Extension of *Drosophila melanogaster* lifespan with a GPCR peptide inhibitor

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Abstract

G protein-coupled receptors (GPCRs) mediate signaling from extracellular ligands to intracellular signal transduction proteins[1]. Methuselah (Mth) is a class B (secretin-like) GPCR, a family typified by their large, ligand-binding, N-terminal extracellular domains[2]. Down-regulation of *mth* increases the lifespan of *Drosophila melanogaster*[3]— inhibitors of Mth signaling would thus be expected to enhance longevity. We used mRNA display selection[4,5] to identify high affinity (K_D = 15 to 30 nM) peptide ligands that bind to the N-terminal ectodomain of Mth. The selected peptides are potent antagonists of Mth signaling, and structural studies suggest that they perturb the interface between the Mth ecto- and transmembrane (TM) domains. Flies constitutively expressing a Mth antagonist peptide exhibit a robust lifespan extension, suggesting that the peptides inhibit Mth signaling *in vivo*. Our work thus provides novel lifespan-extending ligands for a metazoan and a general approach for the design of modulators of this important class of GPCRs.

Because of their participation in numerous cell processes, GPCRs are the targets of approximately half of marketed drugs, and new GPCR ligands continue to be pursued and developed[6]. Naïve approaches toward GPCR ligand identification typically involve high-throughput screening of a molecular library (10^2 to 10^5 unique members) in functional, cell-based assays[6–8]. A powerful, alternative approach for the rapid isolation of novel ligands is *in vitro* peptide selection using mRNA display, which allows access to very high library complexities ($>10^{13}$) in a robust format[4]. High affinity peptide ligands for RNA, small molecule, and protein targets have been identified by mRNA display selection[5].

The crystal structure of the hexahistidine-tagged Mth ectodomain was previously determined, demonstrating that the mature, N-terminal extracellular domain of Mth is a stably folded, glycosylated protein of 195 residues[9]. Since the ectodomains of other class B GPCRs maintain recognition of their cognate ligands independently of their TM cores[10,11], we targeted the Mth ectodomain for *in vitro* selection to isolate putative modulators of Mth signaling. We expressed

and purified a specifically biotinylated construct of the Mth ectodomain[12] to avoid using the weak hexahistidine epitope as an immobilization tag for selection[13]. Hence, the Mth ectodomain was homogeneously presented, perhaps mimicking the juxtaposition of the ectodomain and TM domain in the context of the full-length receptor.

We constructed a random, 27-mer peptide mRNA display library. After eight rounds of selection, with the final four rounds including preclearing steps on matrix without target and specific elution with free, non-biotinylated Mth, we obtained a final 8th round pool that exhibited high activity for Mth and negligible non-specific binding (Fig. 1). DNA sequencing of individual clones from the final selection round revealed a highly conserved consensus, [R/P]xxWxxR, which we term the RWR motif (Table 1). This motif was not found in the recently identified Mth peptide agonist, Stunted[14], and the shortness of the consensus precluded any statistically significant homology to the *Drosophila* proteome.

We synthesized several selected peptides for binding analysis by surface plasmon resonance (SPR). Peptides containing the RWR motif demonstrated high affinity (K_D <30 nM) to the Mth ectodomain (Table 1). A scrambled version of the R8-12 synthetic peptide, as well as W7A and R10A mutants of R8-14, exhibited no measurable binding by SPR at concentrations up to 5 μM. Additionally, a fluorescently labeled analog of R8-12 was shown to bind full-length Mth expressed in cell culture (Supp. Fig. 2). Hence, despite targeting the Mth ectodomain, selected peptides recognize the full-length GPCR.

It is not clear why arginine and proline should be interchangeable at the first consensus position of the RWR motif. Proline could provide a conformational anchor, whereas arginine makes favorable electrostatic contacts that net result in two peptides with similar binding affinities to Mth. Analysis of the amino acid types at each position in the aligned peptides also reveals several trends dependent on whether arginine or proline is in the first position (Supp. Fig. 2 and Supp. Table 1).

In vitro competition binding studies suggest that the selected peptides share the same binding site. Synthetic, unlabeled peptide R8-01 (10 μM) competed with radiolabeled, full-length R8-01 and R8-04 for binding to immobilized Mth ectodomain, resulting in 96% and 94% reductions in binding, respectively, compared to the amount bound without competitor. N-Stunted, a 30-mer synthetic peptide previously shown to activate the Mth receptor[14], also competed for binding to the Mth ectodomain: at 30 μM, N-Stunted reduced binding of radiolabeled R8-01 by 79% compared to binding without competitor. These results suggest that the natural ligand binding site is an interaction "hot spot"[15] and at least partially reconstituted by the Mth ectodomain. Alternatively, allosteric competition may occur through Mth conformational changes upon ligand binding.

We determined the crystal structure of the Mth ectodomain in complex with an RWR motif peptide to identify the binding site. Electron density putatively corresponding to the R8-01 15-mer peptide (Table 1) places the binding site near the C terminus of the ectodomain (Fig. 2a). In the context of the full-length receptor, this suggests that the peptides bind at an interface between the Mth ectodomain and extracellular loops (Fig. 2b). These results contradict the hypothesis that the single exposed Trp residue in the Mth ectodomain is the binding site for the natural ligand[9]. Further fluorescence studies with Mth, R8-01, and R8-04 confirm that Trp120 is not required for peptide binding to Mth (Supp. Fig. 3).

The second extracellular loop (EL2) of Mth contains a WxxR peptide sequence (a partial RWR motif), which may interact with the ectodomain in a similar fashion as the RWR motif peptides and form a distinct surface for agonist binding (Fig. 2b). However, at concentrations of up to 150 μM, a synthetic EL2 peptide did not compete with radiolabeled R8-01. This may not preclude an *in vivo* interaction since the affinity may be enhanced in the full-length receptor

where the EL2 sequence and the ectodomain are co-localized. Additionally, a high affinity, optimized interaction may be undesirable for the modulation of natural signaling. Indeed, the bias of *in vitro* selection for high affinity ligands may favor the recovery of antagonists rather than agonists, which are evolved for function rather than binding.

The Stunted peptide was previously identified as an agonist for Mth using a cell-based, fluorescence reporter system for calcium mobilization, a common consequence of GPCR activation[14]. We used the same technique here to determine if the selected peptides antagonize Mth signaling. R8-12 and R8-14 were strong antagonists of N-Stunted–induced Mth signaling, with 50% inhibitory concentration (IC_{50}) values of 45 ± 10 and 115 ± 25 nM, respectively (Fig. 3). The W7A and R10A mutants of R8-14, as well as the R8-12 scrambled peptide, failed to antagonize Mth at concentrations up to $10 \mu M$. No signaling was induced by any of the peptides in non-transfected, control HEK cells. RWR motif peptides likely antagonize Mth by blocking interaction with agonist. However, it is also possible that the peptides desensitize the receptor (e.g., by triggering internalization) or block GPCR oligomerization, which may be required for proper signaling.

Since down-regulation of *mth* increases lifespan[3], we speculated that the *in vivo* application of a Mth antagonist would affect *Drosophila* longevity. We generated transgenic flies that constitutively express the R8-12 peptide using the UAS/GAL4 system[16]. Expression of the R8-12 peptide extended the mean and maximal adult lifespans at 29 °C by 38% and 26%, respectively (Fig. 4a). Two other independent UAS–R8-12 insertion lines showed similar lifespan increases (Supp Fig. 4a). F1 heterozygous lines, each carrying one of the two P-elements (*da*-GAL4 or UAS–R8-12), show similar lifespans to parental controls, suggesting that the longevity seen with peptide expression is not due to heterosis (Supp. Fig. 4b). Additionally, expression of a mutant R8-12 peptide, where critical residues of the RWR motif were mutated to Ala, resulted in no extension of lifespan (Fig. 4b). Male survival curves at 25 °C also show lifespan extension with R8-12 expression (Supp. Fig. 4c). These results suggest that the expressed R8-12 peptide antagonizes Mth *in vivo*. However, because the role of other Mth-like receptors in longevity is unknown, and because other Mth-like extracellular domains share significant sequence similarity to the Mth ectodomain (up to 60% identity), the action of the R8-12 peptide may be through other or multiple proteins. Studying the effects of R8-12 expression on other phenotypes observed in the *mth* mutant may reveal the relationships between aging, synaptic transmission, and fertility [3,17−19].

In vitro selection has emerged as a powerful alternative to screening approaches for the identification of modulators of G protein signaling[20,21]. Our work provides a clear strategy for designing functional ligands for class B GPCRs, a protein family associated with a number of human diseases[2]. New expression and presentation platforms for TM proteins will be needed to broaden the utility of mRNA display selection for developing novel ligands. Selections targeting full-length GPCRs expressed in cells remain an unattractive option due to the large background of cell-surface proteins. The recent successes in assembling pure GPCRs in paramagnetic proteoliposomes[22] or in a reconstituted bilayer on a biosensor surface[23,24] may be favorable alternatives for presenting a TM protein for selection. The method we describe for the identification of GPCR effectors may provide new tools applicable to aging and other fields.

Methods

mRNA display library preparation

The initial DNA pool was generated by PCR [0.1 M initial template, 5 total cycles of PCR amplification using Herculase DNA polymerase (Stratagene)] of the 142.1 template (5′-TTA AAT AGC GGA TGC ACG CAG ACC GCC ACT AGT $(SNN)_{27}$ CAT TGT AAT TGT AAA

TAG TAA TTG TCC C; N = A, C, G, or T; S = C or G) with the primers 47T7FP (5′-GGA TTC TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT AC) and 21.2 (5′-TTA AAT AGC GGA TGC ACG CAG). This library encoded a T7 promoter for transcription, a 5′-UTR sequence, and an ORF for the peptide, M-X_{27}-TSGGLRASAI. The template DNA was transcribed and purified as described previously[25]. Cross-linking of a puromycin-psoralen linker to the mRNA was performed as described using oligo 28A.1 (5′-[Ps]-UAG CGG AUG C-dA_{16}-[S9]$_2$-dCdC-[Pu]; where unlabeled bases are 2′-OMe RNA, Ps = psoralen C6, S9 = spacer phosphoramidite 9, and Pu = puromycin-CPG, Glen Research)[26].

In vitro translation with the mRNA-28A.1 library was performed in Red Nova Lysate (Novagen) as per the manufacturer's instructions with optimized conditions (100 mM KOAc, 0.5 mM MgOAc, 0.4 µM mRNA-28A.1, and ~25 µM overall L-methionine; 10 ml total reaction volume) and ^{35}S-methionine (0.5 mCi ml^{-1} final). RNA-peptide fusions were salt-treated and purified as described previously[25]. Fusions were reverse-transcribed (oligo 21.2) and desalted into Mth buffer [50 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) BSA, 1 µg ml^{-1} yeast tRNA and 0.05% Tween 20] by gel filtration (NAP-25, GE Healthcare). Based on the ^{35}S-methionine incorporated into the RNA-peptide fusions and an average of 1.3 methionine residues per peptide, the initial complexity of the library was approximately 1.5×10^{13}.

In vitro selection

RNA:cDNA-peptide fusions were incubated with ~0.1 ml of Mth-agarose at 4 °C for 1 h, then filtered and washed with 4×1 ml Mth buffer followed by 2×1 ml Mth buffer without BSA or tRNA. Bound fusions were eluted with 2×100 µl of 0.15% SDS. After removal of the SDS (SDS-OUT, Pierce), fusions were isopropanol-precipitated (50 µg ml^{-1} linear acrylamide, 1/40 volume of 3 M NaOAc at pH 5.2, and 1 volume of isopropanol). The reduced salt used for isopropanol precipitation was necessary to prevent inhibition of subsequent PCR, due to the high salt introduced by the SDS-OUT reagent. Precipitated cDNA was PCR-amplified to produce a new dsDNA pool.

Further rounds of selection were performed as described for the initial round except that *in vitro* translation reactions were smaller (~0.3 ml), less immobilized Mth was used for the selective step (~20 µl), and in rounds 5 through 8, bound fusions were eluted by competition with non-biotinylated Mth (0.5 mg ml^{-1}) in Mth buffer without BSA or tRNA. Additionally, rounds 5 through 8 included a preclearing step where the precipitated RNA:cDNA-peptide fusions were passed through columns containing NeutrAvidin-agarose and/or protein G-sepharose to remove peptides with high non-specific binding for the immobilization matrix.

Kinetics determination by surface plasmon resonance (SPR)

SPR measurements were performed at 25 °C on a BIAcore 2000 instrument equipped with research-grade SA (streptavidin) sensor chips. Biotinylated Mth was immobilized to a surface density of 450 to 700 response units (RU). HBS-EP [10 mM HEPES at pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20 (Tween 20)] was used as the running buffer for all experiments. To collect kinetics data, a concentration series of each peptide was injected for at least 60 s at a flow rate of >45 µl min^{-1}. Raw data was processed with Scrubber and globally fit with CLAMP using a 1:1 bimolecular interaction model[27]. Sensorgrams for the R8-04 peptide are shown in Supp. Fig. 5.

Cell-based GPCR signaling assay

Calcium response assays were performed with HEK 293 cells stably expressing Mth-B essentially as described[14]. Fluorescence spectra were divided by a baseline average, calculated

from the region of data prior to the addition of N-Stunted. Further details are provided in the Supplementary Methods online.

Lifespan assays

P-element transformants with the full-length R8-12 peptide transgene were generated in a w^{1118} background by standard techniques[28]. R8-12^{Ala2} is a W5A and R8A mutant of the full-length R8-12 sequence. UAS-controlled R8-12 lines (UAS–R8-12) were crossed with *da*-GAL4, which had previously been outcrossed with w^{1118}. F1 heterozygous adults (0–4 days old) were transferred to new bottles, aged for 2 days, and males were separated under CO_2 anesthesia (30 males per vial). After allowing the flies to recover overnight at 25 °C, flies (3–7 days old) were incubated in a light- and humidity-controlled environment at the indicated temperature. The UAS–R8-12 and *da*-GAL4 lines were each crossed with w^{1118} to produce F1 heterozygous animals for controls. Flies were transferred to fresh food vials [(0.45% agar, 5% dextrose, 2.5% sucrose, 8.3% corn meal, and 1.5% dried yeast, all by weight/volume) with phosphoric and propionic acids supplemented to prevent mold, as previously described29] every 3–4 days and scored for survival.

Other methods

Additional methods are provided in the Supplementary Methods online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Strader CD, Fong TM, Tota MR, Underwood D, Dixon RAF. Structure and function of G protein-coupled receptors. Annu Rev Biochem 1994;63:101–132. [PubMed: 7979235]

2. Harmar AJ. Family-B G-protein-coupled receptors. Genome Biol 2001;2:REVIEWS3013. [PubMed: 11790261]

3. Lin YJ, Seroude L, Benzer S. Extended life-span and stress resistance in the *Drosophila* mutant *methuselah*. Science 1998;282:943–946. [PubMed: 9794765]

4. Roberts RW, Szostak JW. RNA-peptide fusions for the *in vitro* selection of peptides and proteins. Proc Natl Acad Sci USA 1997;94:12297–12302. [PubMed: 9356443]

5. Takahashi TT, Austin RJ, Roberts RW. mRNA display: ligand discovery, interaction analysis and beyond. Trends Biochem Sci 2003;28:159–165. [PubMed: 12633996]

6. Howard AD, et al. Orphan G-protein-coupled receptors and natural ligand discovery. Trends Pharmacol Sci 2001;22:132–140. [PubMed: 11239576]

7. Milligan G. Strategies to identify ligands for orphan G-protein-coupled receptors. Biochem Soc Trans 2002;30:789–793. [PubMed: 12196198]

8. Szekeres PG. Functional assays for identifying ligands at orphan G protein-coupled receptors. Receptor Channel 2002;8:297–308.

9. West AP Jr, Llamas LL, Snow PM, Benzer S, Bjorkman PJ. Crystal structure of the ectodomain of Methuselah, a *Drosophila* G protein-coupled receptor associated with extended lifespan. Proc Natl Acad Sci USA 2001;98:3744–3749. [PubMed: 11274391]

10. Grauschopf U, et al. The N-terminal fragment of human parathyroid hormone receptor 1 constitutes a hormone binding domain and reveals a distinct disulfide pattern. Biochemistry 2000;39:8878–8887. [PubMed: 10913300]

11. Wilmen A, Göke B, Göke R. The isolated N-terminal extracellular domain of the glucagon-like peptide-1 (GLP)-1 receptor has intrinsic binding activity. FEBS Lett 1996;398:43–47. [PubMed: 8946950]

12. Schatz PJ. Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. Bio-Technol 1993;11:1138–1143.

13. Ja WW, Olsen BN, Roberts RW. Epitope mapping using mRNA display and a unidirectional nested deletion library. Protein Eng Des Sel 2005;18:309–319. [PubMed: 15980016]

14. Cvejic S, Zhu Z, Felice SJ, Berman Y, Huang XY. The endogenous ligand Stunted of the GPCR Methuselah extends lifespan in *Drosophila*. Nat Cell Biol 2004;6:540–546. [PubMed: 15133470]

15. Clackson T, Wells JA. A hot spot of binding energy in a hormone-receptor interface. Science 1995;267:383–386. [PubMed: 7529940]

16. Phelps CB, Brand AH. Ectopic gene expression in *Drosophila* using GAL4 system. Methods 1998;14:367–379. [PubMed: 9608508]

17. Baldal EA, Baktawar W, Brakefield PM, Zwaan BJ. Methuselah life history in a variety of conditions, implications for the use of mutants in longevity research. Exp Gerontol 2006;41:1126–1135. [PubMed: 17064868]

18. Mockett RJ, Sohal RS. Temperature-dependent trade-offs between longevity and fertility in the Drosophila mutant, methuselah. Exp Gerontol 2006;41:566–573. [PubMed: 16677788]

19. Song W, et al. Presynaptic regulation of neurotransmission in *Drosophila* by the G protein-coupled receptor Methuselah. Neuron 2002;36:105–119. [PubMed: 12367510]

20. Ja WW, Roberts RW. G-protein-directed ligand discovery with peptide combinatorial libraries. Trends Biochem Sci 2005;30:318–324. [PubMed: 15950876]

21. Ja WW, Wiser O, Austin RJ, Jan LY, Roberts RW. Turning G proteins on and off using peptide ligands. ACS Chem Biol 2006;1:570–574. [PubMed: 17168552]

22. Mirzabekov T, Kontos H, Farzan M, Marasco W, Sodroski J. Paramagnetic proteoliposomes containing a pure, native, and oriented seven-transmembrane segment protein, CCR5. Nat Biotechnol 2000;18:649–654. [PubMed: 10835604]

23. Bieri C, Ernst OP, Heyse S, Hofmann KP, Vogel H. Micropatterned immobilization of a G protein-coupled receptor and direct detection of G protein activation. Nat Biotechnol 1999;17:1105–1108. [PubMed: 10545918]

24. Stenlund P, Babcock GJ, Sodroski J, Myszka DG. Capture and reconstitution of G protein-coupled receptors on a biosensor surface. Anal Biochem 2003;316:243–250. [PubMed: 12711346]

25. Ja WW, Roberts RW. *In vitro* selection of state-specific peptide modulators of G protein signaling using mRNA display. Biochemistry 2004;43:9265–9275. [PubMed: 15248784]

26. Kurz M, Gu K, Lohse PA. Psoralen photo-crosslinked mRNA-puromycin conjugates: a novel template for the rapid and facile preparation of mRNA-protein fusions. Nucleic Acids Res 2000;28:e83. [PubMed: 10982894]

27. Myszka DG, Morton TA. CLAMP©: a biosensor kinetic data analysis program. Trends Biochem Sci 1998;23:149–150. [PubMed: 9584619]

28. Rubin GM, Spradling AC. Genetic transformation of *Drosophila* with transposable element vectors. Science 1982;218:348–353. [PubMed: 6289436]

29. Lewis EB. A new standard food medium. Drosophila Inf Serv 1960;34:117–118.

30. Palczewski K, et al. Crystal structure of rhodopsin: a G protein-coupled receptor. Science 2000;289:739–745. [PubMed: 10926528]
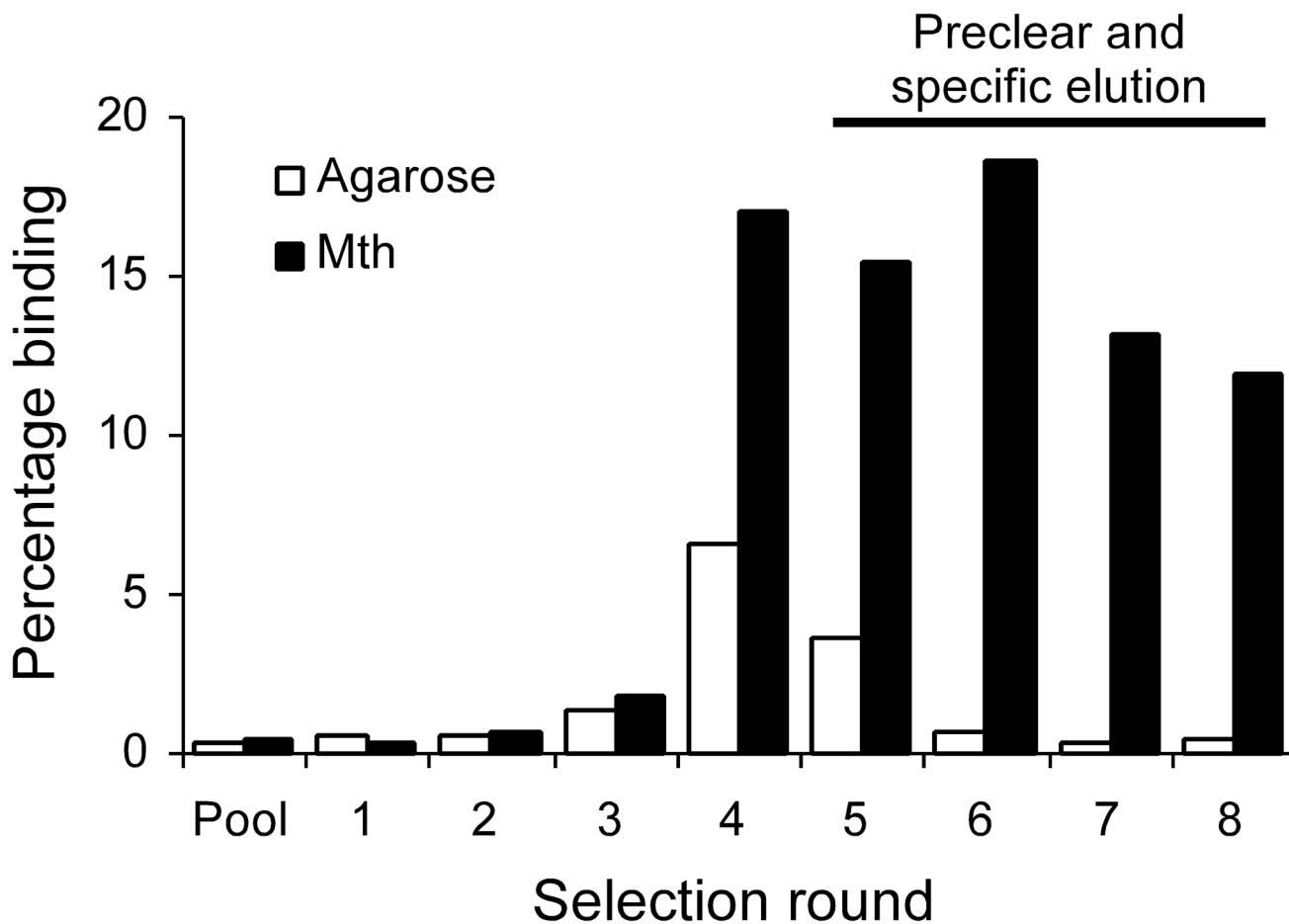
Fig. 1.
Selection of a 27-mer peptide library against the Mth ectodomain. RNase-treated, ^{35}S-methionine–labeled mRNA displayed peptides from each round of selection were assayed for binding to immobilized Mth (black) or to matrix alone (white). Preclearing and competitive elutions were performed in the 5th through 8th rounds to eliminate non-specific binding peptides.
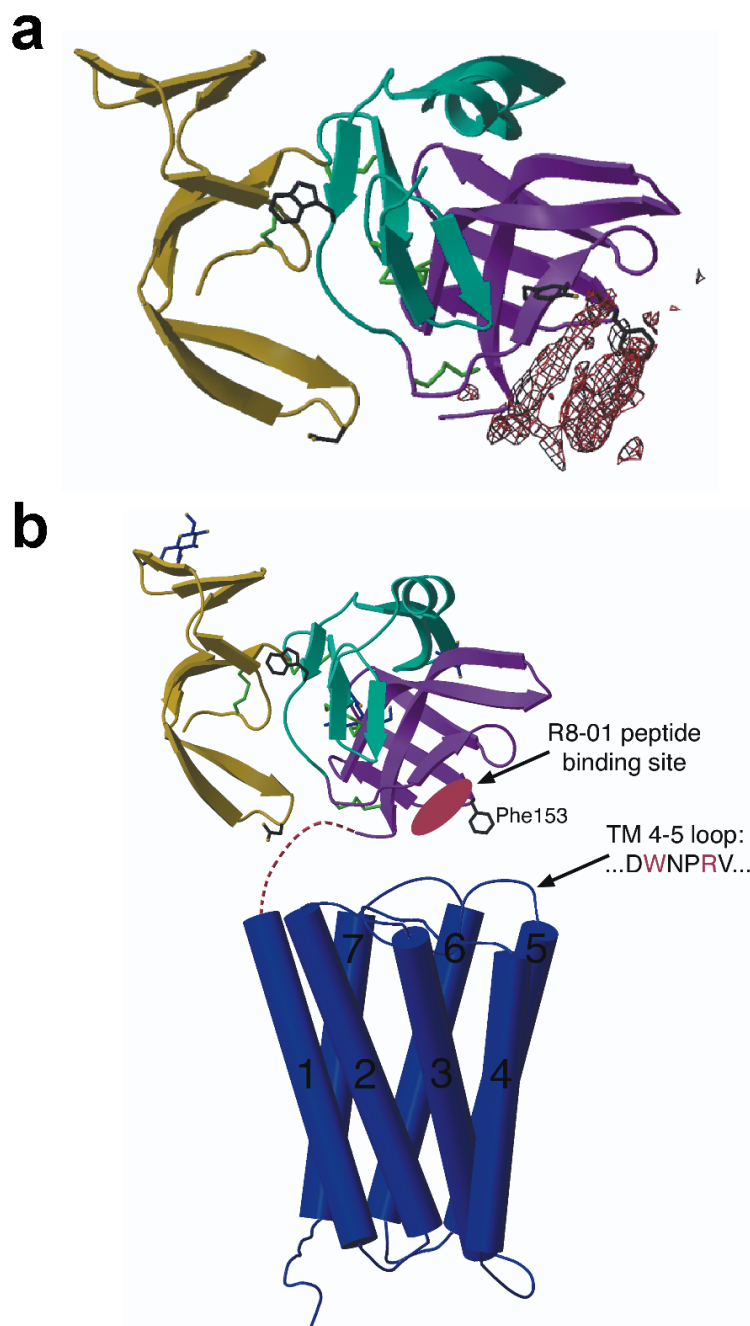
Fig. 2.
Structure of the Mth ectodomain in complex with the R8-01 15-mer peptide. (**a**) Electron density reveals the putative peptide binding site on the Mth ectodomain (shown as a ribbon diagram) from an averaged 3.5 Å $F_O - F_C$ map contoured at 9 σ. Trp120 (a previously proposed natural ligand binding site9), Tyr130 (at the R8-01 binding site), and Asp46 and Phe153 (suggested to interact with the extracellular face of the TM domain9) are shown as stick models. (**b**) Scaled model depicting the full-length structure of Mth (adapted from 9). The TM domain is depicted by the structure of rhodopsin30 as a representative GPCR.
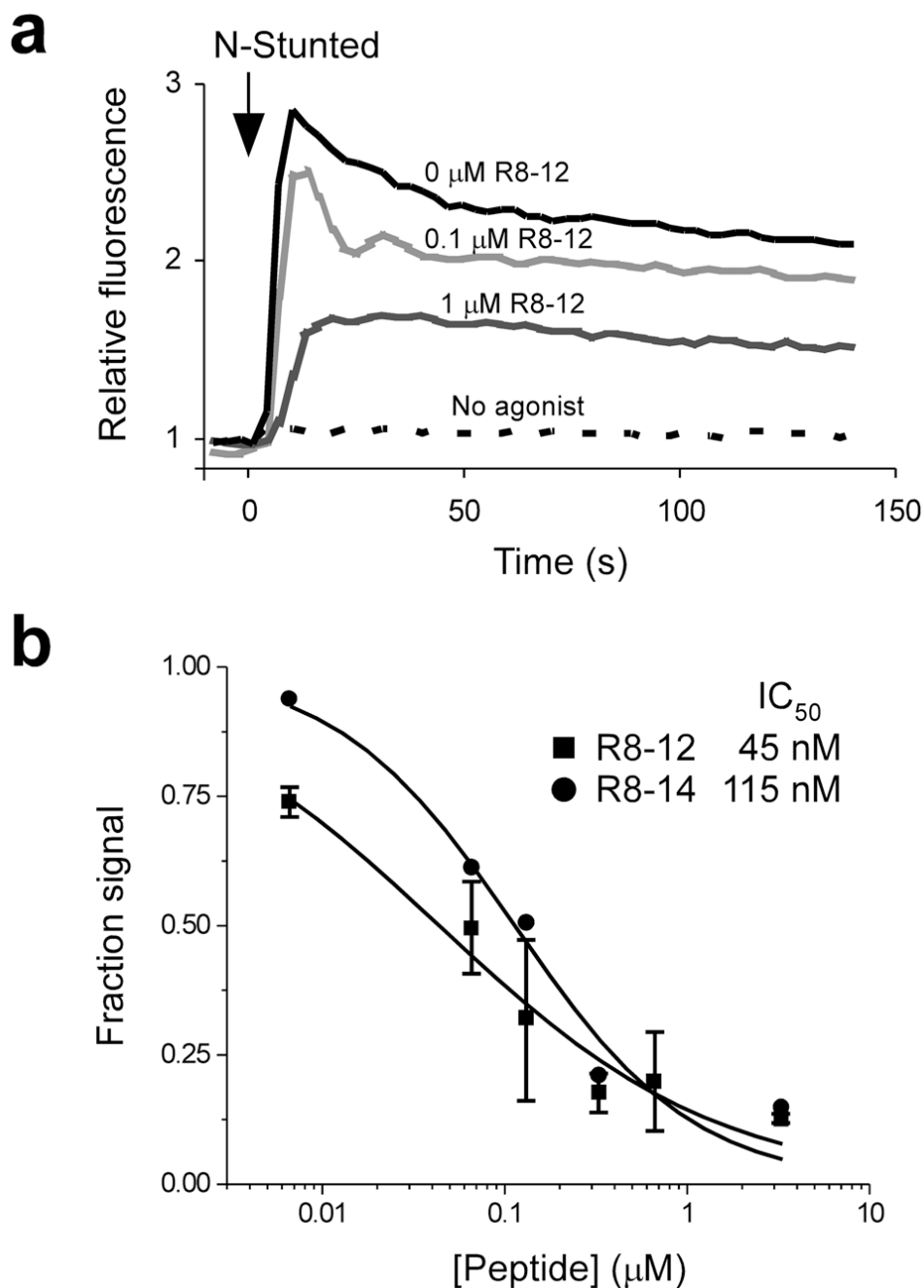
Fig. 3.
RWR motif peptides are antagonists of Mth signaling. (**a**) Mth activation by Stunted results in intracellular calcium mobilization and enhanced fluorescence. The N-Stunted agonist peptide (20 μM final) was added to HEK-Mth cells pre-incubated with and without the indicated concentration of R8-12 peptide. The dashed line indicates a control where only buffer (without Stunted agonist) was added. (**b**) Concentration dependence of the inhibition of Mth signaling by the R8-12 and R8-14 peptides. The fluorescence values at a time point ~13 sec after the addition of N-Stunted agonist (10 μM final) are expressed as a fraction of the fluorescence observed in the absence of antagonists (± s.d. when more than one trial was performed).
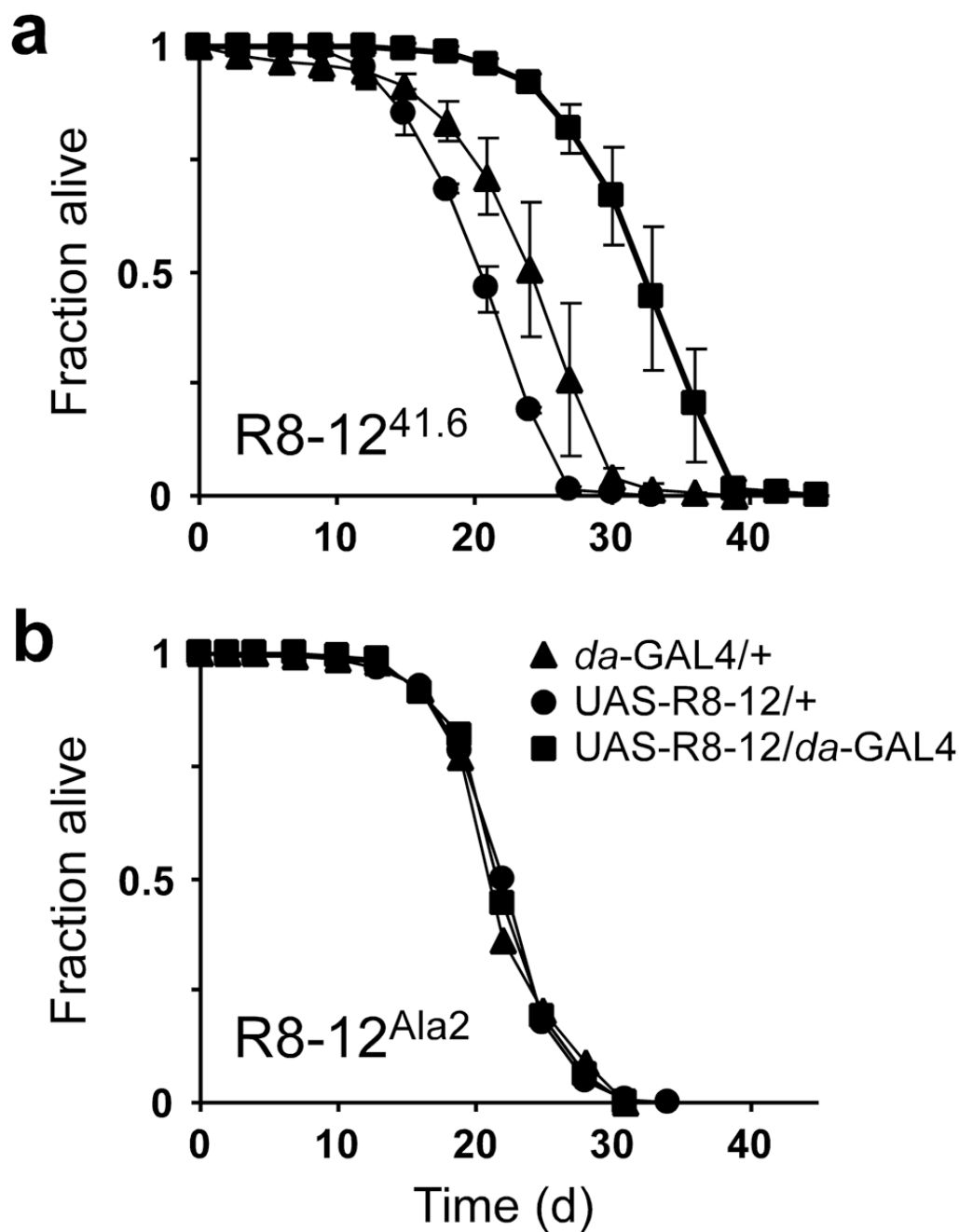
Fig. 4.
Expression of the Mth antagonist R8-12 peptide extends fly lifespan. (**a**) Male lifespan for the UAS–R8-12 transgene insertion line (41.6). (▲) Heterozygous control for the *daughterless*-GAL4 driver (*da*-GAL4/+). (●) Heterozygous control for the UAS–R8-12 peptide construct in the absence of the driver (UAS–R8-12/+). (■) The combination in which the *da*-GAL4 driver, by activating UAS promoter elements, drives expression of the R8-12 peptide (UAS–R8-12/*da*-GAL4). Data are shown ± s.d. (**b**) Male lifespan for a mutant UAS–R8-12 transgene (Ala2). Flies were maintained at 29 °C, and approximately 90 to 120 flies were used for each trial.

inal constant region (TSGGLRASAI), which was frameshifted or
netic peptides are underlined (two peptides, a 22- and a 15-mer, were
s obtained from surface plasmon resonance experiments.

Sequence	k_a $M^{-1}s^{-1}$ $(\times 10^5)$	k_d s^{-1} $(\times 10^{-2})$	K_D[b] nM	χ^2
M L V I W R S R H F G P R L R M A L L G S D R K M W	4.1	0.72	18	1.3
P A V I W R A I Q A M F L R L A S R Q E S K A F N S A	7.0	1.2	18	1.7
M Y V Y W R Y T K H R S L R S A C A R G S S A				
Y P I V W R V M N L A Q L A C A				
F S S L W R S L Y L Y A R V D E A T L R	6.3	1.9	31	1.5
Y D T L W R L A Y S L S R K S A Q G K S V	9.5	5.4	57	0.46
L Y Q W R Y T I S R T T R A L V				
R S T S W G T P S W V T P A R T G				
G M N G P R N G I M S I R L M I T	6.1	19.9	326	0.46
S S W P S R P A R A T P T				