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The Role of the Coiled-Coil Motif in Interactions Mediated by TPD52

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TPD52 (D52)-like proteins are small coiled-coil motif-bearing proteins first identified through their expression in human breast carcinoma that mutually interact in hetero- and homomeric fashions. However, it has been unclear whether the coiled-coil motif is sufficient, or even necessary, for these interactions to occur. We have therefore examined the binding activities of a panel of C-terminally deleted D52 proteins in both the yeast two-hybrid system and pull-down assays. In the yeast two-hybrid system, interactions were only detected when regions C-terminal to the coiled-coil motif were also present. However, using pull-down assays, interactions were detected for all deletion mutants which included the coiled-coil motif. This suggests that the coiled-coil motif is indeed necessary for interactions mediated by D52 proteins, but that C-terminal protein regions facilitate and/or stabilize these interactions. © 2001 Academic Press

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The TPD52 (or D52) family (1) represents a conserved group of small hydrophilic polypeptides and consists of the members D52 (2), D53 (1, 3), and D54 (4, 5). The human *D52* (*hD52*) gene was identified as being overexpressed in human breast (2) and lung cancer (6) and has been subsequently identified as a gene amplification target (7). The *hD52* gene is currently the only known target gene at chromosome band 8q21, a region gained in many forms of human cancer. SEREX screening has also identified hD52 as a tumour-associated antigen in breast carcinoma, along with previously known antigens such as MAGE-3, MAGE-6, NY-ESO-1, ERBB2, and p53 (8, 9). In other species, D52 proteins have been identified as being encoded by a retrovirally transduced gene in proliferating cells (10), or as being phosphorylated in response to increased intracellular calcium (11, 12). Little is known of the biochemical basis of D52-like protein function, although D52 (4, 10, 13) and related proteins (4) are known to mutually interact. We have also recently identified a novel member of the MAL proteolipid family, MAL2, as the first heterologous partner for D52-like proteins (14). As MAL2 is most similar to MAL, an integral component of the apical transport machinery in polarized cell types (15–17), D52-like proteins may cooperate with or regulate MAL2 function in similar processes (14). A role for D52 in vesicle transport was initially suggested by its localization around exocrine secretory granules in pancreatic acinar cells (18) and has been more recently demonstrated by the marked enhancement of Ca^{2+}-dependent amylase secretion produced by the introduction of recombinant D52/CRHSP-28 into permeabilized acinar cells (19).

The presence of a coiled-coil motif in D52-like proteins suggested that this would likely mediate interactions between these proteins, and the binding domain of mouse D52 (mD52) was initially mapped to mD52 residues Met^{1}–Ala^{95}, which includes the coiled-coil motif from Glu^{27}–Leu^{71} (4). However, an independent study found that a Met^{1}–Leu^{71} N8/hD52 protein neither self-associated, nor associated with wild-type N8/hD52 (13). These combined results suggested that regions C-terminal to the coiled-coil motif may also be necessary, or even sufficient, for such interactions to occur. As the Gly^{72}–Ala^{95} D52 region contains part of a 14 residue D52 motif found in all D52-like proteins (5), we derived a panel of C-terminally deleted D52 proteins which, in addition to the coiled-coil motif, included C-terminal regions with and without the D52 motif. We then determined the relative contributions of these regions to D52-like protein interactions in both the Y2H system and pull-down assays. This confirmed that while the coil-coil motif is necessary for interac-

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tions to occur, these are likely facilitated and/or stabilized by additional C-terminal regions. We also carried out sequence analyses of expressed sequence tags (ESTs) deriving from D52-like genes, to determine the significance of sequence variations previously noted in regions encoding the coiled-coil motif. Polymorphisms were found to be relatively frequent in sequences encoding murine but not human D52-like coiled-coil motifs and may therefore provide an additional mechanism to modulate D52-like protein function in rodents.

MATERIALS AND METHODS

Plasmid constructs. For the production of C-terminally deleted D52 fusion proteins (Fig. 1), mouse and human *D52* cDNA sequences were PCR-amplified using the listed primer pairs (Table 1). Both 5′ PCR primers allowed the conversion of a cryptic *Nco*I site flanking the mD52 and hD52 Met^1 codons to a full *Nco*I site, whereas each 3′ PCR primer introduced a stop codon and *Sma*I site after the relevant truncation point (Table 1). PCR cycle conditions involved initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation (94°C for 45 s), annealing (55°C for 1 min), and extension (72°C for 1 min), followed by a final elongation step at 72°C for 5 min.

Resulting PCR products were digested with *Nco*I and *Sma*I, and subcloned into the corresponding restriction sites of expression vectors. Bait proteins for use in the Y2H system were expressed from the pAS2-1 vector (Clontech), which contains the selectable marker *TRP1,* and permits the expression of fusion proteins with the $GAL4_{(1–147)}$ DNA-binding domain. Prey proteins were expressed from the pACT2 vector (Clontech), which contains the selectable marker *LEU2,* and permits the expression of fusion proteins with the $GAL4_{(768–881)}$ activation domain. The pAS2-1mD52 and pACT2hD53 constructs have been described previously (4). For the production of recombinant D52 proteins, the pET32a vector was employed which produces N-terminally thioredoxin-tagged proteins, which also include a 6-His tag for purification (Novagen, U.S.A.). These were derived by subcloning *Nco*I–*Sal*I ($mD52_{(1–103)}$, $mD52_{(1–111)}$, $mD52_{(1–131)}$ $mD52_{(1–151)}$, $mD52_{(1–171)}$), *Nco*I–*Bam*HI ($mD52_{(1–87)}$, $mD52_{(1–89)}$), or *Nco*I–*Sma*I ($mD52_{(1–79)}$) inserts from corresponding bait constructs into the same or compatible sites in pET32a. In the case of the pET32a-$hD52_{(1–71)}$ construct, *hD52* sequences were PCR-amplified (Table 1), and subcloned directly into the *Nco*I and *Eco*RV sites of pET32a. The pET32a-$mD52_{(95–185)}$ plasmid was obtained by subcloning a *Nco*I–*Pvu*II fragment from pAS2-$1mD52_{(95–185)}$ (4), where the *Pvu*II site had been blunt-ended, into the *Nco*I and *Eco*RV sites of pET32a. Minipreparations of all plasmid DNAs were purified by NaCl and polyethyleneglycol 6000 precipitation, and sequenced with *Taq* polymerase and dye-labeled ddNTPs for detection on an Applied Biosystems 373A automated sequencer.

Yeast two-hybrid system. Yeast cultures of the *S. cerevisiae* strain Y190 strain (20) were grown at 30°C in standard liquid or solid media, based upon either rich YPD media (2% bacto-peptone, 1% yeast extract, 2% dextrose), or minimal SD medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, with appropriate amino acid supplements). In the case of SD/-Leu-Trp-His media, this also included 35 mM 3-amino-1, 2, 4-triazole (3-AT).

For the direct testing of interactions, paired bait (pAS2-1) and prey (pACT2) constructs were cotransfected into Y190 cells as previously described (4). Interactions between baits and preys were assessed by qualitatively determining *HIS3* reporter activity, and in some cases quantitatively assessing *LacZ* reporter activity, as previously described (4). In all Y2H experiments, both positive and negative control transfections were performed, which consisted of full-length mD52 bait paired with hD53 prey, and baits paired with the pACT2 vector, respectively.

In vitro transcription/translation. Constructs for *in vitro* transcription/translation were made using the pTL1 vector, a derivative of pSG5 (21), which permits *in vitro* transcription from the T7 promoter. The pTL1 construct employed for *in vitro* transcription/translation of hD52 has been previously described (4). Coupled *in vitro* transcription/translation was performed using the TNT T7 wheat germ lysate system (Promega Corp.) as described (4). Protein product sizes were verified by electrophoresing 2.5 μl of each reaction through 15% denaturing polyacrylamide gels which were fixed, dried, and exposed to autoradiographic film (BioMax, Kodak) overnight at −80°C.

Pull-down assays. Recombinant thioredoxin-6His-tagged truncated D52 proteins and thioredoxin-6-His protein were produced in the BL21 *Escherichia coli* strain following induction of log-phase cultures with 25 μg/ml IPTG for 2 h at 30°C. Proteins were isolated by incubating *E. coli* samples in binding buffer (50 mM potassium phosphate, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, plus inhibitors) for 15 min on wet ice, followed by brief sonication. Protein product sizes were verified by electrophoresing pre- and postinduction protein samples on 15% SDS–polyacrylamide gels, followed by Coomassie brilliant blue staining. Pull-down experiments were conducted basically as previously described (4), with minor modifications. Recombinant proteins (2–5 μg) bound to 25 μl Ni–NTA agarose (Qiagen) were incubated with 20 μl of each *in vitro* translation reaction, and 3.2 mg soluble BL21 protein extract, prepared as described (22), for 1.5 h at 4°C. Matrixes were washed three times with 1 ml cold binding buffer, and bound proteins were eluted in 15 μl SDS–loading buffer by boiling for 5 min, and separated on 15% SDS–polyacrylamide gels. These were stained with Coomassie brilliant blue, dried, and exposed to autoradiographic film (BioMax MS, Kodak) for 5 days at −80°C.

DNA sequence analyses. For analyses of sequence variations within D52-like sequences encoding coiled-coil motifs, 48-bp sequences encoding mouse and human D52 residues L^{31}–V^{46}, mouse and human D53 residues L^{31}–L^{46} and mouse and human D54 residues L^{41}–V^{56} genes were employed in BLAST-N analyses (23) of the respective dbEST divisions. These regions had been found to contain sequence variations in preliminary analyses, and were frequently included in 5′-EST sequences. These were verified as deriving from particular mouse and human D52-like genes by verifying that these were contained within relevant Unigene entries (Hs. 2384/Mm. 2777 for *h/mD52,* Hs. 16611/Mm. 7821 for *h/mD53,* and Hs. 154718/Mm. 27872 for *h/mD54*). Only ESTs which contained entire test sequences were analyzed. Sequence variations were noted if at least two separate ESTs predicted the same amino acid substitution. A full list of ESTs analyzed is available from the authors.

RESULTS AND DISCUSSION

C-Terminal Protein Regions Facilitate Interactions Mediated by D52 in the Yeast Two-Hybrid System

Before proceeding with interaction testing, truncated D52 baits which included between 8 and 100 residues C-terminal to the coiled-coil motif (Fig. 1, Table 1) were tested for their inability to activate Y190 reporter gene expression alone. Of these, $mD52_{(1–79)}$, $mD52_{(1–87)}$, and $hD52_{(1–89)}$ included the coiled-coil motif without the D52 motif, whereas $hD52_{(1–103)}$, $mD52_{(1–111)}$, $mD52_{(1–131)}$, $mD52_{(1–151)}$, $mD52_{(1–171)}$ and full-length mD52 included both motifs (Fig. 1). Introduction of each bait cDNA into Y190 cells gave rise to no growth on SD/-His media after 3–5 days incubation at 30°C (data not shown). We then assayed the binding activity of full-length and deleted D52 proteins toward full-

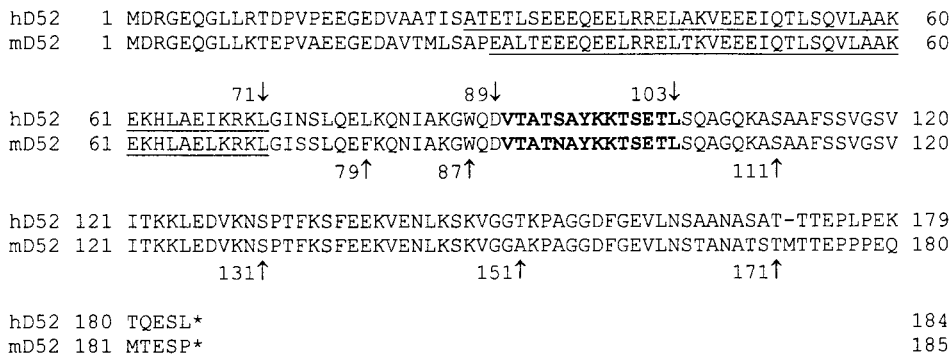

FIG. 1. Alignment of human and mouse D52 sequences, with C-terminal truncation points indicated by arrows above (hD52) or below (mD52) these sequences. The amino acid residue after which each truncation was introduced is shown to the left of each arrow. Coiled-coil motifs are shown underlined, whereas D52 motifs are shown in bold. Numbers at the beginning and end of each sequence line refer to the positions of the first and last amino acids shown, respectively. A single gap (−) was inserted in the hD52 sequence to maintain sequence alignment.

length hD53, as D52 baits had previously interacted maximally with hD53 in the Y2H system (4). Growth on solid SD/-Leu-Trp-His + 35 mM 3-AT media was detected after 2–3 days incubation at 30°C for cotransfectants including full-length mD52 and hD53, and after 4 days for cotransfectants including $mD52_{(1–171)}$ and hD53 (Table 2). All other cotransfectants gave rise to no detectable growth after 6-10 days (Table 2). A subset of these interactions were assayed for β-galactosidase activity (4). Very low β-galactosidase activity levels were measured in cotransfectants including $mD52_{(1–111)}$, $mD52_{(1–131)}$, and $mD52_{(1–151)}$ baits and hD53 prey (Table 2), whereas levels of β-galactosidase activity in cotransfectants including $D52_{(1–171)}$ and hD53 were higher than those measured in the positive control (Table 2).

Pull-Down Assays Demonstrate Binding of Recombinant D52 Fusion Proteins Including the Coiled-Coil Motif and in Vitro-Translated hD52 Protein

We analyzed binding between thioredoxin-6His-tagged deleted D52 proteins ($hD52_{(1–71)}$, $mD52_{(1–79)}$, $hD52_{(1–89)}$, $mD52_{(1–111)}$, $mD52_{(1–131)}$, $mD52_{(1–151)}$, $mD52_{(1–171)}$, and $mD52_{(95–185)}$) and *in vitro*-translated hD52 protein using a pull-down assay system. *In vitro* translation of the *hD52* coding sequence gave rise to single 26-kDa ^{35}S-labeled protein, as predicted (Fig. 2A). Recombinant thioredoxin-6His-D52 proteins or the thioredoxin-6His tag alone were produced in BL21 *E. coli* cells, bound to Ni–NTA agarose, and incubated with *in vitro* translated proteins in a large excess of complex competitor. Following washing of matrices and elution of bound material, autoradiographs of Coomassie brilliant blue-stained gels (Fig. 2B) indicated that ^{35}S-labeled hD52 protein was retained on matrices to which all D52 proteins including the coiled-coil motif were bound (Fig. 2C). ^{35}S-labeled hD52 was not retained by the thioredoxin-6His tag alone, or by thioredoxin-6His-tagged $mD52_{(95–185)}$ (Fig. 2C), which does not include the coiled-coil motif (4).

The fact that all D52 proteins including the coiled-coil motif displayed binding activity in pull-down assays, whereas only those including significant C-terminal regions displayed binding the Y2H system, indicates that while the coiled-coil motif is necessary for interactions to occur between D52-like proteins, these are facilitated and/or stabilized by the presence of C-terminal regions. This is supported by previous

TABLE 1

PCR Primer Sequences Employed to Generate Deleted Human and Mouse D52 Proteins Employed in Interaction Testing

Protein	5′ PCR primer	3′ PCR primer
$mD52_{(1–171)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAGGTACTGGTAGCGTT-3′
$mD52_{(1–151)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAGGCTCCTCCTACTTT-3′
$mD52_{(1–131)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAGGAGTTTTTCACGTC-3′
$mD52_{(1–111)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAGGAGGCCTTCTGCCC-3′
$hD52_{(1–103)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTATAAGGTTTCAGATGTC-3′
$hD52_{(1–89)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAGTCTTGCCACCCTTTG-3′
$mD52_{(1–87)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTACCACCCTTTGGCAATG-3′
$mD52_{(1–79)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAGAACTCCTGAAGCGAG-3′
$hD52_{(1–71)}$	5′-CGCATGCCATGGACCGCGGCGAG-3′	5′-TCCCCCGGGGGATTAAAGTTTCCGCTTGATC-3′

TABLE 2

Interactions Measured in the Y2H System between Full-Length $mD52_{(1–185)}$ Bait or Deleted D52 Bait Proteins and Either hD53 Prey Protein or the pACT2 Vector-Encoded GAL4 AD Domain

Prey	*LacZ*[a]		*HIS3*[b]	
Bait	hD53	pACT2	hD53	pACT2
$mD52_{(1–185)}$	4.44 ± 0.94	0.49 ± 0.30	++	−
$mD52_{(1–171)}$	14.11 ± 7.69	0.23 ± 0.10	+	−
$mD52_{(1–151)}$	1.73 ± 0.49	0.38 ± 0.24	−	−
$mD52_{(1–131)}$	1.43 ± 0.66	0.34 ± 0.05	−	−
$mD52_{(1–111)}$	0.94 ± 0.94	0 ± 0	−	−
$hD52_{(1–103)}$	ND[c]	ND	−	−
$hD52_{(1–89)}$	ND	ND	−	−
$mD52_{(1–87)}$	ND	ND	−	−
$mD52_{(1–79)}$	ND	ND	−	−

[a] *LacZ* reporter gene activity was measured by quantitative β-galactosidase assays. Between 3 and 13 colonies from each cotransfected Y190 strain were cultured in SD/-His-Trp-Leu + 35 mM 3-AT medium and assayed separately for β-galactosidase activity. The mean normalized number of β-galactosidase activity units (4) obtained for each cotransfection is presented ± the standard error.

[b] *HIS3* reporter gene activity was measured by assessing Y190 cotransfectant colony growth on solid SD/-His-Trp-Leu + 35 mM 3-AT medium at 30°C. (−) indicates no growth after 6–12 days, (+) indicates visible growth after 4–8 days, and (++) indicates visible growth after 2–3 days.

[c] ND, not done.

findings where alternative splicing events involving N- and/or C-terminal hD53 regions affected interaction preferences of a hD53 isoform (5). It is notable however that the presence or absence of the D52 motif did not affect the binding characteristics of D52 proteins in either the Y2H system or in pull-downs, arguing against a direct role for this motif in mediating interactions between D52-like proteins.

Variations within Murine Sequences Encoding D52-like Coiled-Coil Motifs

Previous small-scale analyses of ESTs deriving from murine D52-like genes prior to the existence of dbEST had indicated unexpected variations within sequences encoding mouse D52-like coiled-coil motifs (unpublished data). Given the significance of this motif to D52 binding activity, we carried out larger-scale analyses of D52-like ESTs from both human and mouse genes. Whereas no consistent sequence variations were noted in 26 *hD52* ESTs encoding hD52 L^{31}–V^{46}, variations were noted in 5/35 (14.3%) *mD52* EST sequences. A E^{37}/D^{37} variation was predicted by 2/41 (4.9%) *mD52* ESTs, whereas E^{42}/D^{42} and L^{43}/V^{43} variations were predicted by 3/41 (7.3%) *mD52* ESTs (Fig. 3). Similarly, whereas no consistent sequence variations were noted in 43 *hD53* ESTs encoding hD53 L^{31}–L^{46}, variations predicting a mD53 I^{44}/V^{44} substitution were found in 4/7 (57.1%) *mD53* ESTs (Fig. 3). This I^{44}/V^{44} substitution also occurs between mD53 and hD53 sequences (3). Finally, while no consistent sequence variations were noted in 66 *hD54* ESTs encoding residues L^{41}–V^{56}, variations were noted in 10/49 (20.4%) *mD54* ESTs. A E^{43}/D^{43} variation was predicted by 8/49 (16.3%) ESTs, and a E^{46}/V^{46} variation was predicted by 2/49 (4.1%) ESTs (Fig. 3). Furthermore, a E^{47}/D^{47} variation was predicted by 4/49 (8.2%) ESTs, and a E^{48}/* variation was predicted by 2/49 (4.1%) ESTs (Fig. 3). This premature stop codon predicts a protein including only

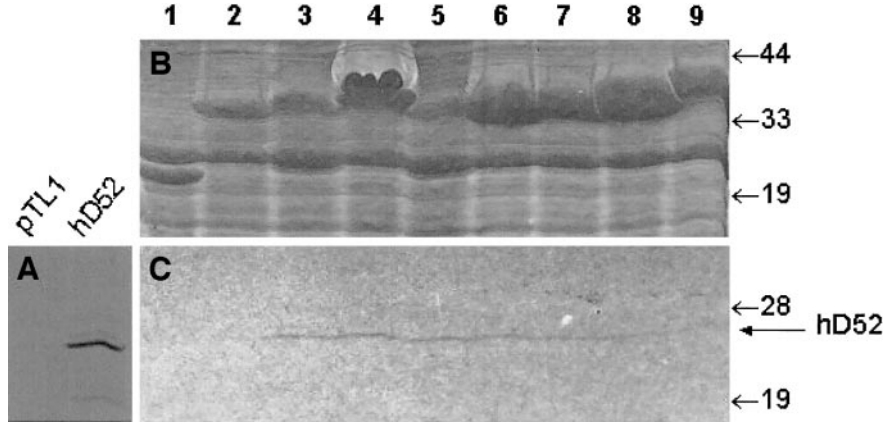

FIG. 2. Interactions between thioredoxin-6His-D52 and *in vitro*-translated hD52 protein in pull-down assays. The positions of molecular weight markers (in kilodaltons) is shown on the right. (A) ^{35}S-labeled proteins resulting from *in vitro* translation of pTL1 constructs bearing either no insert, or *hD52* coding sequences. *In vitro* translation of *hD52* coding sequences gave a single 26-kDa ^{35}S-labeled protein. (B) Coomassie blue-stained thioredoxin-6His tag (lane 1, 20 kDa) and thioredoxin-6His-D52 proteins (lane 2, $mD52_{(95–185)}$; lane 3, $hD52_{(1–71)}$; lane 4, $mD52_{(1–79)}$; lane 5, $hD52_{(1–89)}$; lane 6, $mD52_{(1–111)}$; lane 7, $mD52_{(1–131)}$; lane 8, $mD52_{(1–151)}$; lane 9, $mD52_{(1–171)}$; size range 33–42 kDa), eluted from Ni–NTA agarose. (C) Autoradiographic exposure (5 days) of the gel shown in B indicated that hD52 was retained by all thioredoxin-6His-D52 proteins which included the coiled-coil motif (lanes 3–9), but not by the thioredoxin-6His tag alone (lane 1) or by thioredoxin-6His-$mD52_{(95–185)}$ (lane 2).

```
            cdefgabcdefgabcd
mD52    31 LTEEEQEELRRELTKV 46
                 D    D   L
mD53    31 LSEEEKEELKAELIQL 46
                         V
mD54    41 LTEGEEEELRAELAKV 56
               D  VD*
```

FIG. 3. Regions of murine D52-like coiled-coil motifs examined for sequence variations. Affected residues are shown in bold and alternative residues encoded shown in bold below each sequence. The predicted positions of residues within coiled-coil heptads are indicated in lower-case letters (*a*–*g*) above the mD52 sequence. Numbers at the beginning and end of each sequence line refer to the positions of the first and last amino acids shown, respectively. The mD52 E^{37}/D^{37} variation was predicted by 2/41 ESTs analyzed (GenBank Accession Nos. BB582412 and BB581998), and the mD52 E^{42}/D^{42} and L^{43}/V^{43} variations were predicted by 3/41 ESTs (GenBank Accession Nos. AA245630, AA623838, and AA793673). The mD53 I^{44}/V^{44} variation was predicted by 4/7 EST sequences analyzed (GenBank Accession Nos. AW412134, BE532183, BE570205, and BF164539). The mD54 E^{43}/D^{43} variation was predicted by 8/49 EST sequences analyzed (GenBank Accession Nos. W54810, W66669, W75292, W82290, AA008731, AA124904, AA255184, and AA277778), the mD54 E^{46}/V^{46} variation was predicted by 2/49 ESTs (GenBank Accession Nos. W66669 and AA124904), the mD54 E^{47}/D^{47} variation was predicted by 4/49 ESTs (GenBank Accession Nos. W75292, AA004043, AA124904, and AA103819), and the mD54 $E^{48}/*$ variation was predicted by 2/49 ESTs (GenBank Accession Nos. W66669 and AA103819).

two of seven heptad repeats of the coiled-coil motif, and as such is the shortest D52-like protein predicted to date. Sequence polymorphisms within the coiled-coil motif may therefore provide an additional mechanism to modulate D52-like protein function in rodents. Furthermore, these may also generate C-terminally truncated proteins, which in humans have been reported to be generated by alternative splicing events (4, 5).

In summary, this study has confirmed the necessity of the D52 coiled-coil motif for interactions with similar proteins and has indicated that additional C-terminal regions may facilitate and/or stabilize these binding events. However, the centrally located D52 motif does not appear to play a direct role in the binding of D52-like proteins.

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