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Total Chemical Synthesis and Folding of All-L and All-D Variants of Oncogenic KRas(G12V)

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ABSTRACT: The Ras proteins are essential GTPases involved in the regulation of cell proliferation and survival. Mutated oncogenic forms of Ras alter effector binding and innate GTPase activity, leading to deregulation of downstream signal transduction. Mutated forms of Ras are involved in approximately 30% of human cancers. Despite decades of effort to develop direct Ras inhibitors, Ras has long been considered 'undruggable' due to its high affinity for GTP and its lack of hydrophobic binding pockets. Herein, we report a total chemical synthesis of all L- and all D-amino acid biotinylated variants of oncogenic mutant KRas(G12V). The protein is synthesized using Fmoc-based solid-phase peptide synthesis and assembled using combined native chemical ligation and isonitrile-mediated activation strategies. We demonstrate that both KRas(G12V) enantiomers can successfully fold and bind nucleotide substrates and binding partners with observable enantiodiscrimination. By demonstrating the functional competency of a mirror-image form of KRas bound to its corresponding enantiomeric nucleotide triphosphate, this study sets the stage for further biochemical studies with this material. In particular, this protein will enable mirror-image yeast surface display experiments to identify all-D peptide ligands for oncogenic KRas, providing a useful tool in the search for new therapeutics against this challenging disease target.

INTRODUCTION

The Ras family of GTPase proteins plays a crucial role in cellular signal transduction, acting as a molecular switch to propagate extracellular signals to intracellular phosphorylation cascades that drive cellular growth and survival mechanisms.[1] Ras activity is controlled by a bound guanine nucleotide cofactor: GTP-bound ("on") Ras adopts a conformation that binds to Ras effector proteins, such as B-Raf and PI3 kinase, with high affinity and leads to downstream pathway activation, whereas GDP-bound ("off") Ras adopts an inactive conformation that does not bind to effectors. The Ras nucleotide state is primarily regulated by Guanine Exchange Factors (GEFs), which favor GTP loading, and GTPase activating proteins (GAPs), which catalyze hydrolysis of GTP to GDP.[2] Single point mutations at key residues in the primary sequence of Ras are capable of disrupting GTP hydrolysis by blocking interactions with GAPs and by reducing the intrinsic GTPase activity of Ras, thereby trapping Ras in the GTP-bound "on" state and leading to constitutive pro-growth signaling.[3,4] Such inappropriately activated Ras mutants are found in approximately 30% of human cancers, with the KRas isoform representing an overwhelming majority (>80%) of these cancer types.[5] Direct inhibition of mutant oncogenic Ras represents a "holy grail" in the development of cancer therapeutics; one which has been met with tremendous difficulty since the initial correlation between the RAS oncogene and human cancers was described in the early 1980's.[6]

A number of approaches aimed at modulating oncogenic Ras signaling via small-molecule inhibitors have been only partly successful, and no such molecules have yet reached the clinic.[7] Inhibition of the nucleotide binding site of Ras has proven largely futile due to the unusually

high affinity of Ras for its GDP and GTP substrates and the high concentrations of these molecules in the cell. Moreover, inhibition of Ras-effector interactions or Ras-GEF interactions with small molecules is challenging due to difficulties associated with perturbing pertinent protein-protein interactions (PPIs), and the limited successes reported thus far have been hampered by moderate potency.[8-10] Attempts to chemically perturb Ras post-translational processing and localization have failed thus far in the clinical setting due to the existence of promiscuous and alternative lipidation pathways.[11] Recent efforts aimed at the selective inhibition of KRas(G12C) via covalent-binding inhibitors have shown considerable promise.[12] However, these approaches target only a small population of Ras-driven cancers (albeit a relatively high proportion of lung cancers) and do not represent a general solution toward inhibiting constitutive Ras activity.

Peptide-derived and peptidomimetic inhibitors have great potential in targeting PPIs.[13,14] Of note, α-helical stabilized peptides, including hydrocarbon stapled peptides, represent a promising class of cell-penetrating modulators of PPIs with biological stability and have been successfully applied in targeting Ras.[14a-b,15,16] Recently, one of our labs has developed a class of miniproteins that bind to the Ras effector domain *in vitro* with high affinity and inhibit Ras-effector interactions.[17] These miniproteins were discovered by screening unbiased combinatorial libraries using yeast surface display (YSD), demonstrating that it is possible to identify high-affinity Ras ligands from naïve libraries.[18] With these results in mind, we wondered if mirror-image YSD could be used to identify high-affinity Ras binders composed of all D-amino acids. Unnatural, D-residue peptides have been shown to possess improved stability and bioavailability over their L-residue peptide counterparts, and mirror-image display has successfully afforded useful peptide ligands in the context of various disease types, including HIV/AIDS, Alzheimer's Disease, and cancer.[19,20] Additionally, synthetic all-D proteins have other biochemical research value, such as racemic protein crystallography.[21] Critical to such studies is the total chemical synthesis of an all D-residue version of the naturally occurring all-L residue protein. In this report, we describe the chemical synthesis and folding of all-L residue and all-D residue versions of the oncogenic mutant KRas(G12V)[1-166] (**1**), which bear a biotin affinity tag for use in YSD and other biochemical experiments. We conducted a series of *in vitro* assays to demonstrate the biochemical viability of each protein enantiomer, thus establishing this mirror-image protein pair as a potentially valuable tool for the future discovery of anti-Ras therapeutics.

RESULTS AND DISCUSSION

The primary sequence of KRas(G12V), a 166-residue protein, is shown in **Figure 1** with highlighted points of assembly. The synthesis of HRas has been previously described utilizing Boc-based solid phase peptide synthesis (SPPS) combined with native chemical ligation (NCL) assembly as well as via a semisynthesis approach.[22] Our synthetic approach toward KRas(G12V) relies on the use of Fmoc-based SPPS to generate five peptidyl sequences, which are subsequently assembled via combined NCL and isonitrile-mediated activation strategies to access a biotinylated variant of KRas(G12V).[23, 24] Peptide thioesters were synthesized via Sakakibara elongation of protected peptides with pre-formed C-terminal residue thioesters following SPPS or alternatively by C-terminal hydrazide oxidation.[25,26] In a retrosynthetic fashion, taking primary advantage of the native cysteine residues within the full sequence reduces the synthetic challenge to three NCLs at assembly points Cys51, Cys80, and Cys118. However, in our hands, preliminary attempts to synthesize KRas[118-166] in its entirety by Fmoc-based SPPS proved inefficient and resulted in poor recovery of the desired peptide. Therefore, this 49-mer sequence was further dissected to enable a more efficient preparation. In the absence of available cysteine residues to permit NCL, two alternative assembly points were hypothesized. Utilizing an established strategy, we envisioned a potential NCL-desulfurization approach at Ala146, requiring the installation, ligation, and subsequent desulfurization of a non-native Cys146.[27] Alternatively, the assembly of large peptides via the chemoselective activation of C-terminal thiocarboxylic acids using a hindered isonitrile represents an attractive complementary strategy that is independent of cysteine.[24,28-30] We have recently reported the use of this method to generate challenging hydrophobic peptidyl sequences greater than 100 amino acids in length.[30] Here, we hypothesized that a C-terminal thioacid at Gly138 could serve as a potential disconnection site to eradicate concern for epimerization during activation. However, this instance would require us to demonstrate the potential of a hindered β-branched nucleophile at Ile139 with a combined strategy to orthogonally protect and deprotect side-chain nucleophiles at Lys128, Lys147, and Lys165. This target sequence and associated challenges present a worthwhile opportunity to expand the utility of this promising strategy.

Our synthesis initially focused on completion of the protein using all-L amino acids, and our optimized synthetic route was repeated using all D-residues. Yields are provided in the schemes for both of these syntheses, distinguished by the color-coding. We began our studies with the challenging C-terminal sequence, KRas[118-166], assembled using independent ligation strategies compared in a side-by-side fashion in **Scheme 1A**. In each series, a PEGylated biotin label (referred herein as PEG2-Biotin) was appended at the C-terminus for projected biochemical studies. For the isonitrile-mediated activation approach, we chose to orthogonally mask the ε-amines of lysine residues with allyloxycarbonyl (Alloc) groups.

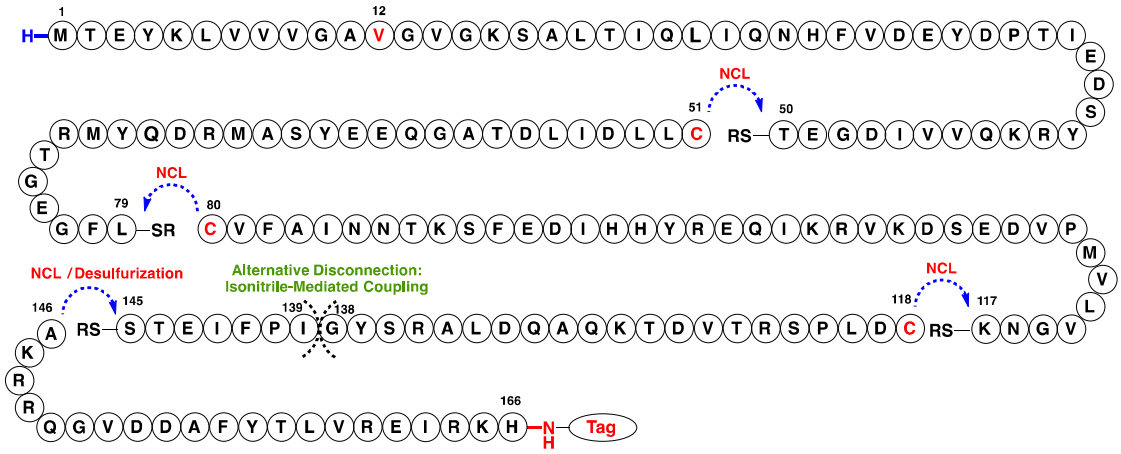
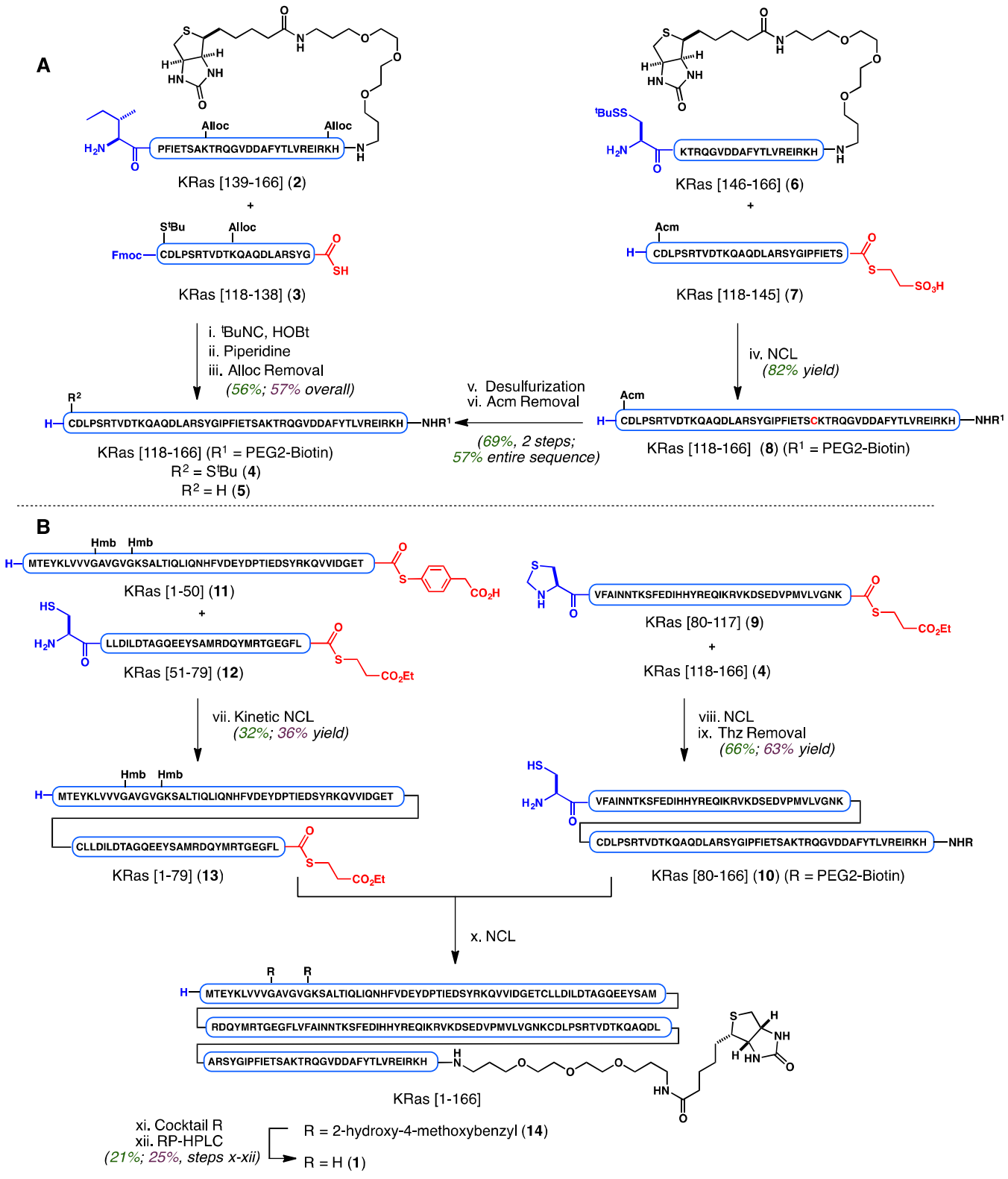


Figure 1: Retrosynthetic disconnection sites for KRas(G12V)[1-166]



Scheme 1: (A) Alternate approaches for the synthesis of biotinylated KRas[118-166]. i) tBuNC (3.2 equiv), HOBt (10.2 equiv), DMA, room temp, 48 hours. ii) Piperidine (20 vol%), 30 min room temp. iii) $PdCl_2$(dppf) (50 mol%), $PhSiH_3$ (50 equiv.), DMF, room temp. iv) 6M Gnd HCl, 0.2 M Na_2HPO_4, 0.2 M 4-mercaptophenylacetic acid, 40 mM TCEP HCl, pH = 7.0, 6 hr, room temp. v) VA-044, tBuSH, TCEP, Gnd HCl buffer, 4 hours, room temp. vi) AgOAc (100 equiv.), 70% aqueous AcOH, 2.5 hr, room temp. (B) Completion of the synthesis of KRas[1-166]. vii) 6M Gnd HCl, 0.2 M Na_2HPO_4, pH = 7.0, 6 hr, room temp. viii) 6M Gnd HCl, 0.2 M Na_2HPO_4, 0.2 M 4-mercaptophenylacetic acid, 40 mM TCEP HCl, pH = 7.0, 4 hours, room temp. ix) NH_2OMe HCl added, pH = 4.5-5.0, 4.5 hours, room temp. x) 6M Gnd HCl, 0.2 M Na_2HPO_4, 0.2 M 4-mercaptophenylacetic acid, 20 mM TCEP HCl, pH = 7.0, 6.5 hours, room temp. xi) TFA/thioanisole/ethanedithiol/anisole

(90:5:3:2), 2 hours, room temp. xii) solubilization in 1:1 MeCN/H_2O (0.1% TFA) with sonication, then TCEP, pH = 8.0, and RP-HPLC purification. Yields are reported in the following manner (Green = all L-residue peptides; Purple = all D-residue peptides)

Under previously described conditions, the activation of C-terminal thioacid **3** with *tert*-butyl isocyanide in the presence of HOBt and N-terminal nucleophilic partner **2** afforded the desired Alloc-protected product.[30] Moreover, concentrations comparable to NCL conditions (in this case, 4-5 mM) provided nearly full conversion within 48 hours. Further experimentation found that the N-terminal Fmoc could be removed in the same pot by the subsequent addition of piperidine to the crude reaction mixture, allowing for a clean, two-step, one-pot ligation procedure.[31] The intermediate product, KRas[118-166], was isolated in crude form following precipitation with diethyl ether and was directly subjected to reductive Alloc deprotection. Brief exposure of the precipitate to $PdCl_2$(dppf) and $PhSiH_3$ in DMF afforded **4** following purification by HPLC.[32]

In a direct comparison of strategies, the projected NCL between **6** and **7** proceeded in high yield as anticipated (82% isolated), with the N-terminal cysteine of **7** protected as S-acetamidomethyl (S-Acm) to impart selectivity in the NCL and the subsequent desulfurization step.[27b] The resultant ligation product (**8**) was subjected to desulfurization and Acm deprotection to afford KRas[118-166] (**5**) in 69% isolated yield over two steps following HPLC purification. Overall, the three-step conversion of **6** and **7** to common ligation product KRas[118-166] proceeded in 57% yield. The comparable yields and ease of reaction set-up in each study suggests that in certain cases, isonitrile-mediated activation of C-terminal Gly thioacids can also provide facile access and should be considered as a complementary strategy to NCL-desulfurization, especially for hydrophobic sequences lacking available or median cysteine residues. Moreover, although both of these highlighted approaches require multi-step assembly and deprotection, we contend that the isonitrile-mediated activation approach may be preferable due to intermediary purification ease—requiring only precipitation upon addition of diethyl ether with no desalting or HPLC necessary to isolate intermediate peptides—in cases where the overall yields are similar.

Having validated the synthetic practicality of subtarget KRas[118-166] (**4**), we turned our attention to the overarching goal of completing a total synthesis of the entire oncogenic KRas sequence (**Scheme 1B**). We further extended **4** via NCL with thioester, KRas[80-117] (**9**), followed by direct addition of methoxylamine hydrochloride to remove the N-terminal thiazolidine (Thz), furnishing KRas[80-166] (**10**). Next focusing on the N-terminal portion of the protein, we found synthesis and handling of KRas[1-50] (**11**) to be challenging due to its hydrophobic nature. Solubility issues and synthesis efficiency could be drastically improved through incorporation of acid-labile 2-hydroxy-4-methoxybenzyl (Hmb) groups within the amide backbone of **11**.[33] Presumably, the incorporation of tertiary amides at Gly10 and Gly15 serves to beneficially disrupt secondary structural elements of this hydrophobic sequence, thereby augmenting general solubility to facilitate handling and purification. The Hmb group also holds an advantage in that it can be rendered temporarily acid-stable upon acylation (for synthesis of this peptide, see Supporting Information). Becker and co-workers also note similar challenges associated with hydrophobicity in the synthesis of wild-type HRas.[22a,34] In our hands, **11** could be subjected to kinetic chemical ligation with **12** to selectively afford alkyl thioester, KRas(G12V)[1-79] **13**.[35] Finally, NCL between **13** and **10** was found to be efficient, with near complete consumption of limiting reagent **13** after 6-8 h at neutral pH. The corresponding Hmb-protected sequence, KRas[1-166] (**14**), could be conveniently isolated via its direct precipitation from the ligation buffer upon the addition of cold water followed by centrifugation of the resultant precipitate. Here, solubility differences of full sequence **14** were exploited. Excess **10** remained largely in the supernatant with minimal solubilization of target **14** (**Figure 2**). The subsequent exposure of this precipitated material to acidic deprotection conditions (TFA/thioanisole/1,2-ethanedithiol/anisole) afforded **1** following purification by HPLC, with excellent agreement by high-resolution mass analysis (**Figure 3**).

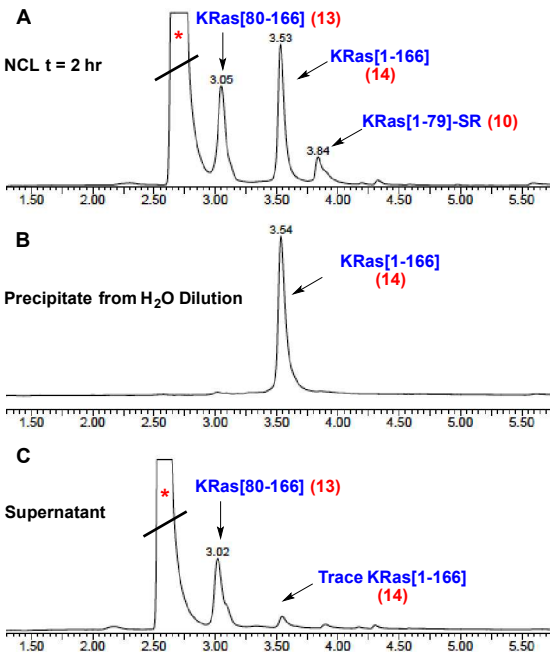


Figure 2. UPLC traces of the NCL between **10** and **13** and isolation of **14**. (A) NCL after 2 hours; (B) Only **14** is obtained in the precipitate from H_2O dilution after the NCL is complete (t = 6.5 hours); (C) Only trace **14** remained in the supernatant after dilution of the NCL with H_2O, while excess **13** remains entirely dissolved. *denotes absorption peak derived from MPAA.

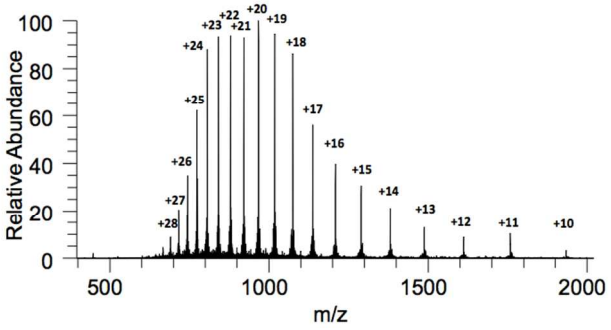
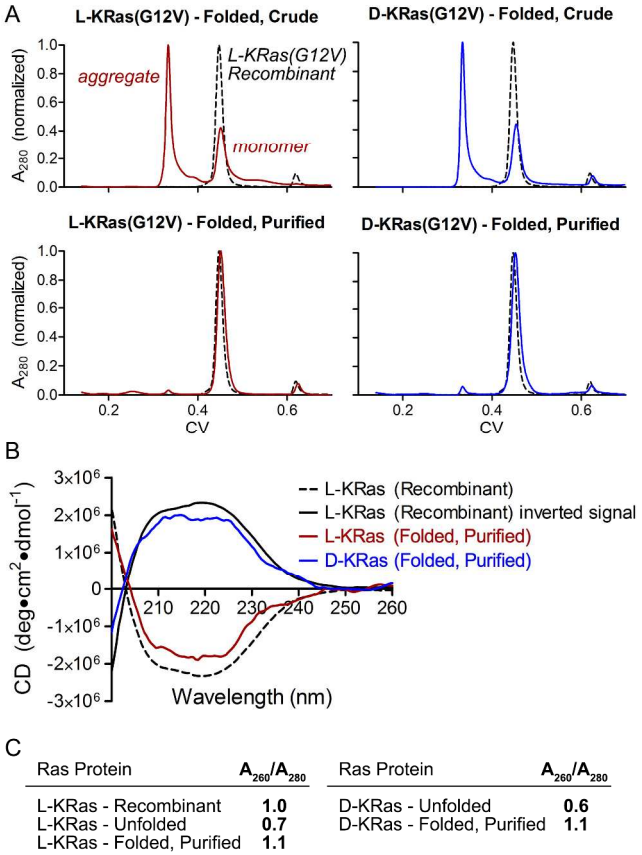


Figure 3. High-resolution mass spectrum of biotinylated, all D-residue KRas(G12V) (**1**).

With a reproducible synthetic route established, we turned our attention to establishing biochemical competency of the synthetic protein pair. We adopted a previously reported folding protocol, which entailed solubilization by guanidine hydrochloride and rapid folding into denaturant-free buffer.[22a] After optimization, we found both enantiomers of synthetic KRas to consistently display moderate folding efficiency based on gel filtration analysis (**Figure 4A**), using either D-GppNHp (for the all L-residue protein) or L-GppNHp (for the all D-residue protein) as folding templates.[36,37] This folding procedure was conducted on a 100 μg (5 nmol) scale and afforded approximately 30-40 μg of monomeric, nucleotide-bound protein per preparation. This is sufficient material to conduct a yeast or phage display selection at 5 μM protein in a 250 μL binding volume, which is a typical scale performed in our laboratory. In each case, further purification by size-exclusion chromatography afforded a monomeric species with a retention time equivalent to recombinant KRas(G12V), as assessed by analytical gel filtration. Moreover, circular dichroism analysis of this pair of folded proteins showed good agreement with that of recombinant KRas(G12V), with the all D-residue protein exhibiting an opposite sign compared to the all L-residue proteins. (**Figure 4B**). Comparison of A_{260}/A_{280} values showed an increase from 0.6-0.7 for the unfolded proteins to 1.0-1.1 for the recombinant and folded proteins, consistent with association with guanine nucleotides, which possess an absorption maximum at 252 nm (**Figure 4C**).



C

Ras Protein	A_{260}/A_{280}	Ras Protein	A_{260}/A_{280}
L-KRas - Recombinant	**1.0**	D-KRas - Unfolded	**0.6**
L-KRas - Unfolded	**0.7**	D-KRas - Folded, Purified	**1.1**
L-KRas - Folded, Purified	**1.1**		

Figure 4: Folding analysis of synthetic proteins. A) Crude gel filtration traces and purified synthetic all L- and all D-KRas(G12V) after folding with GppNHp; B) Circular dichroism analysis of folded and purified synthetic proteins and recombinant KRas(G12V); C) Comparison of A_{260}/A_{280} values for synthetic proteins and recombinant KRas(G12V).

To further characterize the synthetic proteins, we folded both in the presence of fluorescent mant-GppNHp ((2'/3')-*O*-(*N*-methylanthraniloyl)guanosine-5'-*O*-[(β,γ)-imidotriphosphate]) nucleotide analogs, which exhibit an increase in fluorescence upon binding to Ras and have been used to study Ras-ligand binding interactions.[38] The enantiomeric pair of proteins folded with mant-GppNHp nucleotides of corresponding stereochemistry exhibited comparable nucleotide dissociation behavior to recombinant KRas, marked by a decrease in fluorescence upon the addition of excess unlabeled GppNHp of correlated stereochemistry. In contrast, addition of the incorrect antipode of nucleotide showed no significant nucleotide exchange for either material.[39] In the case of synthetic L-KRas, the addition of the Ras-binding domain (RBD) of B-Raf, a canonical Ras effector known to inhibit nucleotide dissociation, slowed dissociation in a similar manner to that observed with recombinant KRas.[38] As expected, the addition of the B-Raf RBD had no observed effect on nucleotide dissociation from all D-residue KRas. Similarly, addition of the Ras-binding miniprotein 225-44 (all L-amino acid residues) slowed nucleotide dissociation for recombinant and synthetic L-KRas, with no effect observed on all D-KRas.[17] Conversely, as anticipated, addition of an all D-residue variant of miniprotein 225-44 does

indeed slow nucleotide dissociation for all-D KRas but had no effect on recombinant or synthetic L-KRas (**Figure 5**).

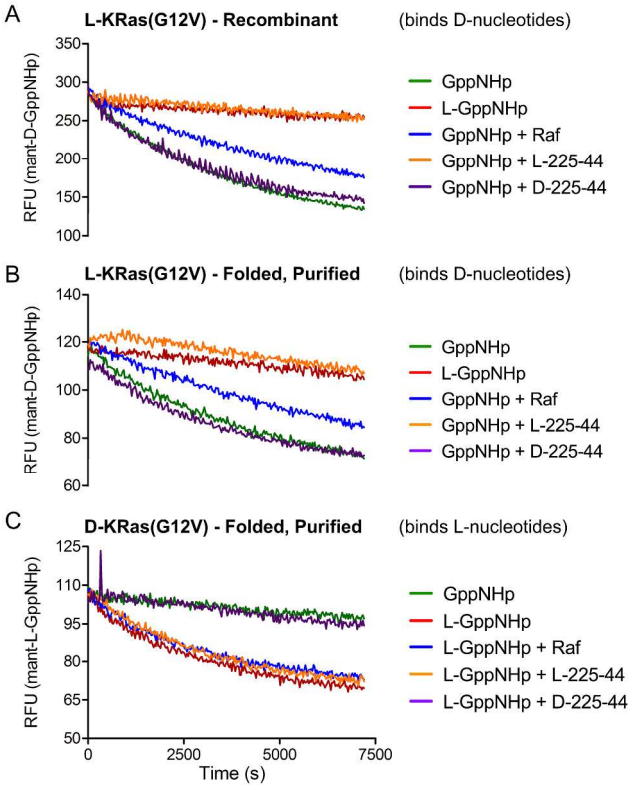


Figure 5: Unlabeled nucleotide displacement of mant-GppNHp-loaded KRas(G12V) in the presence of Ras-binding miniproteins. A) Recombinant L-KRas(G12V); B) Synthetic L-KRas(G12V); C) Synthetic D-KRas(G12V).

Finally, we performed surface plasmon resonance (SPR) binding assays to confirm direct binding of synthetic, folded KRas to Ras-binding miniproteins (**Figure 6**). We found that the 225-44 miniprotein exhibited high nonspecific binding to the SPR sensor chip, so we prepared all-L and all-D variants of the 225-11 miniprotein, which has been previously co-crystallized with KRas(G12V) and shown by SPR to bind with low-nanomolar affinity.[17] Recombinant and synthetic all-L KRas bound to all-L 225-11 with K_d values of 6 nM and 7 nM, respectively, comparable to the reported value of 3.6 nM, but did not bind to all-D 225-11. Conversely, synthetic all-D KRas bound to all-D 225-11 with a K_d of 2 nM but did not bind to all-L 225-11. A small amount of non-specific binding of the all-D 225-11 miniprotein to L-KRas was observed in this SPR assay, as were slight differences in the sensogram curvature. These discrepancies may potentially result from the presence of some protein aggregate and/or microheterogeneity within the synthetic materials, or as a consequence of the microscale nature of the refolding procedure. Regardless, selective low-nanomolar binding to all-D KRas is clearly observed. These combined biophysical experiments demonstrate that folding, nucleotide association, and miniprotein-binding profiles of both mirror-images of synthetic KRas(G12V) resemble that of recombinant KRas(G12V) and exhibit the expected enantiodiscrimination.[40, 41]

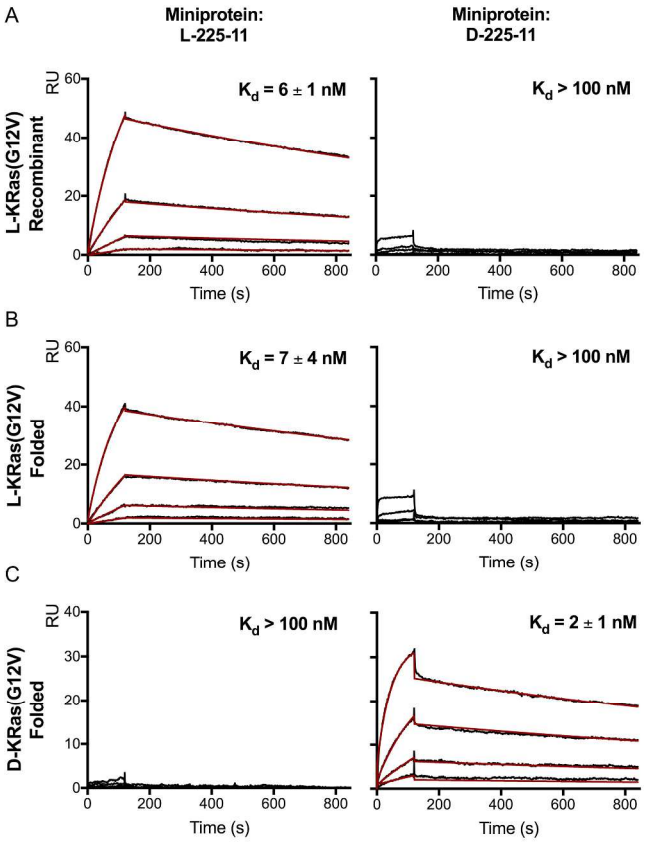


Figure 6: Surface plasmon resonance (SPR) binding analysis of Ras proteins with Ras-binding miniproteins. A) Binding of miniproteins with recombinant KRas; B) Binding of miniproteins with synthetic all-L KRas; C) Binding of miniproteins with synthetic all-D KRas. K_d values are mean ± SEM for triplicate runs.

CONCLUSION

In conclusion, we have described the total chemical synthesis and folding of both mirror-image forms of biotinylated oncogenic KRas(G12V). Overall ligation yields of the linear synthetic route **11** + **12** + **13** were 6.7% and 9.0% for all- L and all-D peptides, respectively. Following assembly of five peptidyl fragments accessible by Fmoc-based SPPS and *in vitro* folding, the all-L residue variant of KRas(G12V) showed a similar nucleotide and miniprotein binding profile to that of recombinant KRas(G12V). In contrast, the all-D residue variant of KRas(G12V) exhibited similar behavior but with opposite enantiorecognition with respect to nucleotide and miniprotein binding. The synthesis, folding, and biochemical verification of all-D KRas(G12V) provides the crucial reagent needed for the identification of all D-amino acid Ras inhibitors by mirror-image display screening, affording a valuable tool for the ongoing effort to develop direct-acting Ras therapies.

CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures and characterization data for all new compounds and synthetic peptides, analytical characterization of all L- and all D-KRas(G12V), and details of nucleotide displacement studies are included (PDF).

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Author Contributions

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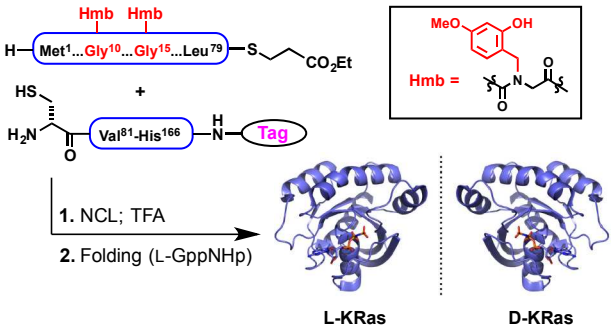
40. The overlay of our combined biophysical data suggests that synthetic KRas(G12V) variants are properly folded and behave similarly to that of recombinant material. We note slight discrepancies in the biophysical comparison of both synthetic all-L and all-D KRas variants with that of recombinant material. Folding and biophysical characterization were generally performed on microscale – thus slight changes in actual protein quantities may have an effect on the overall data. Additionally, the presence of some protein aggregate within the folded synthetic materials cannot be excluded and may be reflected in the experimental results. Nonetheless, the combination of these experiments clearly demonstrates 1) the enantiomeric relationship of the synthetic proteins, 2) nucleotide binding with enantiodiscrimination, and 3) peptide ligand binding with enantiodiscrimination.
41. We have demonstrated that little to no epimerization occurs during thioester synthesis of pertinent peptides (For details see Supporting Information), and regard this as an unlikely factor in the observed folding efficiency of these synthetic proteins. For related C-terminal thioester synthesis methods with particular focus on the potential of epimerization at the C-terminal residue upon activation, see: (a) Futaki, S.; Sogawa, K.; Maruyama, J.; Asahar, T.; Niwa, M.; Hojo, H. *Tetrahedron Lett.* **1997**, *35*, 6237-6240. (b) Quibell, M.; Packman, L. C.; Johnson, T. *J. Chem. Soc., Perkin Trans. 1* **1996**, 1219-1225. (c) Bray, B. L. Nat. Rev. Drug Discov. **2003**, *2*, 587-593. (d) Nagalingam, A. C.; Radfors, S. E.; Warriner, S. L. *Synlett* **2007**, *16*, 2517-2520. (e) Flemer Jr.; S. *J. Pept. Sci.* **2009**, *15*, 693-696.


Hmb Hmb
H–Met1...Gly10...Gly15...Leu79–S–CO2Et
HS
+
H2N
Val81-His166
H
N
Tag
O
1. NCL; TFA
2. Folding (L-GppNHp)
MeO
OH
Hmb =
L-KRas
D-KRas

TOC Graphic