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Drosophila Raf's N Terminus Contains a Novel Conserved Region and Can Contribute to Torso RTK Signaling

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ABSTRACT

Drosophila Raf (DRaf) contains an extended N terminus, in addition to three conserved regions (CR1–CR3); however, the function(s) of this N-terminal segment remains elusive. In this article, a novel region within Draf's N terminus that is conserved in BRaf proteins of vertebrates was identified and termed **c**onserved **r**egion **N**-terminal (CRN). We show that the N-terminal segment can play a positive role(s) in the Torso receptor tyrosine kinase pathway *in vivo*, and its contribution to signaling appears to be dependent on the activity of Torso receptor, suggesting this N-terminal segment can function in signal transmission. Circular dichroism analysis indicates that DRaf's N terminus (amino acids 1–117) including CRN (amino acids 19–77) is folded *in vitro* and has a high content of helical secondary structure as predicted by proteomics tools. In yeast two-hybrid assays, stronger interactions between DRaf's Ras binding domain (RBD) and the small GTPase Ras1, as well as Rap1, were observed when CRN and RBD sequences were linked. Together, our studies suggest that DRaf's extended N terminus may assist in its association with the upstream activators (Ras1 and Rap1) through a CRN-mediated mechanism(s) *in vivo*.

EVOLUTIONARILY conserved receptor tyrosine kinase (RTK) signaling pathways function in fundamental cellular processes including differentiation, proliferation, and cell survival in eukaryotes (Schlessinger 2000). The Raf serine/threonine kinase, as a key component of RTK signaling modules, plays a central role in transmitting upstream stimuli to the nucleus (Daum *et al.* 1994). Cyclic control of Raf depends on activities of GTPases, kinases, phosphatases, and scaffold proteins (Kolch 2000; Chong *et al.* 2001; Morrison 2001; Dhillon *et al.* 2002; Raabe and Rapp 2002). Clues to these regulatory events were derived from the identification of conserved regions/motifs/sites. However, the mechanisms that modulate Raf serine/threonine kinases are complicated and remain elusive. Mammals have three Raf isoforms, ARaf, Braf, and CRaf. They share a similar primary structure consisting of three conserved regions (CR1, CR2, and CR3). Conserved region 1 (CR1), where a Ras binding domain (RBD) and a cysteine-rich domain (CRD) reside, is required for Ras–Raf interaction. CR2, a serine/threonine-rich region, contains a 14-3-3 binding site. CR1 and CR2 are embedded in the regulatory N-terminal half of Raf proteins, while CR3, including the catalytic kinase region and an additional 14-3-3 binding site, resides in the C terminus (reviewed by Wellbrock *et al.* 2004). In addition to these three conserved regions, BRaf has an extended amino-terminal segment followed by CR1 (Terai and Matsuda 2006; Fischer *et al.* 2007). However, studies of BRaf regulation have mainly focused on CR1, CR2, and CR3 with little attention, thus far, given to the role of this N-terminal region. Translocation of Raf proteins to the membrane, a critical step in their activation, can be mediated through different mechanisms. It is reported that direct interaction between a basic motif in CRaf's kinase region and phosphatidic acid (PA) can recruit Raf to the membrane (Rizzo *et al.* 2000; Kraft *et al.* 2008). This PA-binding site is conserved in ARaf and BRaf proteins. Also, association with Ras, a major regulator of Raf kinases, plays a crucial role(s) in translocation and activation of Raf. However, the molecular mechanisms of Ras–Raf coupling are not completely understood. Raf's RBD can directly interact with the switch 1 region of GTP–Ras and is thought to be the core element for Ras binding (Nassar *et al.* 1995). CRD is involved in Ras–Raf coupling, as well, through interaction between its hydrophobic patch and the lipid moiety of Ras (Williams *et al.* 2000; Thapar *et al.* 2004). Thus, both RBD and CRD contribute to Ras–Raf interaction and the effects are likely additive. Disabling either RBD or CRD is thought to reduce but not completely eliminate Raf activity (Hu *et al.* 1995). Recently, Fischer *et al.* (2007) found BRaf's interaction with HRas was also facilitated by the extended N terminus, *in vitro*. At the present time, however, the identity of residues/sites that participate in this process are unknown and the biological implications of this N-terminal region *in vivo* have not been defined.

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Drosophila has one *Raf* gene first described genetically as *l(1) pole hole*, and later referred to as *DRaf* or *Raf*. As a member of the MAP kinase signaling module, DRaf plays an essential role in numerous RTK pathways in Drosophila development (Perrimon 1994; Van Buskirk and Schüpbach 1999; Duffy and Raabe 2000; Brennan and Moses 2000). On the basis of its primary structure, the DRaf protein is more similar to BRaf than either ARaf or CRaf (Morrison and Cutler 1997; Dhillon and Kolch 2002; Chong *et al.* 2003). DRaf and BRaf have two acidic residues (E420–E421 in DRaf; D447–D448 in BRaf) preceding the kinase region that correspond to residues Y301–Y302 in ARaf and Y340–Y341 in CRaf, respectively. These negative charged acidic residues mimic constitutive phosphorylation and are thought to be related to the higher basal activity of BRaf (Mason *et al.* 1999; Mishra *et al.* 2005). Both DRaf and BRaf have an extended amino terminus, when compared to ARaf and CRaf, in addition to CR1, CR2, and CR3. DRaf and BRaf also share parallels in their modes of regulation. Rap1 can activate both BRaf and DRaf, but not ARaf or CRaf (Ohtsuka *et al.* 1996; Mishra *et al.* 2005). Like the Raf proteins in mammals, the activity of DRaf is regulated through phosphorylation/dephosphorylation (Baek *et al.* 1996; Rommel *et al.* 1997; Radke *et al.* 2001; Laberge *et al.* 2005), interaction with scaffold proteins or other binding partners (Roy *et al.* 2002; Roy and Therrien 2002; Douziech *et al.* 2003, 2006; Roignant *et al.* 2006; Rajakulendran *et al.* 2008). These regulatory events occur within the three conserved regions (CR1–CR3) of Draf; however, the role of DRaf's N-terminal region has not been elucidated.

Development of both embryonic termini in Drosophila is dependent on DRaf-mediated Torso RTK signaling. Binding of Trunk or Torso-like with the Torso receptor initiates Ras1–DRaf–MEK signaling at the poles of early staged embryos, and in turn, triggers expression of at least two gap genes, *tailless* and *huckebein*, which specify terminal structures and help to establish segmental identities in the embryo (reviewed by Furriols and Casanova 2003). The domain of *tailless* (*tll*) expression in the embryonic posterior region has been used as a quantitative marker to measure the strength of the Torso RTK signal in early embryos. At the cellular blastoderm stage, embryos from wild-type (WT) mothers show posterior *tll* expression from approximately 0–15% embryo length (EL). At a later stage embryos exhibit normal internal head structures, three thoracic segments (T1–T3), eight abdominal denticle belts (A1–A8), as well as the Filzkörper (Fk) tail structure. Decreased or loss of Torso RTK pathway activity results in a reduced posterior expression domain of *tll* and consequently absence of embryonic tail structures. In contrast, gain-of-pathway activity can lead to expanded *tll* expression domains at both poles, and subsequently enlarged head and tail structures, accompanied by deletion of central abdominal segments (Ghiglione *et al.* 1999; Jiménez *et al.* 2000).

In this study, using the Drosophila embryonic termini as both a qualitative and quantitative *in vivo* assay system, we examined the role played by DRaf's N terminus in Torso signaling in different genetic backgrounds. We observed a subtle, but consistent, higher signaling potential for full-length DRaf proteins when compared with those lacking amino-terminal residues 1–114 ($DRaf^{\Delta N114}$). Furthermore, a novel region within DRaf's N terminus that is conserved in RAF genes of most invertebrates and BRaf genes of vertebrates was identified and termed **c**onserved **r**egion **N**-terminal (CRN). Our studies suggest that DRaf's extended N terminus may assist in its association with the upstream activators Ras1 and Rap1 *in vivo* and thus, potentially play a regulatory role(s) in DRaf's activation through a CRN-mediated mechanism(s). Minor adjustment by CRN on Ras1 and Rap1 binding may help to fine tune DRaf's activity and consistently provide optimal signal output.

MATERIALS AND METHODS

Drosophila strains and genetics: In this study, *y w*, $Draf^{11\text{-}29}$ ($Draf^{-}$; DRaf protein null, Melnick *et al.* 1993), $trunk^{1}$ (trk^{-}; loss-of-function allele, lacks C-terminal 16 amino acids, Schüpbach and Wieschaus 1989; Casanova *et al.* 1995), $torso^{XR1}$ (tor^{-}; Torso protein null allele, *tor* gene deletion, Sprenger *et al.* 1989), and $torso^{RL3}$ (tor^{RL3}; gain-of-function allele, H242L amino acid replacement in the extracellular domain, Sprenger *et al.* 1993) strains were used. The flippase dominant female sterile (FLP-DFS) technique was utilized to generate $Draf^{11\text{-}29}$ germline clones (Chou and Perrimon 1996). Drosophila stocks were raised at 25° on standard cornmeal medium. To study the gain-of-function effects of the temperature-sensitive tor^{RL3} allele (Figure 3), virgin females were collected and mated with wild-type males at 25° for 3–4 days and then moved into a 29° incubator. Eggs were collected at 29° during the first 1–2 days for Western analysis and phenotypic characterization.

Transgene design: Full-length and truncated DNAs were amplified using wild-type DRaf cDNA (GenBank no.AY089490, obtained from Drosophila Genomics Research Center) as template, and inserted into the polylinker site of the *P*-element transformation vector pCaSpeR-HS83. The full-length cDNA sequence (FL DRaf) encodes a DRaf protein with 739 residues, while the truncated cDNA sequence ($DRaf^{\Delta N114}$) corresponds to amino acids 115–739 of the FL DRaf protein. The constitutively active heat-shock 83 gene (HS83) promoter was used to drive the expression of DRaf transgenes to simplify the generation of transgenic lines with various genetic backgrounds. Transgenic lines were generated by Genetic Services (Sudbury, MA).

Multiple lines derived for each transgene were used in this study. $DRaf^{\Delta N114}$ (L1, #a and #b), FL DRaf (#a and #b) were used to generate germline clones bearing females (Figure 1). Lines #1, #2, #3 of $DRaf^{\Delta N114}$ and #1, #2, #3 of FL DRaf were used in tor^{RL3}, trk^{1}, and tor^{XR1} backgrounds. The $DRaf^{\Delta N114}$ line #1 is homozygous lethal, thus we generated trk^{1}/trk^{1}; $DRaf^{\Delta N114}$#1/$DRaf^{\Delta N114}$#3, and tor^{XR1}/tor^{XR1}; $DRaf^{\Delta N114}$#1/$DRaf^{\Delta N114}$#3 lines that produce DRaf protein levels equivalent with other lines (Figure 4, Table 1).

Western analysis: To produce protein extracts, 100 eggs were collected and homogenized in 36-μl lysis buffer containing 20 mm Tris-Cl (pH 8.0), 150 mm sodium chloride, 0.2% Triton-X 100, 0.2% Nonidet P-40, 10 mm EDTA, 1 mm EGTA,

1 mM phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin and 20 mM leupeptin. Insoluble material was removed by centrifugation (13,000 rpm, 10 min) at 4°. Protein extracts were separated by 8% SDS–PAGE, and electrophoretically transferred to nitrocellulose membrane. DRaf proteins were probed with rabbit anti-Raf antibody (70.1, Sprenger *et al.* 1993) and horseradish peroxidase-coupled goat anti-rabbit secondary antibody (Thermo Scientific). α-Tubulin proteins probed with mouse antibody (Sigma) and horseradish peroxidase-coupled goat anti-mouse secondary antibody (Thermo Scientific) were used as an internal control. The membranes were developed using SuperSignal West Pico kit (Thermo Scientific). Protein level was quantified with Image J.

***In situ* hybridization:** *tailless* and *engrailed* probes were generated from wild-type cDNA clones (*tailless*: GenBank no. BT022195; *engrailed*: GenBank no. AY069448, obtained from the Drosophila Genomics Research Center) using the PCR DIG probe synthesis kit (Roche Applied Science). Whole-mount mRNA *in situ* hybridizations were performed in embryos according to the protocol of Tautz and Pfeifle (1989) with minor modifications.

Circular dichroism spectral measurement: DNA corresponding to amino acids 1–117 of DRaf ($DRaf^{N117}$) was recombined into the pGEX vector. The GST–$DRaf^{N117}$ fusion protein was produced by expression in *Escherichia coli* BL21 and purified by standard affinity chromatography. Purified GST–$DRaf^{N117}$ protein was digested with thrombin. The $DRaf^{N117}$ protein (~13 kDa) was purified by a size-exclusive column (Amersham Biosciences) and verified by mass spectra and N-terminal sequencing. Protein sample (0.05 mg/ml in 10 mM sodium phosphate buffer) was loaded to 0.1-cm quartz circular dichroism (CD) cuvette. CD spectra was measured by Jasco J-710 spectropolarimeter (Protein facility at Iowa State University) at room temperature. Data were collected with 0.2-nm resolution and at a scan rate of 1.5 nm min^{-1}. The ellipcity value of the blank buffer at each wavelength was substracted from each point.

Yeast two-hybrid analysis: The R174 to L mutation in DRaf ($DRaf^{R174L}$) was generated by PCR-based site-directed mutagenesis and confirmed by sequencing. DNA sequences corresponding to amino acids 1–117 (N), 1–212 (NRBD), 18–212 (Δ17NRBD), 78–212 (Δ77NRBD), and 115–212 (RBD) were obtained by PCR using wild-type DRaf as the template, while DNAs encoding $NRBD^{R174L}$ and RBD^{R174L} were amplified from $DRaf^{R174L}$ DNA. Amplified DNAs were cloned into pGADT7 vector (Clontech). DNA sequences encoding amino acids 1–183 of Ras1 (Ras1ΔCAAX) and 1–180 of Rap1 (Rap1ΔCAAX) were amplified from cDNAs of wild-type Ras1 and Rap1 (Ras1: GenBank no. AF186648; Rap1: NCBI Reference no. NM_057509, obtained from the Drosophila Genomics Research Center), respectively, and inserted into the pGBKT7 vector (Clontech).

Constructed pGADT7 and pGBKT7 plasmids were transformed into yeast Y187 strain. Protein–protein interactions were tested by β-galactosidase assays using X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma; solid-support assay) or ONPG (ortho-nitrophenyl-b-D-galactopyranoside, Sigma; liquid quantitative assay) as substrates. β-Galactosidase units in quantitative assays were calculated according to the Yeast Protocol Handbook (Clontech). All yeast two-hybrid experiments are confirmed by reciprocal bait–prey assays and repeated at least four times.

RESULTS

To study the potential function of DRaf's N-terminal residues (amino acids 1–114), we generated transgenic flies expressing full-length DRaf (FL DRaf) or DRaf proteins lacking amino-terminal residues 1–114 ($DRaf^{\Delta N114}$; Figure 1A). The constitutive heat-shock 83 (HS83) promoter was selected to drive transgene expression to simplify the generation of complex genetic backgrounds required to test the functionality of N-terminal residues. We used the Torso pathway to test the signaling potential of these maternally expressed DRaf proteins. Since the Torso signaling system is solely dependent on activity of maternal DRaf proteins, we could readily determine and verify the quantity of DRaf proteins available for Torso signal transduction in early staged embryos by Western blot analysis. Thus, at equivalent protein concentrations, we compared the signal potential of FL DRaf and $DRaf^{\Delta N114}$ proteins to characterize the role of the N terminus in a well-defined RTK pathway *in vivo*.

DRaf's N terminus can contribute to RTK signaling in Drosophila embryos: Embryos that were deficient for maternal DRaf protein (derived from $Draf^{11\text{-}29}/Draf^{11\text{-}29}$ female germ cells, see MATERIALS AND METHODS) lack posterior *tll* expression at ~2.5 hr after egg deposition and subsequently exhibit abnormal cuticle pattern with deletion of posterior structures due to loss of Torso RTK signaling (Figure 1D, ii and ii′). We generated females with germ cells homozygous mutant for the $Draf^{11\text{-}29}$ allele ($Draf^{-/-}$) but expressing FL DRaf or $DRaf^{\Delta N114}$ proteins using the "FLP-DFS" technique (Chou and Perrimon 1996). Cuticles of embryos produced by $Draf^{-/-}$; $DRaf^{\Delta N114}$ female germline clones expressing maternal truncated proteins equal to or greater than endogenous wild-type DRaf levels were essentially equivalent to those of wild-type embryos with only one (1/245) lacking posterior Filzkörper (line L1, Figure 1, B and E). However, when $DRaf^{\Delta N114}$ was expressed at low maternal levels (~1/4 of endogenous DRaf level; two independent transgenic lines #a and #b; Figure 1B), ~4.5% of the embryos assayed lacked posterior Filzkörper (Figure 1, Diii and E). At such a reduced expression level (two independent lines #a and #b, Figure 1B), FL DRaf showed rescue of posterior pattern with Filzkörper development observed for a higher percentage of embryos (~98.5%, $\chi^2 = 9.91976318$, $P < 0.01$; Figure 1, D and E). In agreement with the cuticle phenotype, an abnormal posterior *tll* expression pattern (<13% EL) was observed more often for embryos that inherited truncated $DRaf^{\Delta N114}$ (21.2%, $n = 52$) rather than full-length DRaf proteins (9.0%, $n = 78$; $\chi^2 = 3.9386844$, $P < 0.05$, Figure 1D), suggesting that $DRaf^{\Delta N114}$ was less active than FL DRaf in Torso RTK signaling.

To test our protein quantification assay, a more rigorous examination was conducted using Western blot analysis. Three samples representing lysates of 6, 12, and 18 eggs from each line ($Draf^{-/-}$; $DRaf^{\Delta N114}$#a and $Draf^{-/-}$; *FL DRaf* #a) were loaded on to a SDS–PAGE gel. As shown in Figure 2A, the intensity of DRaf and corresponding tubulin bands exhibits a roughly linear

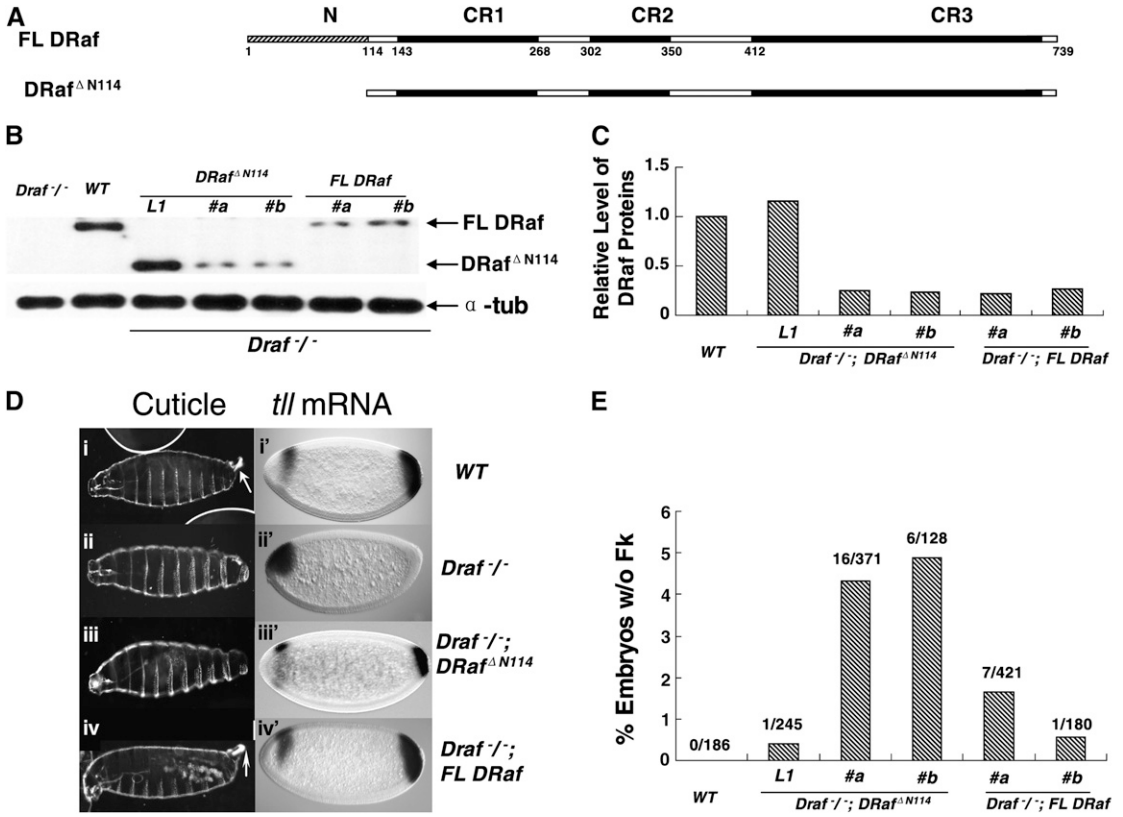

FIGURE 1.—Rescue of posterior structures in embryos derived from *Draf*$^{-/-}$ female germ cells by expression of full-length *DRaf* or truncated *DRaf*$^{\Delta N114}$ transgenes. (A) Schematic representations of full-length DRaf (FL DRaf) with 739 amino acids and truncated DRaf$^{\Delta N114}$ proteins. In addition to the three conserved regions (CR1, CR2, and CR3), FL DRaf has an extended N terminus. (B) Western analysis of embryonic DRaf proteins from eggs produced by *Draf* $^{11\text{-}29}$/*Draf* $^{11\text{-}29}$ (*Draf*$^{-/-}$), wild type (WT), *Draf*$^{-/-}$; *DRaf* $^{\Delta N114}$ (three independent lines, L1 with ~1× endogenous DRaf level, #a and #b with ~1/4 endogenous DRaf level) and *Draf*$^{-/-}$*; FL DRaf* (two independent lines, #a and #b with ~1/4 endogenous DRaf level) germline clone bearing females. Lysate was prepared from eggs at 0–3 hr after egg deposition. Full-length DRaf (~90 kDa) and DRaf$^{\Delta N114}$ (~77 kDa) proteins are denoted by arrows. Lysate of eggs from *Draf*$^{-/-}$ germ cells was used as a negative control. α-Tubulin (α-tub) was used as the loading control. (C) A bar graph representing relative levels of DRaf proteins normalized with α-tubulin. (D) Cuticles of mature embryos derived from wild type (WT), *Draf*$^{-/-}$, *Draf*$^{-/-}$*; DRaf* $^{\Delta N114}$, and *Draf*$^{-/-}$*; FL DRaf* female germ cells (left). Accumulation of *tll* mRNA in cellular blastoderm embryos was detected by *in situ* hybridization (right). Posterior *tll* expression is solely dependent on the Torso pathway and used as a marker for Torso RTK signaling. Anterior expression of *tll* is regulated by another pathway(s) in addition to Torso signaling, is more complex, and is used as an internal control for staining here. Wild-type embryos show (i) normal Filzkörper (Fk) structure (arrow), (i′) *tll* mRNA accumulation at the posterior (~0–15% embryo length, EL), and an anterior head "stripe" (~75–85% EL). Embryos derived from *Draf*$^{-/-}$ germ cells lack (ii) posterior structures (A8 denticle belt, Filzkörper) and (ii′) posterior *tll* mRNA expression. (iii) An embryonic cuticle derived from *Draf*$^{-/-}$*; DRaf* $^{\Delta N114}$ germ cell lacks the Filzkörper structure. (iii′) A reduced posterior *tll* expression domain is at ~0–8% EL in an embryo from *Draf*$^{-/-}$*; DRaf* $^{\Delta N114}$ germline clone bearing mother. (iv) Filzkörper structure (arrow) and (iv′) normal expression pattern of *tll* mRNAs are rescued by FL DRaf expression for embryos derived from *Draf*$^{-/-}$*; FL DRaf* maternal germ cells. (E) A bar graph showing the percentage of embryos without Fk structures. When expressed at low maternal level (~1/4 endogenous level), embryos without Fk were found more often for those that inherited truncated Draf$^{\Delta N114}$ rather than full-length DRaf proteins ($\chi^2 = 9.91976318$, $P < 0.01$).

correlation with the number of eggs lysed (Figure 2B). In addition, the normalized DRaf protein level was consistent among the three samples loaded for the same transgenic line (Figure 2C), suggesting our Western blots analysis was reliable. Importantly, we also addressed the question of maternal DRaf protein stability and whether deletion of the N-terminal region altered DRaf accumulation levels during Torso signal transduction. Embryonic lysates from eggs collected at 0–1, 1–2, and 2–3 hr after deposition were prepared (*Draf*$^{-/-}$*; DRaf* $^{\Delta N114}$#a and *Draf*$^{-/-}$*; FL DRaf* #a). As shown in Figure 2, D and E, DRaf protein levels remained roughly constant, indicating both FL DRaf and DRaf$^{\Delta N114}$ proteins are stable throughout the 0- to 3-hr period when the Torso pathway is active.

Next, we genetically altered the Torso pathway to create a sensitized signaling environment and compared the potential of DRaf$^{\Delta N114}$ and FL Raf proteins in this background. *tor*^{RL3} is a temperature-sensitive, recessive, gain-of-function allele of the Torso receptor. At the nonpermissive temperature 25–29°, *tor*^{RL3}/*tor*^{RL3} mothers produce embryos that show broad *tll* expression at both anterior and posterior ends. These embryos develop and show deletion of central abdominal seg-

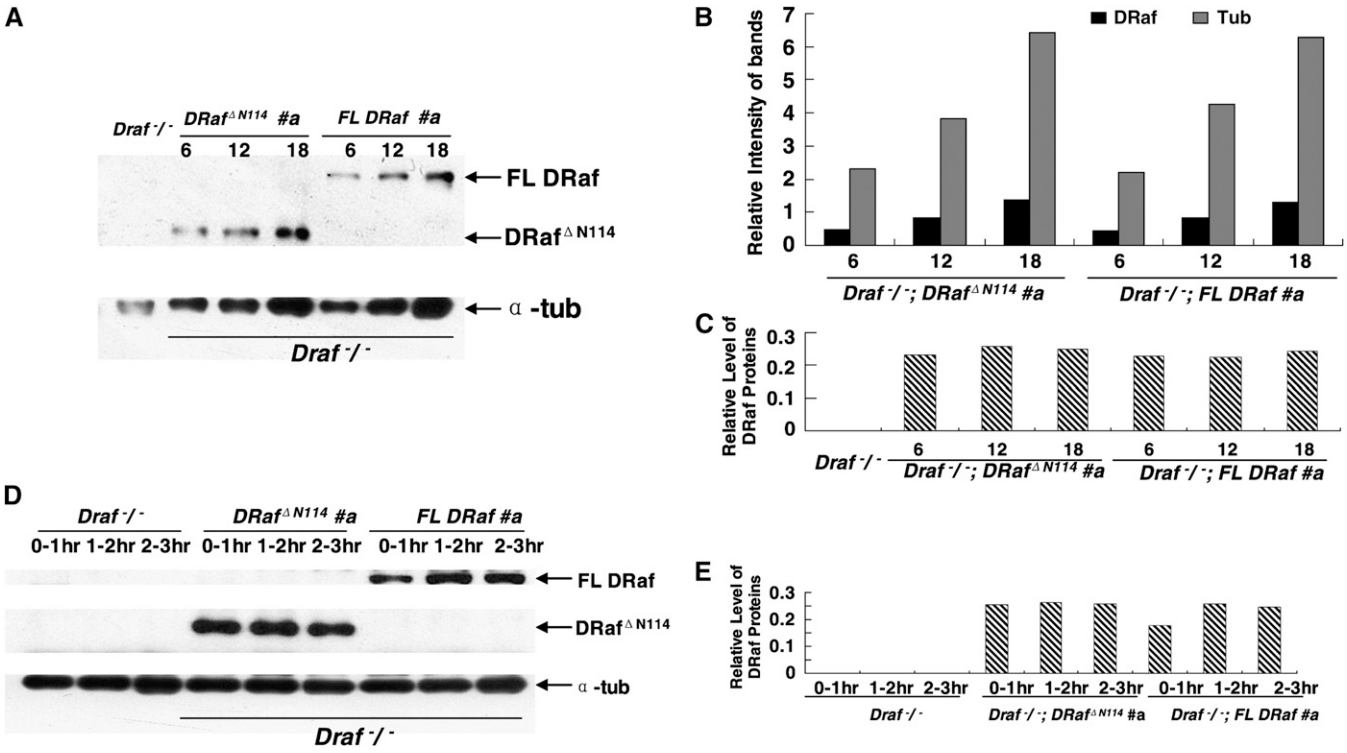

FIGURE 2.—Verification of DRaf protein quantitation assays and stability of DRaf proteins in early embryos. (A) Three samples representing lystes of 6, 12, and 18 eggs for each line (*Draf*$^{-/-}$*; DRaf*$^{\Delta N114}$#a and *Draf*$^{-/-}$*; FL DRaf* #a) were loaded for Western blot analysis. Full-length DRaf (~90 kDa) and DRaf$^{\Delta N114}$ (~77 kDa) proteins are denoted by arrows. Lysate of eggs from *Draf*$^{11\text{-}29}$*/Draf*$^{11\text{-}29}$ (*Draf*$^{-/-}$) germ cells was used as a negative control. α-Tubulin levels were probed as a loading control. (B) Bar graph showing relative intensity of DRaf (solid bar) and α-tubulin (shaded bar) bands. (C) A bar graph depicting normalized DRaf protein level from A. (D) Western analysis of embryonic DRaf proteins from eggs collected at 0–1, 1–2, and 2–3 hr after deposition and produced by *Draf*$^{-/-}$, *Draf*$^{-/-}$*; DRaf*$^{\Delta N114}$ (line #a) and *Draf*$^{-/-}$*; FL DRaf* (line #a) germline-bearing females. Full-length DRaf (~90 kDa) and DRaf$^{\Delta N114}$ (~77 kDa) proteins are denoted by arrows. α-Tubulin was used as the loading control. (E) Normalized DRaf protein level from D is shown in this bar graph depicting the stable accumulation of these DRaf proteins.

ments and do not hatch (STRECKER *et al.* 1989). Eggs derived from females heterozygous for *tor*^{RL3} (*tor*^{RL3}/+) can hatch as larvae; however, some of these larvae show a gain-of-function phenotype with deletion of an abdominal segment(s). At 29°, we found 7.4% ($n = 553$, Figure 3D) of the larvae from *tor*^{RL3}/+ mothers showed deletion, fusion, or broken abdominal denticle bands. In this genetic background, when expressed at comparable protein levels (Figure 3, A and B), FL DRaf enhanced the *tor*^{RL3} phenotype much more significantly than DRaf$^{\Delta N114}$, resulting in a greater number of embryos with central abdominal defects. We found 31.8% ($n = 422$) embryos with FL DRaf proteins showed the gain-of-function phenotype (Figure 3, C and D), while only 17.1% ($n = 450$) of the DRaf$^{\Delta N114}$ embryos showed such defects (Figure 3, C and D). We repeated these experiments using two additional, independently derived transgenic *FL DRaf* and *DRaf*$^{\Delta N114}$ lines and observed similar results ($\chi^2 = 51.063876$, $P < 0.001$; Figure 3D).

To test whether the cuticle phenotypes observed were due to alterations in the embryonic fate map, we determined the mRNA accumulation pattern for the *engrailed* (*en*) segmentation gene in approximately stage 11 embryos. The *en* mRNA wild-type pattern is dependent on normal signaling in the Torso pathway. We found 36.1% ($n = 169$) of the embryos from *tor*^{RL3}/+*; FL DRaf* mothers had at least one deleted, fused, or broken *en* central abdominal stripe(s) (Figure 3E, iv). The segmentation defects observed were most likely due to the expansion of head and/or tail domains and indicative of the gain-of-function phenotype. In contrast, only 33 of 141 (23.4%) embryos from *tor*^{RL3}/+*; DRaf*$^{\Delta N114}$ mothers had such defects ($\chi^2 = 6.38030206$, $P < 0.02$). Consistent with the cuticular phenotypes and *en* expression patterns, expansion in the domain of *tll* expression was observed more often for embryos from *tor*^{RL3}/+*; FL DRaf* mothers (32.0%, $n = 50$) compared with those from *tor*^{RL3}/+*; Draf* $^{\Delta N114}$ females (14.5%, $n = 76$; $\chi^2 = 5.50220096$, $P < 0.02$, Figure 3E, iii′ and iv′). Together, these *in vivo* studies consistently indicated that deletion of the N terminus reduces the ability of DRaf to enhance the ectopic gain-of-function effects of *tor*^{RL3} and that these N-terminal residues could participate in Torso RTK signaling.

The contribution of DRaf's N terminus to signaling appears to be dependent on the activity of the Torso receptor: Embryos lacking normal maternal Trunk (Trk) activity show little or no posterior *tll* expression (occa-

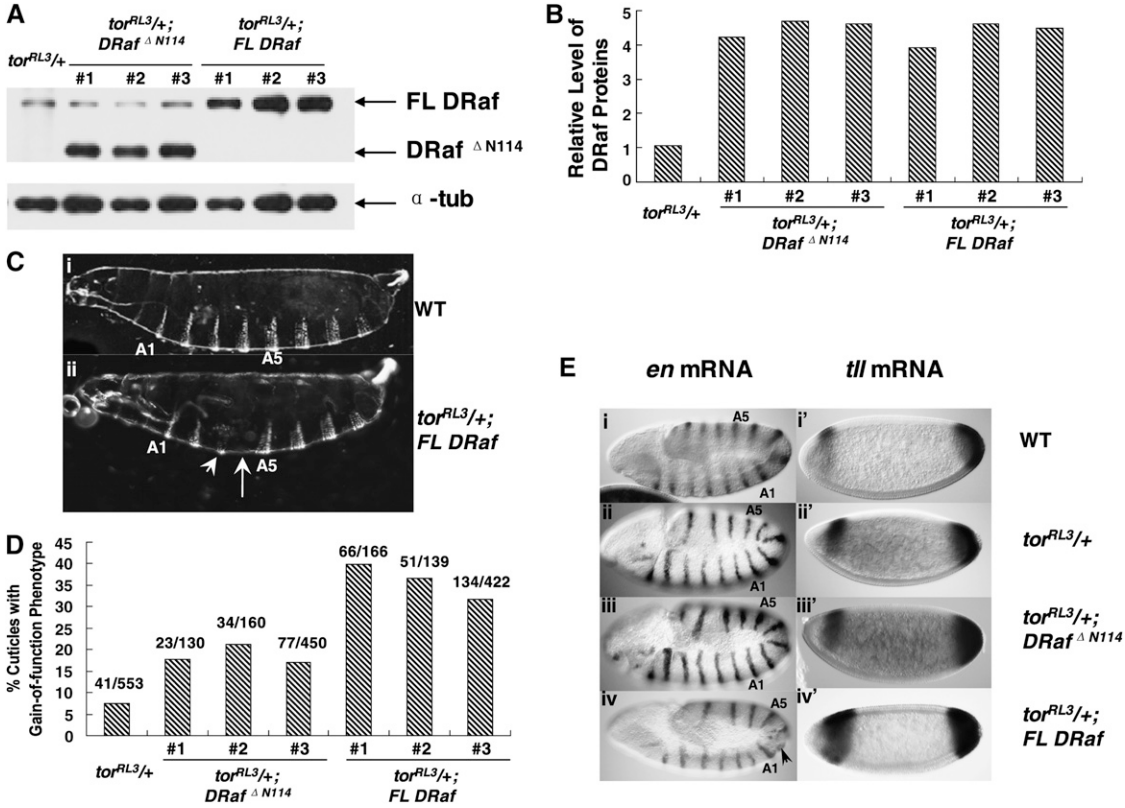

FIGURE 3.—Gain-of-function effects of tor^{RL3} are differentially enhanced by expression of *FL DRaf* and $DRaf^{\Delta N114}$ transgenes. (A) Western analysis of embryonic DRaf proteins from eggs (0–3 hr) produced by $tor^{RL3}/+$, $tor^{RL3}/+$; $DRaf^{\Delta N114}$ (three independent lines, #1, #2, and #3), or $tor^{RL3}/+$;*FL DRaf* (three independent lines, #1, #2, and #3) females at 29°. Full-length DRaf (~90 kDa) and $DRaf^{\Delta N114}$ (~77 kDa) proteins are denoted by arrows. α-Tubulin was used as the loading control. (B) Normalized DRaf protein level from A is shown as a bar graph. (C) Cuticles of mature embryos are shown. (i) A wild-type (WT) embryo exhibits normal cuticle pattern with 8 abdominal denticle belts. (ii) An embryonic cuticle derived from $tor^{RL3}/+$; *FL DRaf* mother has one broken abdominal denticle band (arrow head) and is missing one central abdominal denticle belt (arrow). (D) Percentage of embryonic cuticles with gain-of-function phenotypes is shown. Gain-of-function effects of tor^{RL3} were differentially enhanced by FL DRaf and $DRaf^{\Delta N114}$ proteins ($\chi^2 = 51.063837$, $P < 0.001$). (E) Expression of *engrailed* (*en*) at approximately stage 11 (left) and accumulation of *tailless* (*tll*) mRNA at cellular blastoderm stage (right) in embryos from WT, $tor^{RL3}/+$, $tor^{RL3}/+$;$DRaf^{\Delta N114}$, or $tor^{RL3}/+$;*FL DRaf* mothers: Examples of embryos derived from (i) WT, (ii) $tor^{RL3}/+$, and (iii) $tor^{RL3}/+$;$DRaf^{\Delta N114}$ mothers exhibit normal *en* mRNA pattern with three thoracic (T1–T3) and nine abdominal (A1–A9) expression stripes. (iv) An embryo from a $tor^{RL3}/+$; *FL DRaf* mother is shown with partial deletion of *en* stripes (arrow) in a region that gives rise to central abdominal segmental pattern. Examples of embryos derived from (i′) WT and (ii) $tor^{RL3}/+$ mothers exhibiting a normal *tll* mRNA pattern. (iii′) An embryo from a $tor^{RL3}/+$; $DRaf^{\Delta N114}$ mother shows slightly expanded posterior expression domain of *tll*. (iv′) An embryo derived from $tor^{RL3}/+$;*FL DRaf* females exhibits expanded domain of *tll* expression for both anterior and posterior regions.

sionally, trace-level *tll* expression was detected in 3–4 cells at the posterior embryo tip) and exhibit terminal defects with deletion of all posterior structures (A8 denticle belt and Filzkörper; Figure 4Cii). Interestingly, overexpression of FL DRaf partially restores the A8 denticle belt structure in embryos from $trunk^1/trunk^1$ ($trk^-/^-$; lacks the last 16 amino acids) mothers (Figure 4Civ). This result is consistent with our unpublished findings (L. AMBROSIO and K. H. BAEK) using the trk^3 allele (encodes the first 89 amino acids) and a different method. Rescue of posterior structures for some Trk-deficient embryos was found after injection of wild-type *DRaf* mRNA, also suggesting that accumulation of exogenous DRaf proteins promotes signaling in this trk^- background. However, expression of the $DRaf^{\Delta N114}$ transgene at a similar level failed to rescue the A8 denticle band defect in embryos from $trk^-/^-$ mothers (Figure 4, A and Ciii). We repeated these experiments using two additional *FL DRaf* and $DRaf^{\Delta N114}$ transgenic lines and observed similar results ($\chi^2 = 82.8574882$, $P < 0.001$; Figure 4D). This indicated that addition of FL DRaf but not $DRaf^{\Delta N114}$ proteins partially restored posterior Torso RTK signaling in the trk^- background and FL DRaf appears to possess greater activity compared with $DRaf^{\Delta N114}$. Consistent with this hypothesis, as shown in Figure 4C, iii′ and iv′, partial rescue of posterior *tll* mRNA expression was detected in some cellular blastoderm embryos derived from $trk^-/^-$; *FL DRaf/FL DRaf* females (8.1%, $n = 37$) but not for those derived from $trk^-/^-$; $DRaf^{\Delta N114}/DRaf^{\Delta N114}$ mothers ($n = 52$, $\chi^2 = 4.36329353$, $P < 0.05$). Together, these data consistently suggest that the absence of the N-terminal

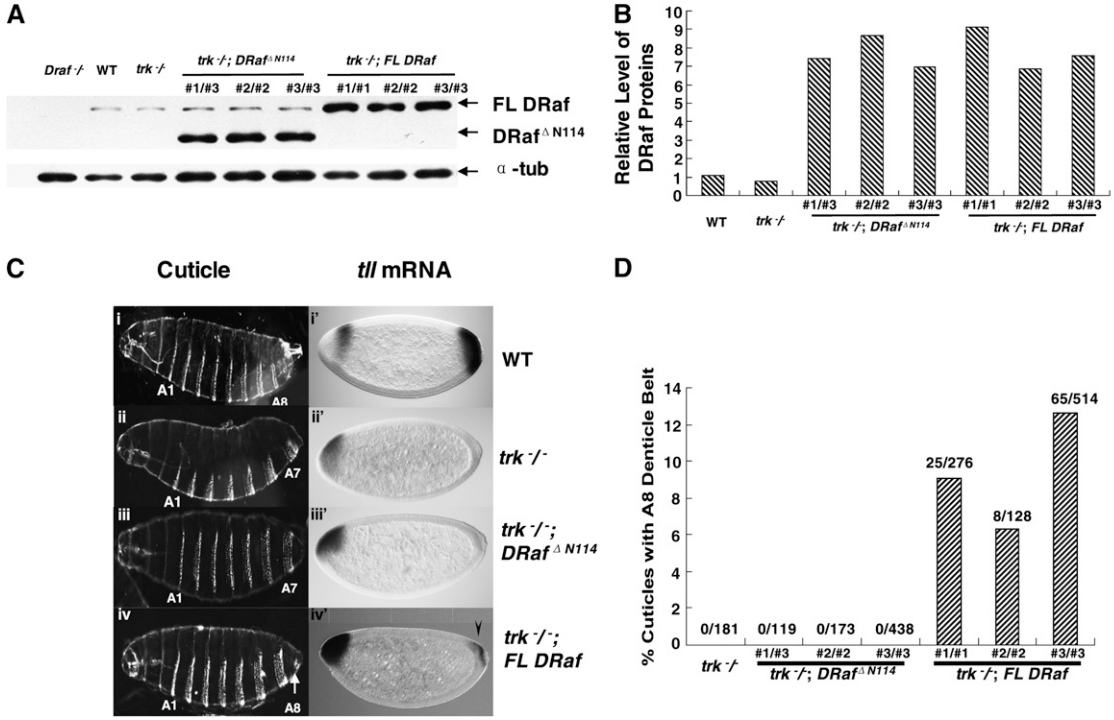

FIGURE 4.—Effects of FL DRaf and DRaf$^{\Delta N114}$ expression on posterior development in embryos derived from *trk* ^{1}/*trk* ^{1} mothers. (A) Western analysis of embryonic DRaf proteins from eggs (0–3 hr) produced by *Draf* $^{11\text{-}29}$/*Draf* $^{11\text{-}29}$ (*Draf*$^{-}$/$^{-}$), wild type (WT), *trk* ^{1}/*trk* ^{1} (*trk*$^{-}$/$^{-}$), *trk*$^{-}$/$^{-}$; *DRaf*$^{\Delta N114}$/*DRaf*$^{\Delta N114}$ (three lines, #1/#3, #2/#2, and #3/#3), and *trk*$^{-}$/$^{-}$; *FL DRaf/FL DRaf* (three lines, #1/#1, #2/#2, and #3/#3) females. Full-length DRaf (~90 kDa) and DRaf$^{\Delta N114}$ (~77 kDa) proteins are denoted by arrows. Embryonic lysate from *Draf*$^{-}$/$^{-}$ germline clone females was used as a negative control. α-Tubulin was used as the loading control. (B) Normalized DRaf protein level from A is shown in the bar graph. (C) Representative cuticles of mature embryos derived from wild type (WT), *trk*$^{-}$/$^{-}$, *trk*$^{-}$/$^{-}$; *DRaf*$^{\Delta N114}$/*DRaf*$^{\Delta N114}$, or *trk*$^{-}$/$^{-}$; *FL DRaf/FL DRaf* females are shown (left). Accumulation of *tll* (right) mRNAs was detected by *in situ* hybridization. (i) A wild-type (WT) embryo has normal cuticle pattern with eight abdominal denticle belts and Filzkörper structure. (i′) A WT embryo at cellular blastoderm stage exhibits a normal posterior expression domain of *tll*. (ii) Cuticle of a mature embryo from a *trk*$^{-}$/$^{-}$ mother is missing posterior structures (A8 segment, Filzkörper). (ii′) A cellular blastoderm embryo from a *trk*$^{-}$/$^{-}$ mother lacks posterior *tll* expression. (iii) An embryonic cuticle from a *trk*$^{-}$/$^{-}$; *DRaf*$^{\Delta N114}$/*DRaf*$^{\Delta N114}$ mother lacks posterior structures (A8 denticle belt, Filzkörper). (iii′) A cellular blastoderm embryo from a *trk*$^{-}$/$^{-}$; *DRaf*$^{\Delta N114}$/*DRaf*$^{\Delta N114}$ mother lacks posterior expression of *tll* mRNA. Expression of FL DRaf (iv) restores the A8 denticle belt (arrow) and (iv′) posterior *tll* expression (arrow) in embryos lacking maternal Trk activity. (D) Effect of *FL DRaf* or *DRaf*$^{\Delta N114}$ transgene expression on A8 denticle development in embryos derived from *trk*$^{-}$/$^{-}$ mothers (percentage of embryonic cuticles with A8 denticle belt). Shown are results using transgenic *DRaf*$^{\Delta N114}$ or *FL DRaf* lines that express DRaf protein at similar levels. Expression of exogenous FL DRaf, but not DRaf$^{\Delta N114}$, results in partial rescue of A8 denticle belt in some embryos derived from *trk*$^{-}$/$^{-}$ mothers ($\chi^2 = 82.8574882$, $P < 0.001$).

segment reduces the signaling potential of DRaf and the N terminus can contribute to Torso RTK terminal signaling in a positive manner.

SPRENGER *et al.* (1993) previously observed a low level of Torso receptor phosphorylation in eggs derived from *trk* loss-of-function, but not *tor* loss-of-function females. Therefore, a small amount of Torso signal activity may exist in our *trk*$^{-}$ background. This may be due to (1) the presence of active Torso-like (Tsl) ligand; (2) potential residual Trunk activity, considering the molecular lesion of the *trk*^{1} allele we used (lacks only the last 16 amino acids); or (3) the intrinsic activity of the Torso receptor. This activity could allow rescue of posterior structures by *FL DRaf* expression. If so, the contribution of the N terminus to terminal signaling is likely sensitive to such upstream events. Thus, we examined the consequences of DRaf expression in embryos from *tor*^{XR1}/*tor*^{XR1} (*tor*$^{-}$/$^{-}$; protein null) mothers that lacked the Torso receptor. We found that expression of FL DRaf or DRaf$^{\Delta N114}$ failed to restore the posterior structure (A8 denticle belt) for these embryos (Table 1). This indicates the contribution of DRaf's N-terminal residues to rescue the A8 denticle belt is dependent on activity of the receptor.

The N terminus of DRaf contains a novel conserved region and has a high content of helical secondary structure: We analyzed the amino acid sequence of DRaf's N terminus using several bioinformatics tools to obtain hints regarding its structure, and perhaps mechanism(s) of its functional role(s). A PROSITE motif search showed a putative protein kinase C (PKC) phosphorylation site within the "T-S-K" motif of the N terminus (positions 60–62; SIGRIST *et al.* 2002). Phosphorylation site prediction by NetPhos 2.0 suggested

TABLE 1

Expression of FL DRaf or DRaf$^{\Delta N114}$ did not result in rescue of the A8 denticle belt in embryos produced by *tor^{XR1}/tor^{XR1}* females

Maternal genotype	No. (%) embryos whose most posterior structure belongs to: A6/A7 segment	A8 denticle belt	Total no.
tor^{XR1}/tor^{XR1}	117 (100)	0 (0)	117
tor^{XR1}/tor^{XR1}; DRaf$^{\Delta N114}$#1/DRaf$^{\Delta N114}$#3	72 (100)	0 (0)	72
tor^{XR1}/tor^{XR1}; DRaf$^{\Delta N114}$#2/DRaf$^{\Delta N114}$#2	77 (100)	0 (0)	77
tor^{XR1}/tor^{XR1}; DRaf$^{\Delta N114}$#3/DRaf$^{\Delta N114}$#3	98 (100)	0 (0)	98
tor^{XR1}/tor^{XR1}; FL Draf#1/FL DRaf#1	61 (100)	0 (0)	61
tor^{XR1}/tor^{XR1}; FL Draf#2/FL DRaf#2	70 (100)	0 (0)	70
tor^{XR1}/tor^{XR1}; FL Draf#3/FL DRaf#3	81 (100)	0 (0)	81

that the Thr in this T-S-K motif had a high phosphorylation potential (Figure 5A; Blom *et al.* 1999). Predictions of secondary structure for the N-terminal region using GORV, PHD, and Predator indicated a high α-helical propensity (Figure 5A) (Rost *et al.* 1994; Frishman and Argos 1996; Garnier *et al.* 1996; Combet *et al.* 2000). A blastp search of other organisms with DRaf's N-terminal sequence identified honeybee Raf, chick C-Rmil, and BRaf proteins of sea urchin, zebrafish, frog, and human. The region containing amino acids 19–77 of DRaf showed homology between candidates. These sequences were aligned using ClustalW, and are shown in Figure 5A (Combet *et al.* 2000). Overall, the amino acids showed 18.6% identity and 47.5% similarity, and we term this region CRN. Interesting features of CRN include the putative phosphorylation site and a propensity to form two α-helical structures. This suggests that the N-terminal region of DRaf may have function(s) shared by other BRaf proteins.

The conserved structural features, including α-helical propensity, may be related to the functional role(s)/regulatory mechanism(s) of DRaf's N terminus. To confirm the prediction attained by bioinformactics tools, CD spectral measurement of the N-terminal part of DRaf (amino acids 1–117, DRaf^{N117}) was performed after its expression and purification *in vitro* (see materials and methods). As shown in Figure 5B, a bilobed spectrum with local minima at ~209.4 nm and at ~221.4 nm was observed, indicating the relatively high content of helical secondary structure for DRaf's N terminus. The estimated helix content of DRaf^{N117} is ~77% on the basis of the CD spectra data analysis using DICHROWEB (Whitmore and Wallace 2008, http://dichroweb.cryst.bbk.ac.uk/html/home.shtml). This result bolsters the predictions by GORV, PHD, and Predator.

The N terminus assists in association of DRaf's RBD with small GTPases Ras1 and Rap1 *in vitro*: Fischer *et al.* (2007) found that association of BRaf with HRas was facilitated by N-terminal sequences, *in vitro*. To examine whether the presence of DRaf's N terminus can affect Ras1 binding, we tested interaction between Ras1ΔCAAX and DRaf's RBD (Ras binding domain) using the yeast two-hybrid assay. A stronger interaction with Ras1ΔCAAX was detected when N-terminal residues were linked to RBD in both solid-support (data not shown) and liquid quantitative β-galactosidase assays ($P < 0.05$, *t*-test; Figure 6B), suggesting that the N terminus may assist in association of DRaf with Ras1. This is consistent with results obtained for BRaf (Fischer *et al.* 2007).

No direct interaction was detected between Ras1ΔCAAX and isolated N-terminal residues of DRaf (Figure 6B, and solid-support data not shown). Thus, the N terminus appears to contribute to Ras1 binding, but as an isolated protein fragment, cannot directly interact with Ras1. Arg174 located in DRaf's RBD region is essential for its association with Ras1 and substitution of Arg174 to Leu in RBD (RBD^{R174L}) abolishes Ras1 binding (Fabian *et al.* 1994; Li *et al.* 1998). We found that N-terminal residues cannot restore Ras1 interaction when linked with RBD^{R174L} (Figure 6B and solid-support data not shown). This indicated the effects of the N terminus were dependent on interaction between RBD and Ras1.

Moreover, we tested the idea that the conserved CRN region (19–77) might be essential for the contribution of DRaf's N terminus to Ras1ΔCAAX binding. Deletion of the first 17 N-terminal amino acids (Δ17NRBD) did not change Ras1ΔCAAX binding. However, if N-terminal amino acids including CRN were removed (Δ77NRBD), interaction with Ras1ΔCAAX was reduced to a level similar to that observed by deletion of the entire N terminus (amino acids 1–114; Figure 6, A and B). Together, these findings suggested the hypothesis that N-terminal residues of DRaf can assist in Ras1 interaction through a CRN-mediated mechanism(s). The small GTPase Rap1, a close relative of Ras1, is known to interact with DRaf and play a role in Torso RTK signaling *in vivo* (Mishra *et al.* 2005). To examine if DRaf's N terminus affects its association with Rap1, we tested interaction between Rap1ΔCAAX and DRaf's RBD (Figure 6C). A stronger interaction with Rap1ΔCAAX was detected when the N terminus was linked to RBD, similar to our findings with Ras1 ($P < 0.05$, *t*-test). Furthermore, the conserved CRN region (19–77) seems essential for the contribution of

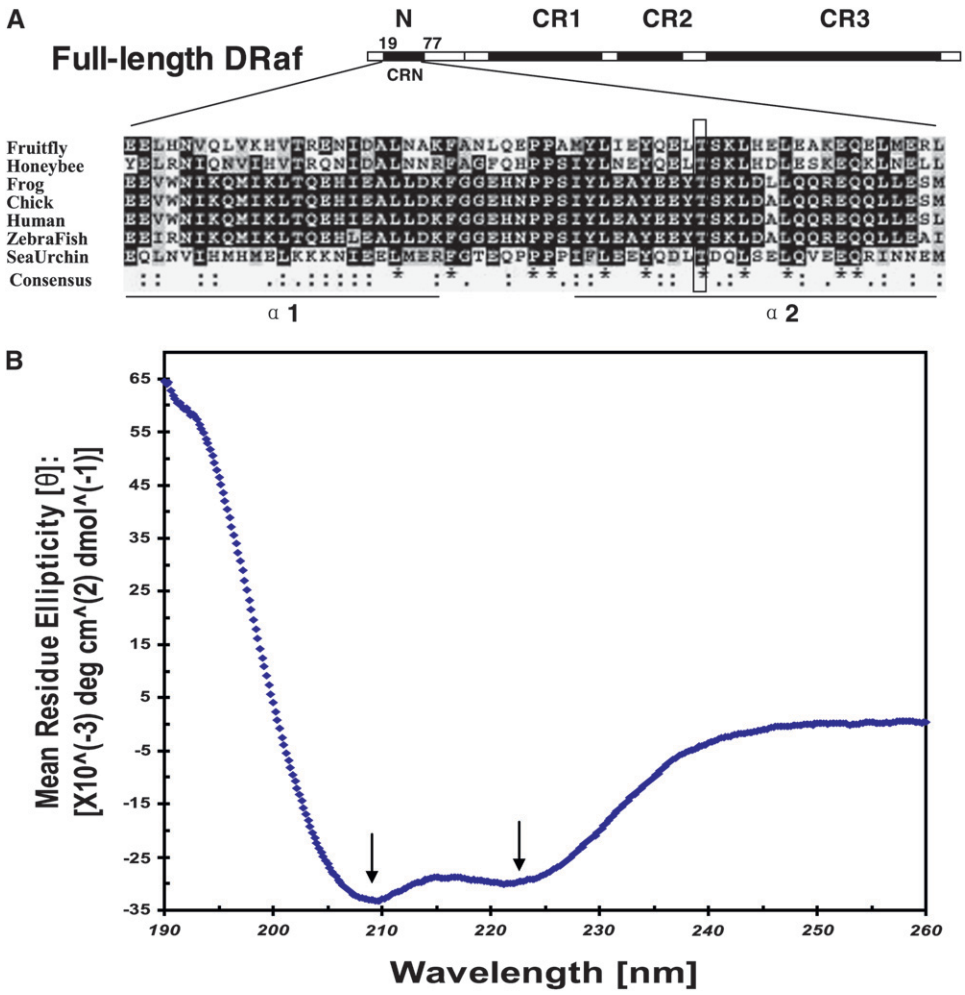

Figure 5.—The N terminus of DRaf contains a novel conserved region and has a high content of helical secondary structure. (A) Drosophila Raf (NP_525047; 739 amino acids) has in addition to its three conserved regions (CR1–CR3), an extensive N terminus. A novel region (amino acids 19–77) within the N terminus is conserved in honeybee Raf (*Apis mellifera* XP_396892), frog BRaf (*Xenopus laevis* AAU29410), chicken C-Rmil (*Gallus gallus* CAA47436), human BRaf (*Homo sapiens* NP_004324), zebrafish BRaf (*Danio rerio* BAD16728), and sea urchin BRaf (*Strongylocentrotus purpuratus* XP_781094) and termed **c**onserved **r**egion **N**-terminal (CRN). Sequences of CRN were aligned using ClustalW (identities were denoted as *; strong and weak similarities were denoted as : and .," respectively, in consensus line, http://www.ebi.ac.uk/tools/clustalw/), and the conserved residues were shaded using BOXSHADE (identities, solid; similarities, shaded, http://www.ch.embnet.org/software/BOX_form.html). Secondary structure prediction with GORV indicates CRN has the propensity to form two α-helices (α1 and α2). The putative PKC phosphorylation site DRaf's Thr60 is framed. (B) Circular dichroism (CD) spectral measurement of DRaf's N terminus (amino acids 1–117) *in vitro*. The bilobed spectrum (arrows, local minima at ~209.4 nm and at ~221.4 nm) indicative of helical secondary structure is shown.

the N terminus to this interaction with Rap1, suggesting a CRN-mediated mechanism(s) may be a general feature for its binding to both Ras1 or Rap1.

DISCUSSION

In our study, a novel region (amino acids 19–77) within Draf's N terminus, conserved for Raf genes of most invertebrates and BRaf genes of vertebrates, was identified and termed CRN. This conserved region has not been described by others, but potential roles for the extended N terminus have been proposed in two reports. Terai and Matsuda (2006) found that in HeLa cells, the N terminus of BRaf may mediate Raf dimerization to generate BRaf–BRaf or BRaf–CRaf complexes, and play an important regulatory role in calcium-induced BRaf activation. However, Fischer *et al.* (2007) reported that deletion of BRaf's N terminus did not affect BRaf–CRaf dimer formation. Instead, they found that N-terminal residues appeared to facilitate interaction with HRas *in vitro*. In accordance with their data, stronger interactions between DRaf's RBD (Ras binding domain) and the small GTPase Ras1ΔCAAX were observed when N-terminal and RBD sequences were linked in our yeast two-hybrid analysis. This suggested that the N terminus might assist in Ras1 binding. Furthermore, the identity of specific residues in the N terminus that might participate in Ras1 binding were mapped to the CRN region (amino acids 19–77). Two known Raf motifs, RBD and CRD, are involved in Raf's interaction with Ras. Our studies, and results obtained by Fischer *et al.* (2007) using BRaf, suggest that the N-terminal residues of DRaf and BRaf proteins, particularly the CRN region, might be another element that plays a role(s) in Ras–Raf coupling.

The small GTPase Rap shares with Ras nearly identical Raf binding regions that comprise switch 1 and the lipid moiety (Hariharan 2005). Rap functions as an antagonist of Ras in regulating CRaf activity (Cook *et al.* 1993), but can activate BRaf in a parallel way with Ras (Ohtsuka *et al.* 1996). Isoform-specific features of different Raf family members may explain their distinct responses to Rap. In flies, both Ras1 and Rap1 can interact with and activate DRaf (Mishra *et al.* 2005).

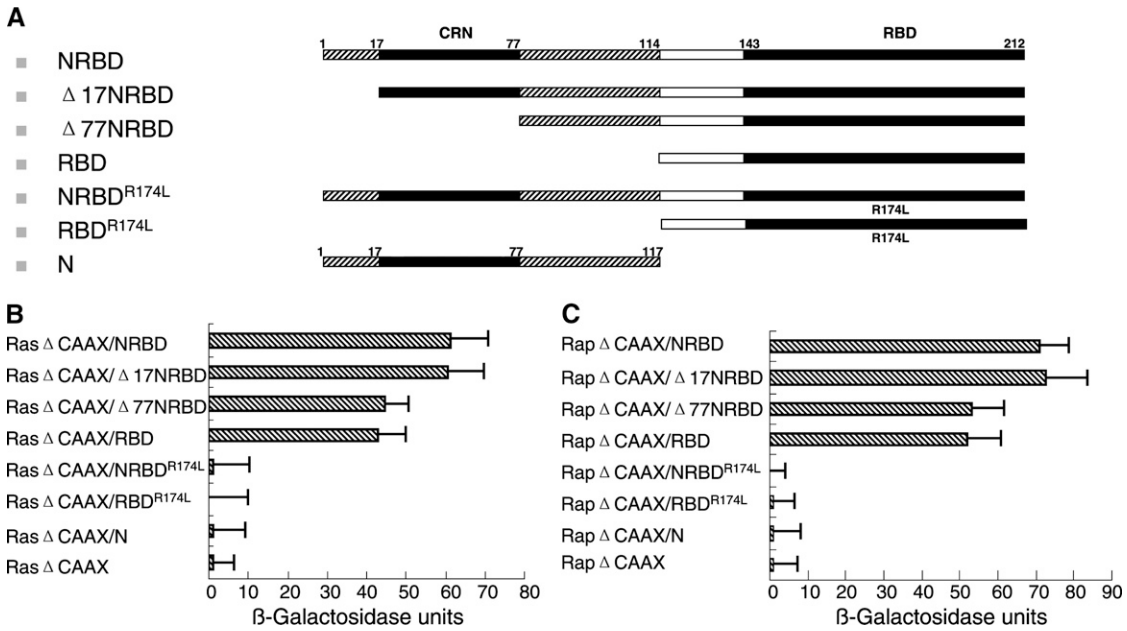

Figure 6.—Effects of the extended N terminus of DRaf on Ras1 and Rap1 binding. (A) Schematic representations of different DRaf constructs used for yeast two-hybrid analysis. (B) Interactions between DRaf's RBDs and Ras1ΔCAAX: Removal of CRN or the entire N-terminal region reduces Ras1ΔCAAX binding ($P < 0.05$, *t*-test). (C) Interactions between DRaf's RBDs and Rap1ΔCAAX: Removal of CRN or the entire N-terminal region reduces Rap1ΔCAAX binding ($P < 0.05$, *t*-test).

Thus, it was reasonable to test whether DRaf's N terminus including CRN might also assist in Rap1 binding. In agreement with this idea, stronger interaction between RBD and Rap1ΔCAAX was observed when DRaf's CRN and RBD sequences were linked *in vitro*, further suggesting that the N terminus may contribute to both Ras1 and Rap1 binding potentially through a CRN-mediated mechanism(s) *in vivo*.

What is/are the molecular mechanism(s)?: No direct interaction between Ras1 or Rap1 and the isolated DRaf N-terminal segment (amino acids 1–117) was detected, or when the N terminus was linked with the Ras1/Rap1 binding-deficient RBD^{R174L}. Thus, the contribution of DRaf's N-terminal residues to Ras1 and Rap1 binding requires the presence of RBD. It is possible that the CRN-containing N terminus may assist in Raf–Ras interaction by making RBD more accessible to Ras1 and/or in a sequential manner, subsequent to RBD–Ras1 interaction, by stabilizing the RBD–Ras1 complex. Deletion of CRN may result in conformational or structural changes that reduce Ras1 binding affinity. Structural analysis of these complexes may provide important clues and help to understand the molecular mechanism(s) by which CRN assists in Ras–Raf interaction. Our computational analysis suggested conserved CRN has the propensity to form two α-helical structures (α1 and α2; Figure 5A) and contains a putative phosphorylation motif T-S-K located in α2. In agreement, DRaf's N terminus (amino acids 1–117) was folded *in vitro* and had a high content of helical secondary structure (Figure 5B). These findings may help to establish a basis for future determination of molecular structure.

Although no verified binding partner(s) for DRaf or BRaf's N terminus has been identified, it is still possible that CRN may interact with other regulatory factors *in vivo*, that may affect Ras or Rap binding and/or function in activation of DRaf and BRaf. If so, the conserved structural features of CRN most likely relate to these regulatory events *in vivo*. Site-directed mutagenesis of conserved sites/motifs could provide useful information regarding the molecular mechanism(s) of CRN's role in the activation of DRaf and BRaf.

Torso RTK signal is differentially elevated by overexpression of FL DRaf and $DRaf^{\Delta N114}$ *in vivo*: We initiated our *in vitro* studies of DRaf's N terminus on the basis of our *in vivo* findings using both loss- and gain-of-function genetic assays that deletion of N-terminal residues consistently reduces DRaf's signal potential in the Torso pathway. When expressed at high levels, FL DRaf enhanced the gain-of-function effects of the tor^{RL3} allele much more significantly than $DRaf^{\Delta N114}$. In embryos from $trk^{-/-}$ mothers, addition of FL DRaf, but not $DRAF^{\Delta N114}$, partially restored the A8 denticle belt structure (Figure 4). These findings indicate that the N terminus can play a positive role(s) in Torso RTK signaling. Interestingly, the contribution of DRaf's N terminus in the Torso pathway appeared to be dependent on upstream receptor activity, suggesting its role in transmission of the signal. Together with our yeast two-hybrid data, as well as the results obtained by Fischer *et al.* (2007) for BRaf, we propose that the presence of N-terminal residues may facilitate the association of DRaf with the upstream regulators Ras1 and Rap1, thereby assisting in transmission of the RTK signal *in vivo*.

For instance, in the *trk*$^{-}$ background, a small amount of active GTP–Ras1 and GTP–Rap1 are likely present, mostly due to activation by residual upstream Trunk activity, the presence of Torso-like ligand, and/or the intrinsic activity of the Torso receptor. The *trk*^{1} mutation used in this analysis results in protein truncation at the last 16 amino acids. It is possible that overexpression of FL DRaf proteins in this background increases the likelihood of interaction between abundant DRaf proteins and membrane bound GTP–Ras1 or GTP–Rap1. This in turn, could elevate the RTK signal and partially restore development of the A8 denticle belt structure in some embryos. On the other hand, deletion of the N terminus could destabilize Ras1–DRaf (or Rap1–DRaf) coupling or decrease the duration of interaction, resulting in reduced DRaf signal transmission. This may explain why expression of DRaf$^{\Delta N114}$ failed to rescue the A8 denticle belt in embryos from *trk*$^{-/-}$ mothers.

Why are only minor differences detected *in vivo* between FL DRaf and DRaf$^{\Delta N114}$: Previously, an auto-inhibitory role had been assigned to residues compromising the first half of the DRaf protein, in addition to their functions in promoting its activity. Deletion of the N-terminal amino acids 1–272 (including the N terminus and CR1) or 1–402 (including the N terminus, CR1, and CR2) of DRaf at least partially relieved these negative effects (Baek *et al.* 1996). Here, although removal of the N-terminal 1–114 residues did not result in constitutive DRaf$^{\Delta N114}$ activity in embryos lacking the maternal Torso receptor (Table 1), it is still possible that the N terminus may contribute to auto-inhibitory effects. Together with CR1 and CR2, these N-terminal residues (1–114) may help maintain DRaf's inactive conformation. If so, the N terminus might play dual roles, both positively and negatively regulating DRaf. Therefore, its contribution to signaling may be neutralized by this auto-inhibition and consequently result in a subtle *in vivo* effect. If so, selective mutagenesis of the "inhibitory" motifs/sites in the N-terminal region or removal of other cofactors involved in its negative regulation may amplify signaling differences between FL DRaf and DRaf$^{\Delta N114}$. Ras binding has been thought crucial to recruit Raf to the membrane and promote its RTK signaling activity. However, the Drosophila Torso pathway appears tolerant of alterations in Ras1–DRaf coupling (Hou *et al.* 1995). Draf^{C110} has a R174L point mutation in the RBD domain and likely comprised for Ras1 binding (Li *et al.* 1998). The RBD^{R174L} is Ras binding deficient in our yeast two-hybrid assay (Figure 6B). However, *tll* expression patterns and cuticles of the embryos derived from mothers with *Draf*^{C110}/*Draf*^{C110} germ cells were indistinguishable from those of wild-type embryos (Melnick *et al.* 1993), suggesting a mechanism(s) independent of RBD–Ras1 interaction might function in recruiting DRaf to the membrane. In agreement with this model, Rizzo *et al.* (2000) found membrane translocation of CRaf could be mediated by its interaction with PA and independent of Ras binding. This PA binding site is also conserved in ARaf, BRaf, and DRaf. Thus, Draf^{C110} could be recruited to the cell membranes by associating with PA. Moreover, it is known that Raf's CRD participates in Ras binding through its interaction with the lipid moiety of Ras (Williams *et al.* 2000; Thapar *et al.* 2004). Once at the membrane, it is also possible that the interaction between Draf^{C110}'s CRD and Ras1 could further promote its membrane attachment and result in relatively normal Torso signal production. In this study, the presence of RBD, CRD, and the potential PA binding site may be sufficient to promote DRaf's activation in Torso signaling. This may explain why at approximately endogenous wild-type protein level maternally expressed DRaf$^{\Delta N114}$ is able to rescue the embryonic terminal defects of *Draf*$^{11\text{-}29}$ mutants (Figure 1, B, C, and E). Together, considering the Torso pathway's tolerance of alterations in Ras1–DRaf coupling and the minor role DRaf's N terminus plays in Ras1 binding, it is reasonable that the phenotypic consequences of removing these N-terminal residues (DRaf$^{\Delta N114}$) are not great in Torso signaling. The subtle phenotypic effects of DRaf's N terminus could also be due to compensation provided by potential autoregulatory feedback or alternative redundant processes in the *in vivo* system. In our study, the expression of DRaf proteins at a low level (~1/4 endogenous wild-type level) appeared to sensitize the assay system. We found deletion of the N terminus seemed to increase the threshold of DRaf protein levels required for normal signaling. Furthermore, by adding one copy of the ectopic *tor*^{RL3} allele or removing wild-type maternal Trunk activity we apparently increased the sensitivity of the Torso pathway. These allowed the embryonic terminal system to display enhanced differences between FL DRaf and DRaf$^{\Delta N114}$ proteins.

The biological implications of the N-terminal region: Why is this N terminus with its "subtle" functional effects conserved during evolution, and what is its biological relevance? There are numerous RTK pathways functioning in Drosophila cellular and developmental processes. In spite of the identical Ras–Raf–MEK signal cassette they share, these RTK pathways can lead to different biological responses. Previous studies indicated that such specificity might be due to the difference in the intensity and/or duration of the signal (Woods *et al.* 1997, 2001; Kerkhoff and Rapp 1998; Ghiglione *et al.* 1999). This suggested that the magnitude of Raf signal could function as a critical determinant of biological responses. Participation of multiple DRaf elements in Ras1 or Rap1 binding could be a good strategy to modulate its activity. Normally, tight association with Ras1 or Rap1 through RBD and CRD regions is required and sufficient to initiate the activation of DRaf, while minor adjustments/regulation of interaction by the CRN region could optimize signaling potential and reduce variability. Thus, the extended N terminus

including CRN may play a role(s) as one element in a multidomain effort to promote DRaf's interaction with Ras1 and Rap1, participating and assisting in regulation to reliably attain maximal signal output.

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