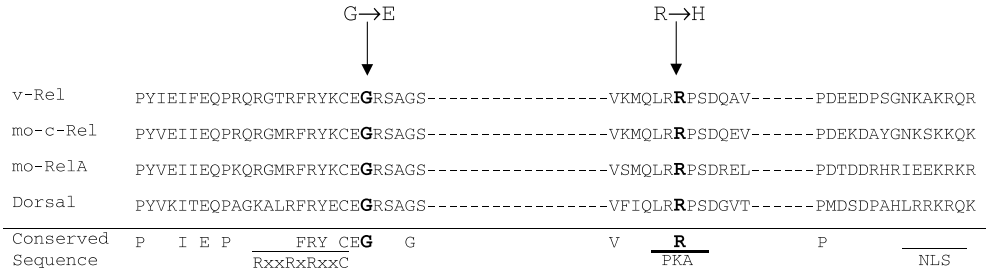

Fig. 1. Relevant target residues are conserved among mouse, viral, and *Drosophila* Rel proteins. Sequences of portions of the Rel homology (RH) domains of v-Rel, mouse c-Rel, mouse RelA, and Dorsal are shown and conserved residues are indicated. Underlined are sequences involved in DNA binding (RxxRxRxxC), phosphorylation by PKA, and nuclear localization (NLS). The G residue is adjacent to residues involved in DNA binding and the R residue is located in the protein kinase A (PKA) consensus site. The G and R residues (bold letters) that can be mutated to glutamate (E) and histidine (H), respectively, to create ts mutants in Dorsal and v-Rel [3,4] are conserved in mouse c-Rel and RelA. Dashes indicate large gaps in the sequences.

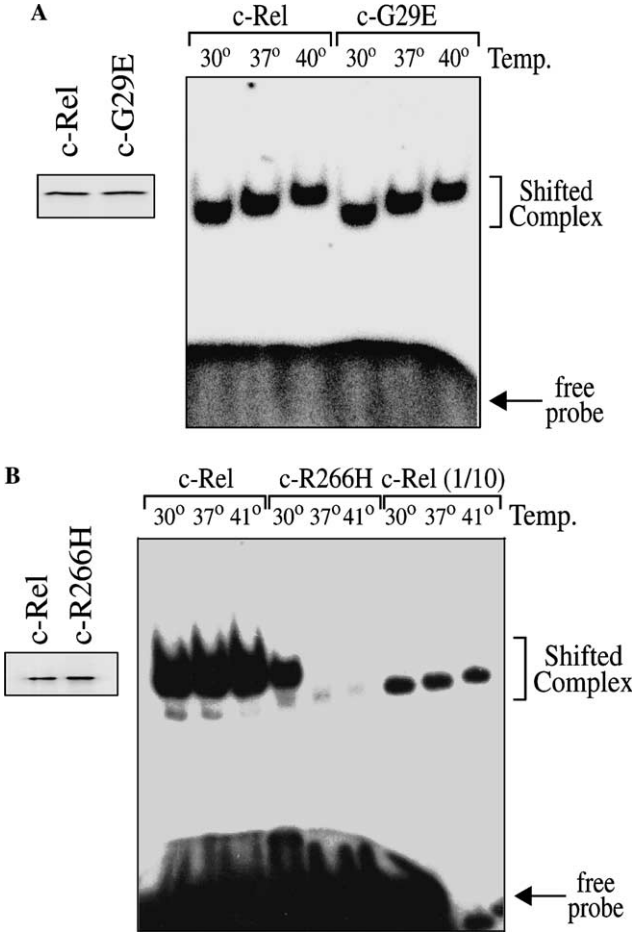

Fig. 2. Mouse c-Rel mutant c-R266H shows a temperature-sensitive ability to bind DNA in vitro. (A) Equalized amounts of in vitro-translated mouse c-Rel and c-G29E (left) were incubated with a ^{32}P-labeled κB site probe at 30 °C for 15 min and then transferred to the indicated temperature for an additional 15 min. Samples were then analyzed on a 5% polyacrylamide gel and complexes were detected with a Bio-Rad phosphorimager (right). The protein–DNA complexes are indicated by an arrow. (B) In vitro translated mouse c-Rel and c-R266H (left) were analyzed in an EMSA (right). The last three lanes are 10-fold dilutions of the first three lanes, respectively.

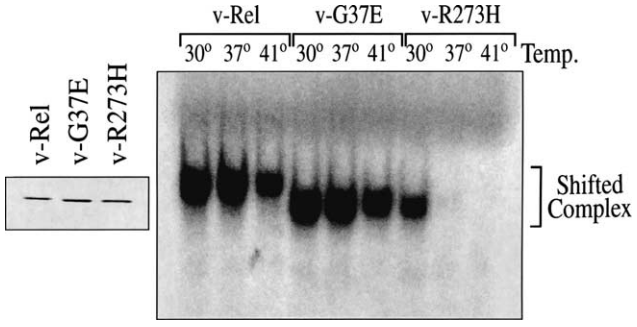

Fig. 3. In vitro DNA-binding activity of v-Rel mutants v-G37E and v-R273H. Equal amounts of in vitro-translated v-Rel, v-R273H, and v-G37E (left) were incubated with a radiolabeled κB site probe at 30 °C for 15 min and then transferred to the indicated temperatures for an additional 15 min. Samples were electrophoresed on a polyacrylamide gel and complexes were detected by phosphorimaging (right).

vivo experiments with mutant v-G37E [4,5,13,14] were performed with the bona fide v-G37E mutant and the overall conclusions of these papers do not change.) Thus, in terms of DNA binding in vitro, mouse c-Rel mutants c-G29E and c-R266H behave similar to the analogous v-Rel mutants v-G37E and v-R273H.

Mouse c-Rel mutant c-R266H shows a reduced ability to bind DNA at low concentrations in vitro and its DNA-binding activity is not reversible upon temperature shift

To further investigate the basis for the conditional DNA-binding activity of mouse c-Rel mutant c-R266H, we analyzed the ability of c-R266H to bind DNA at 30 °C when assayed at different protein concentrations. As shown in Fig. 4, wild-type mouse c-Rel could bind DNA over a range of protein concentrations, whereas c-R266H bound DNA only when present at a relatively high concentration, as compared to wild-type c-Rel. The location of the c-R266H mutation is near, but not within, residues involved in dimerization of chicken

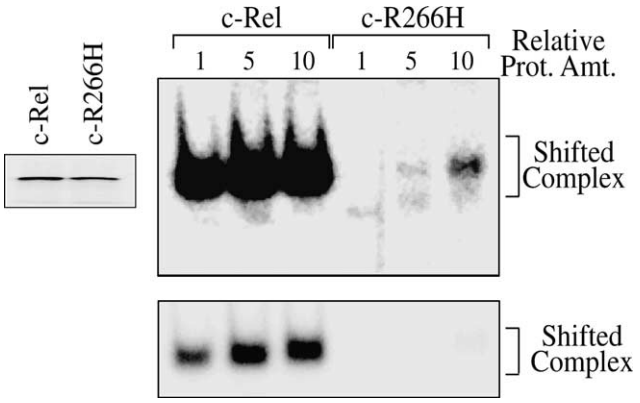

Fig. 4. Mouse c-Rel R266H shows a concentration-dependent ability to bind DNA. In vitro-translated mouse c-Rel and c-R266H were electrophoresed on an SDS–polyacrylamide gel to quantify the amounts of c-Rel and c-R266H (left). Quantitation using a phosphorimager showed that there is a 2:1 ratio of c-Rel:c-R266H and the amounts were then equalized for the EMSA in. Increasing and equal amounts of the in vitro-translated proteins were incubated with radiolabeled κB site probe at 30 °C for 30 min. Samples were subjected to PAGE and complexes were detected by phosphorimaging. Shown at the bottom is a shorter exposure of the EMSA, demonstrating that wild-type c-Rel also shows reduced binding at the lowest concentration.

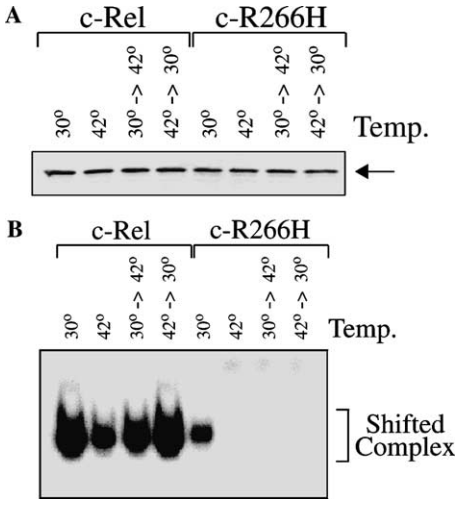

Fig. 5. The DNA-binding activity of c-R266H is irreversibly inactivated at high temperatures. As indicated, in vitro-translated c-Rel proteins (wild-type or c-R266H) were incubated at 30 or 42 °C, were incubated at 30 °C for 15 min and then shifted to 42 °C for an additional 15 min, or were incubated at 42 °C and then shifted to 30 °C. In (A), these proteins were then analyzed on an SDS–polyacrylamide gel and detected by phosphorimaging. In (B), the temperature incubations were performed in the presence of a κB site probe and samples were then analyzed in an EMSA, as described for Fig. 2.

c-Rel [6]. The location of the c-R266H mutation and the concentration-dependent reduction in DNA binding by c-R266H strongly suggest that the mutation in c-R266H reduces its ability to form homodimers. That is, in vitro at high concentration and at a temperature of 30 °C c-R266H can form sufficient numbers of homodimers to bind DNA under standard EMSA conditions.

To ensure that the apparent ts DNA-binding activity of c-R266H was not due to degradation of c-R266H at the higher temperatures, we incubated in vitro-translated c-Rel proteins (wild-type and c-R266H) under several conditions: at either 30 or 42 °C for 30 min; at 30 °C for 15 min and then shifted to 42 °C for another 15 min; or initially at 42 °C for 15 min and then shifted to 30 °C for 15 min. As shown in Fig. 5A, wild-type c-Rel and c-R266H were not degraded under any of these conditions.

Using the same temperature incubation conditions, we also show that high-temperature inactivation of the DNA-binding activity of c-R266H is not reversible (Fig. 5B). That is, when c-R266H is first incubated at 42 °C and then shifted to 30 °C, it cannot bind to DNA. Thus, it appears that c-R266H undergoes a conformational change at 42 °C that either prevents c-R266H homodimers from reforming at 30 °C or, less likely, prevents the c-R266H homodimers from binding to DNA when returned to 30 °C.

Mouse c-Rel mutant c-R266H shows a temperature-sensitive ability to activate transcription

To analyze an in vivo activity of c-Rel mutants c-R266H and c-G29E, expression vectors for wild-type or mutant c-*rel* were co-transfected with a κB-site luciferase reporter plasmid into A293 cells. Reporter gene activity was then measured in cells growing at 33 or 39 °C. c-R266H showed a ts ability to activate transcription. Namely, at 33 °C c-R266H activated transcription from the reporter plasmid, albeit less efficiently than wild-type c-Rel, whereas at 39 °C c-R266H did not activate transcription above basal levels (Table 1). Like wild-type c-Rel, mutant c-G29E activated transcription substantially above basal levels at both 33 and 39 °C (Table 1).

A two amino acid insertion within the protein kinase A recognition sequence in mouse c-Rel specifically disrupts its ability to form DNA binding-competent homodimers

As a further characterization of the effect of mutations near Arg-266 in mouse c-Rel, we created a mutant (c-SPW) that has a two aa insertion (Pro–Trp) just after aa 266 (Arg–Arg–Pro–Trp–Pro–Ser), which disrupts the protein kinase A recognition sequence. Mutant c-SPW is analogous to a v-Rel mutant (v-SPW) that can form heterodimers but not homodimers [7,15,16]. Similar to the v-SPW mutant, mouse c-Rel mutant c-SPW cannot by itself bind to DNA (Fig. 6A), but can form DNA-binding heterodimers with p50 approximately as efficiently as can wild-type c-Rel (Fig. 6B). Thus, it appears that c-SPW is a partially defective mutant that can form heterodimers but not homodimers, again suggesting that mutations within the PKA recognition sequence of Rel/NF-κB proteins affect their abilities to form homodimers and consequently bind DNA.

Table 1
Reporter gene activation by wild-type c-Rel and mutants c-G29E and c-R266H

Protein	Relative luciferase activity[a]		Ratio[b] 39 °C/33 °C
	33 °C	39 °C	
None	1	1	1.0
c-Rel	29 ± 5.8	21 ± 3.8	0.7
c-G29E	110 ± 17.0	42 ± 3.6	0.4
c-R266H	3 ± 0.3	1 ± <0.1	0.3

[a] A293 human kidney cells were co-transfected with expression plasmids for no protein (pcDNA3.1 vector control), c-Rel, c-G40E, or c-R266H and a κB-site luciferase reporter plasmid. After incubating cells for 48 h at either 33 or 39 °C, the luciferase activity was determined as described in Materials and methods. Values are averages of two experiments performed with triplicate samples and in each case are relative to the activity seen with the pcDNA 3.1 vector control.

[b] Values are ratios of the luciferase activities at 39 °C/33 °C. Note that the ratio of activity for c-R266H is likely to be an underestimate because its activity at 39 °C is essentially background (but the value of 1.0 is used for the ratio).

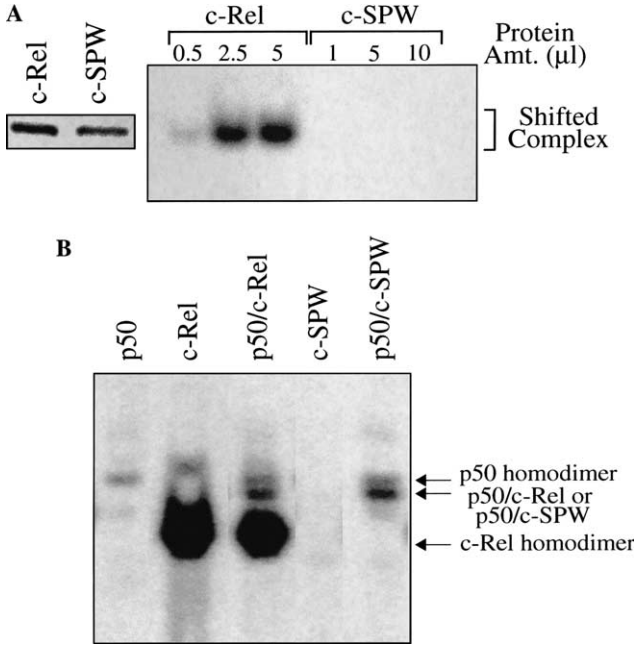

Fig. 6. Mouse c-SPW cannot bind DNA as a homodimer, but can bind DNA as a heterodimer with p50. (A) In vitro-translated c-Rel and mutant c-SPW (left) were incubated with a radiolabeled κB site probe and analyzed by PAGE (right). There was a 1.5:1 ratio of c-Rel:c-SPW, and therefore, equalized amounts of each protein (as indicated) were used for the EMSA. In (B), c-Rel and mutant c-SPW were co-translated with p50 and then incubated with a radiolabeled κB site probe. The positions of the relevant complexes are indicated.

The c-R266H-like mutation in mouse RelA abolishes its ability to bind DNA

To determine the effect of these types of mutations on the related cellular Rel/NF-κB protein RelA, we created the analogous mutations in mouse RelA (RelA–R274H and RelA–G40E), translated the proteins in vitro, and

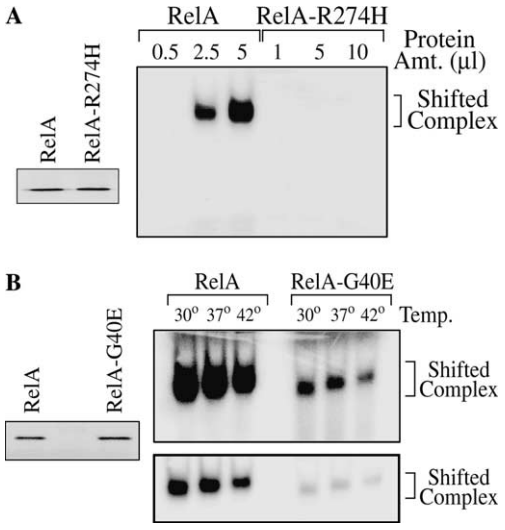

Fig. 7. Mouse RelA mutant R274H cannot bind DNA. (A) In vitro-translated RelA and mutant RelA-R274H (left) were used in an EMSA (right). There was a 1:1.5 ratio of RelA:RelA-R274H (left). Increasing amounts of in vitro-translated RelA and RelA-R274H were incubated with a radiolabeled κB probe at 30 °C for 30 min and were then analyzed in an EMSA, as described for Fig. 4. (B) In vitro-translated RelA and RelA-G40E were analyzed as in (A). The bottom panel is a shorter exposure of EMSA, showing that wild-type RelA also shows slightly reduced binding at 42 °C. The κB site probe (5'GGAAATTTCC3') used in these EMSAs is one that is optimized for DNA binding by RelA homodimers [26].

assessed their abilities to bind DNA in an EMSA. As shown in Fig. 7A, RelA–R274H could not bind DNA over a range of protein concentrations where DNA binding by wild-type RelA could readily be detected. Thus, it appears that the Arg-to-His change has a more drastic effect on the formation of homodimers by RelA than on c-Rel, likely reflecting the different abilities of c-Rel and RelA to form homodimers. Although the RelA–G40E mutant had an overall reduced DNA-binding ability as compared to wild-type RelA, RelA–G40E could nevertheless bind DNA over a range of temperatures (30–42 °C) in vitro.

Discussion

In this report, we have created and characterized a single aa mutant of mouse c-Rel (c-R266H) with temperature-sensitive activity in vitro and in vivo. This mutant is analogous to ts mutants described previously for Dorsal and v-Rel [3,4]. In contrast, introduction of the same Arg-to-His mutation into mouse RelA completed abolished its ability to bind DNA. The different effects of the Arg-to-His substitution on different Rel proteins (i.e., v-Rel, c-Rel, and Dorsal vs. RelA) may reflect different intrinsic abilities of these proteins to form homodimers. Namely, v-Rel and c-Rel form homodimers relatively avidly whereas RelA only weakly

forms homodimers. Indeed, in most cells RelA exists in heterodimers with p52 or p50. This is in contrast to what occurs when RelA is overexpressed or at a high concentration in vitro, where it can be active as a homodimer. Among the subclass of transactivating Rel/NF-κB proteins, there is likely to be a progression in their abilities to form homodimers, with RelB never forming homodimers in vivo, RelA occasionally, and c-Rel most often. Even RelB, which is not known to form homodimers under any circumstances in vivo, can be driven to form homodimers at high concentrations in vitro (G. Ghosh, pers. commun.). Thus, we believe that the Arg-to-His mutations within the PKA recognition sequence affect the activity of Rel family proteins by shifting the equilibrium for homodimer vs. monomer states. For c-Rel, v-Rel, and Dorsal, this equilibrium remains in the physiological range (i.e., homodimers can still form under permissive conditions of temperature and concentration), but for RelA the Arg-to-His mutation causes the equilibrium to shift beyond the physiological range (that we used to measure DNA binding).

The mutant residues in the c-R266H and c-SPW proteins described in this report are not predicted to be located directly within the dimer interface [8] (Fig. 8). If true, these mutations must alter the local structure of c-Rel in such a way that residues within the dimer interface are distorted. The fact that the mouse and chicken c-SPW proteins [16] can still form functional heterodimers indicates that the effect of the two aa insertion on the dimer interface is not apparent when the mutant structure contacts a wild-type dimer interface and possibly that c-Rel heterodimers are more stable than c-Rel homodimers. Moreover, it should be pointed out that some mutations in residues that are predicted, based on X-ray crystallography, to be in the dimer interface (in p50–p50 at least) paradoxically do not affect homodimer formation [17]. Thus, it is also possible that all the details of dimer structure or formation cannot be inferred from X-ray crystallography; for example, there may be dimerization intermediates that involve sequences outside of the final dimerization interface that are important for the final folded dimeric state. Similar to our studies, Malay et al. [18] have identified a mutation in aldolase B that affects tetramer formation and enzyme stability, and this mutant residue does not lie in a predicted subunit interface.

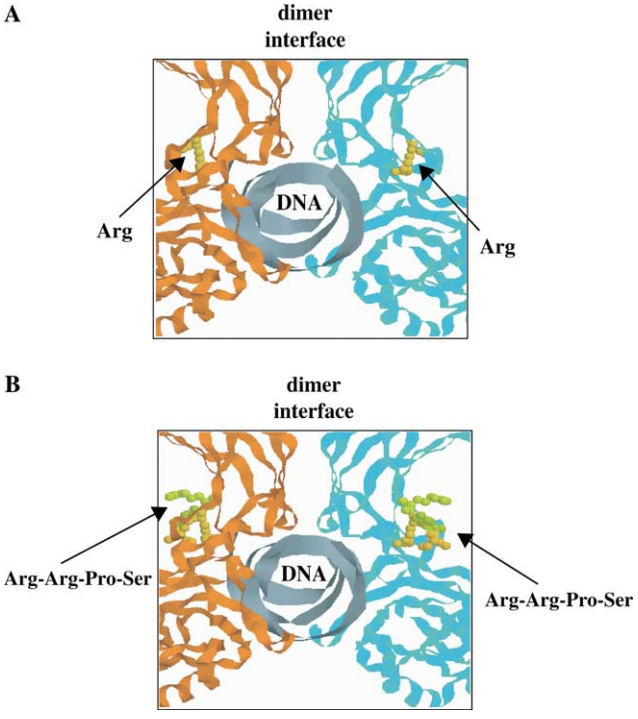

Fig. 8. c-Rel mutants (c-R266H and c-SPW) that affect homodimer formation are not located in the c-Rel dimer interface [6]. (A) Arrows indicate location of the Arg residue at position 266 (balls) on c-Rel homodimer complex bound to DNA (ribbons). In (B), the arrows indicate the PKA recognition site (Arg–Arg–Pro–Ser) (balls) where Pro–Trp was inserted between the Arg-266 and Pro-267 residues in homodimerization-defective mutant c-SPW. Adapted from Ref. [6].

The c-R266H and c-SPW mutations, which disrupt homodimer formation, also disrupt a consensus protein kinase recognition sequence (Arg–Arg–Pro–Ser). Interestingly, this PKA recognition sequences is not present in RelB (Gln–Arg–Leu–Thr), which does not normally form homodimers [19]. We have previously used protein–protein cross-linking to demonstrate that mutations of the serine residue, the site of PKA phosphorylation, to alanine or aspartate/glutamate reduce the ability of chicken c-Rel to form homodimers (and consequently to bind DNA) [7]. Similar mutations at the analogous serine in mouse and human RelA also affect homodimer formation, but not heterodimer formation [20,21]. Thus, the interpretation of results obtained with mutations that disrupt the PKA consensus recognition sequence of Rel/NF-κB proteins is complex. For example, it is not completely clear that the reports of reduced transactivation by Ser-to-Ala mutants at the protein kinase A recognition site are due to reduced phosphorylation by PKA or other kinases (as has often been suggested [22–24]), or are due to reduced formation of homodimers. Indeed, based on the results presented here and elsewhere [7,20,21], one would predict that phosphorylation of the Ser residue would abolish transactivation by homodimers and may shift the equilibrium towards heterodimer formation.

At the permissive temperature, the c-R266H mutant has an approximately 10-fold reduced ability to activate transcription of a κB site-dependent reporter gene as compared to wild-type c-Rel (Table 1). Similarly, v-Rel mutant v-R274H has a greatly reduced transforming efficiency and DNA-binding ability compared to wild-type v-Rel under permissive conditions [4,9]. Nevertheless, both mouse c-R266H and v-R274H are completely

inactive at higher temperatures. Thus, these mutants are true ts mutants.

In contrast, the c-G29E mutation does not render mouse c-Rel functionally ts for DNA binding in vitro or for reporter gene activation in vivo, whereas the analogous mutation does render v-Rel and Dorsal functionally ts in vivo [3,4]. Interestingly, on reanalysis, we now find that the v-G37E mutant, when translated in vitro, is not ts for DNA binding, even though it is clearly ts for transformation of chicken spleen cells [3,5,14]. It is possible that the Gly-to-Glu mutation at this position is not as disruptive to mouse c-Rel function as it is to the activity of v-Rel or Dorsal. Alternatively, the assay (reporter gene activation) that we used to measure the in vivo activity of c-G29E may not be as stringent as the biological assays used to measure the activity of v-G37E (lymphoid cell transformation) or of Dorsal dl^2 (embryonic ventral structure formation). For example, the Gly-to-Glu mutation at this site may affect binding to different κB sites differently, or it may affect other activities of Rel proteins, such as interactions with other transcription factors, in ways that are not measured in the reporter gene assay but that are relevant in more biological assays. Consistent with this model, Menetski [25] has shown that different dimers (e.g., p50–p50 vs. p50–RelA vs. RelA–RelA) bind to different κB sites with strikingly different affinities and that the conformations adopted by a given dimer (e.g., RelA–RelA) on different κB sites can be quite different. Of note, the ratio of reporter gene activation by c-G29E at 39 and 33 °C indicates that c-G29E is more thermolabile than wild-type c-Rel, even though c-G29E clearly activates transcription above control levels at both temperatures (Table 1).

The use of conditional or partially defective mutants in microorganisms, including bacteria and yeast, and in viruses has, in many cases, been important in identifying steps in a variety of complex pathways. As our ability to apply genetic approaches to the study of mammalian systems increases, it will likely to be useful to develop similar types of mutants in key regulatory proteins. The Rel/NF-κB signal transduction pathway is a highly studied, but complex and overlapping, pathway where the contributions of individual components may eventually be revealed through the use of subtle mutants in individual Rel/NF-κB family members. Therefore, our creation and characterization of ts and partially defective mutants of mouse c-Rel may be relevant to the development of such genetic systems.

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