Investigation of Methylation at Site K117 in KRAS Protein

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Abstract

RAS proteins are founding members of the RAS superfamily of GTPases and have been extensively studied as they play key roles in cell growth, differentiation, and survival. They function as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. Mutations in RAS cause disruptions in this cycle and have been identified in various types of human cancer and RASopathies. In addition to mutations, lysine post-translational modifications (PTMs), such as methylation, acetylation, and ubiquitination, can modulate RAS cycling and activity. We have recently identified novel lysine methylation sites in RAS, in collaboration with the Sasaki lab. One of the methylation sites, lysine 117, is strictly conserved in the RAS superfamily. Mutations at this residue have been linked to human disease and cancer. Thus, we hypothesize that methylation at lysine 117 may disrupt KRAS nucleotide cycling. To study the effects of methylation at lysine 117, novel alkylation strategies utilizing "methyl-lysine analogues" and enrichment strategies utilizing "methyl binding domains" are being developed to generate site specific KRAS methylation. To verify the successful methylation of K117, SDS-PAGE gel electrophoresis and mass spectrometric characterization are being implemented.

Introduction

The RAS superfamily of small GTPases act as molecular switches that regulate cellular signal transduction pathways that control cellular growth. When their ability to switch between on and off states is disrupted via mutations or PTMs, RAS becomes "stuck" in an active or inactive state, and lead to cancer and RASopathies.

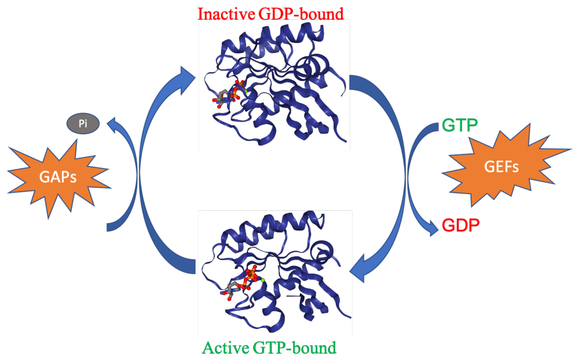

Fig 1. KRAS Utilizes Accessory Factors for Greater Cycle Efficiency. KRAS is a member of the RAS superfamily of small GTPases that transmits signals through pathways that regulate cell proliferation, survival, differentiation, signal transduction, and gene expression. To cycle through "on" and "off" states with greater efficiency, KRAS utilizes accessory factors. Guanine exchange factors (GEFs) exchange GDP for GTP and GTP activating proteins (GAPs) aid in in hydrolysis of GTP to GDP.

The RAS isoform, KRAS, is the most prevalent oncogene in human cancer. Its post-translationally modified form is therefore the focus of our research.

Novel Lysine Methylation Sites

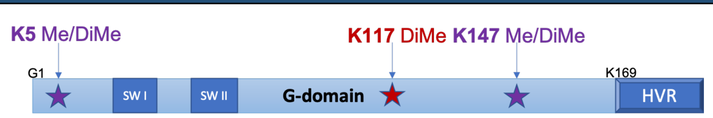

Fig 2. KRAS Undergoes Lysine PTMs. KRAS protein undergoes a number of lysine PTMs, including ubiquitination, methylation, and acetylation. Several sites including K5, K117, and K147, have been identified as novel lysine methylation sites[1]. Of these residues, lysine 117 is strictly conserved due to key interactions it makes with GDP and GTP. Mutations at this site impair guanine nucleotide binding and populate KRAS in its GTP-bound form leading to deregulated activation[2].

We hypothesize that methylation at this site could alter the rate of nucleotide exchange and subsequently affect interaction with downstream effectors. Recent studies indicate mutated KRAS is observed in 95% of pancreatic cancer cases[4]. However, the effects of methylation at lysine 117 remain unclear.

Novel KRAS Alkylation Strategy

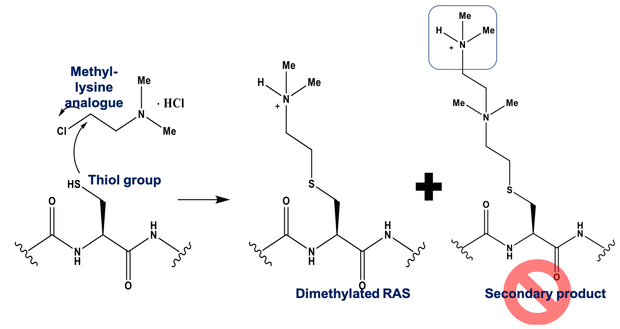

Fig 3. Procedure to Generate Dimethylated KRAS. To obtain dimethylated KRAS, lysine 117 was selectively mutated to cysteine, which has a thiol group. The thiol group turns into a thiolate (S-) group at a higher pH and reacts with the methyl-lysine analogue (MLA) containing the methyl group. The product resembles dimethylated KRAS. However, sometimes it also results in an unwanted secondary linkage product.

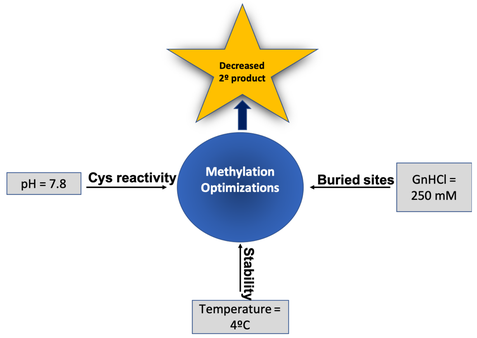

Fig 4. Optimization of KRAS Methylation. To eliminate production of unwanted secondary amines, the pH, temperature, and guanidine hydrochloride (GnHCl) concentration were optimized. Lowering the pH to 7.8 makes the thiolate group less reactive and creates a cleaner product. Lowering the temperature to 4°C allows for greater KRAS stability. Optimizing the GnHCl concentration at 250 mM allows for methylation at buried sites like lysine 117.

Isolation of Methylated KRAS

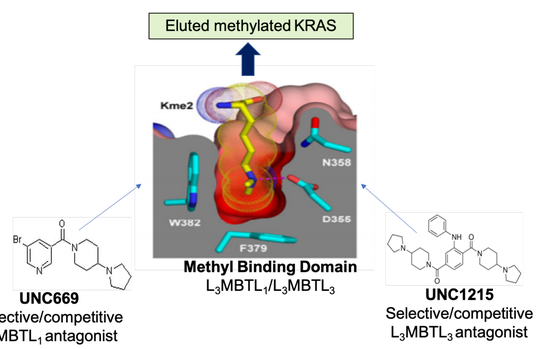

Fig 5. Isolation of Methylated KRAS. As methylation is substoichiometric, we are developing a procedure to separate methylated KRAS from non-modified KRAS, using a protein that recognizes methylated KRAS. For this we used methyl binding domain (L_3MBTL_1 or L_3MBTL_3) from the malignant brain tumor (MBT) protein family. This protein recognizes and binds to the protonated amine of methylated KRAS[3]. The small molecule agonists, UNC669 and UNC1215, have high affinity for MBT and the methylated KRAS is competitively eluted, resulting in its isolation from the reaction mixture. This method is still being optimized.

Protein Purification and Analysis

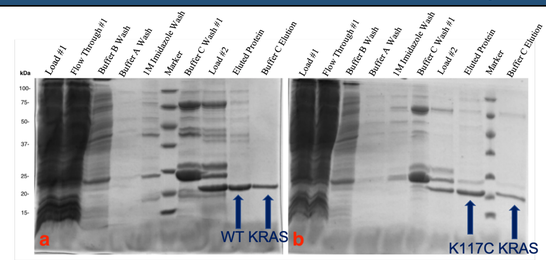

Fig 5. Successful Purification of Wild-type and K117C KRAS Confirmed by SDS-PAGE Electrophoresis (a) Wild-type KRAS purification verified at molecular weight ~19.2 kDa. (b) K117C mutant KRAS purification verified at molecular weight ~19.2 kDa. Purification yield was >90% and was validated by MS spectra of the individual WT KRAS and K117C KRAS samples.

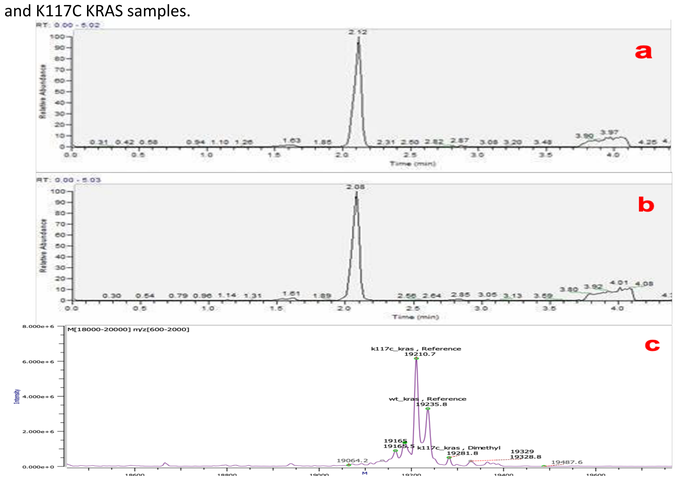

Fig 6. Mass Spectrometric (MS) Analysis of Dimethylated KRAS K117C. MS characterization of (a) WT KRAS and; (b) K117C KRAS shows our starting samples are pure. Dimethylated KRAS K117C (c) is modified at low yield and contains additional protein contaminants, indicating further optimization is needed. The small peak at 19.28 kDa indicates a small fraction (~6%) of dimethylated K117C KRAS protein was obtained.

Conclusions and Future Directions

Conclusions:

- A novel alkylation strategy is being optimized to yield intact methylated K117C KRAS.
- While K117C and WT KRAS purification is feasible, methylation and enrichment techniques are still in need of optimization.
- Small molecules UNC669 and UNC1215 (developed by Stephen Frye lab) can be used to enrich methylated KRAS.
- This alkylation strategy could be applied to other proteins to study the effects of methylation.

Future Directions:

- Once methylation is achieved, we will biochemically characterize methylated KRAS by monitoring a guanine nucleotide exchange to determine whether demethylation at lysine 117 alters nucleotide binding.
- Employ the Drosophila model system to study methylated sites in endogenous KRAS in collaboration with Don Fox lab (Duke).
- Investigate additional PTMs at lysine 5 and 147.

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References

1. Yin, G. *et al.* A KRAS GTPase K104Q mutant retains downstream signaling by offsetting defects in regulation. *J. Biol. Chem.* 292, 4446–4456 (2017).
2. Prior, I. A., Lewis, P. D. & Mattos, C. A comprehensive survey of ras mutations in cancer. *Cancer Res.* 72, 2457–2467 (2012).
3. Simon, M. D., *et al.* (2007). The site-specific installation of methyl-lysine analogs into recombinant histones. *Cell*, *128*(5), 1003–1012.
4. Nagasaka, T. *et al.* Colorectal cancer with mutations in BRAF, KRAS, and Wild-Type with respect to both oncogenes showing different patterns of DNA methylation. *J. of Clin. Oncol.*, 4584-4594 (2004).