The Role of Ubiquitination in Ras Signaling

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Background

- Ras is an essential protein that acts as is a signaling switch to regulate cell growth, differentiation and death.
- Ras activates signaling when bound to GTP, and the enzymatic activity of Ras is to turn itself off by hydrolyzing GTP to GDP.
- Regulators increase rates of GTP hydrolysis and GDP exchange:
  - GTPase activating protein (GAP) increases the rate of hydrolysis
  - Guanine nucleotide exchange factor (GEF) increases the rate of nucleotide exchange upon receptor-mediated activation
- 33% of cancers contain an activating Ras mutation; the most common mutations inhibit GAP-mediated hydrolysis.
- Recent evidence suggests that post-translational modifications (PTMs), in particular monoubiquitination, of Ras may also be important for Ras activation and regulation.
- Monoubiquitination of Ras is isoform and cell-line specific and may represent an alternative target for Ras-driven cancers.
- Preliminary data suggests that isoform specific monoubiquitination may alter Ras activation and interactions with downstream effectors.

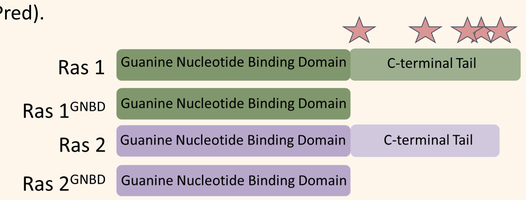
Approach

Overview

Determine the impact of monoubiquitination on Ras1 and Ras2 from *S. cerevisiae* on *in vitro* binding and activation. These data will then inform interpretation of the role of monoubiquitination on Ras in vivo

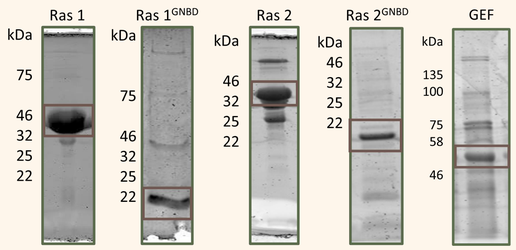
Constructs

The Guanine Nucleotide Binding Domain (GNBD) is highly conserved between Ras 1 and Ras 2. The C-terminal tail differ significantly in sequence. Stars indicated predicted sites of ubiquitination found in the variable tail of Ras1 but not Ras2 (UbPred).



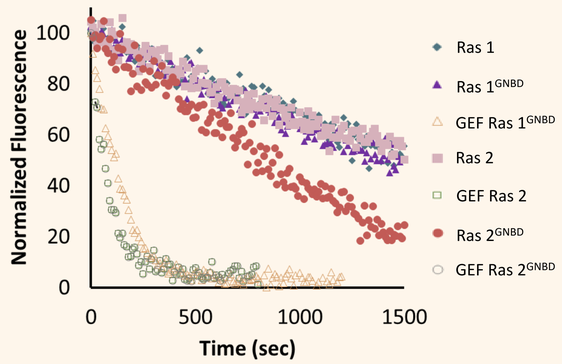
Protein Expression and Purification

All proteins were expressed on recombinant plasmids in *E. coli*, grown at 18°C overnight and purified using Ni-NTA affinity chromatography. Purity was assessed using SDS-PAGE.



Results

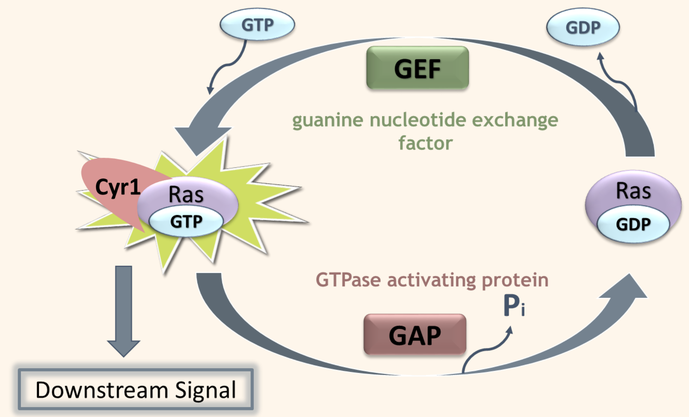
Guanine Nucleotide Dissociation Data



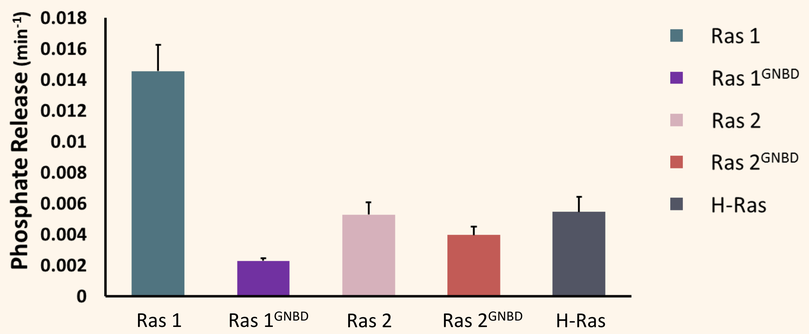
Nucleotide exchange is slow in the absence of GEF (SOS^{cat}). However, removing the C-terminal tail of Ras2 does increase the rate of intrinsic nucleotide exchange. The presence of the c-terminal tail does not significantly alter rates of GEF-mediated nucleotide exchange for either Ras1 or Ras2.

Rate of MANT-GDP release from Ras was used to measure the rate of nucleotide dissociation in the absence and presence of GEF. Ras was pre-loaded with MANT-GDP and dissociation was initiated by the addition of excess unlabeled GDP. The decrease in fluorescence over time indicates loss of MANT-GDP binding to Ras.

Summary of Ras Signaling and Regulation



Guanine Nucleotide Hydrolysis Data

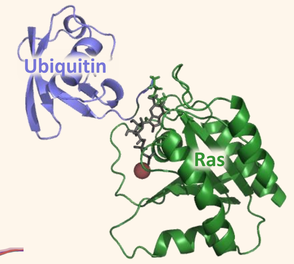


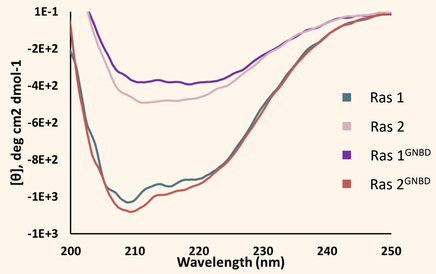
Rate of GTP hydrolysis by Ras measured by detection of free phosphate using a malachite green assay. Data was collected for all four *S. cerevisiae* Ras constructs as well as mammalian Ras (H-Ras) for comparison. Data was collected at 37°C.

The presence of the c-terminal tail significantly alters the rate of GTP hydrolysis for Ras1 but not for Ras2.

Results

Secondary Structure Analysis





CD data collected for the four Ras constructs whose purifications are described above. Key differences in secondary structure content are observed in the absence of the C-terminal tails of both Ras1 and Ras2.

Conclusions

- While Ras1 and Ras2 have historically been considered redundant proteins, *in vitro* analysis indicates that the proteins have different enzymatic activities.
- Differences in their enzymatic activities depend on the presence or absence of the c-terminal tails. Since most PTMs occur in the tail region, this suggests that PTMs may alter or further differentiate the activities of Ras1 and Ras2.
- The binding affinity of Cyr1 for Ras increases in the presence of ubiquitination, suggesting that ubiquitination may alter the response of Ras to signaling and activation.
- Our next question is how ubiquitination alters the biochemical activity of Ras1 and Ras2.
- Together these data will elucidate the role of monoubiquitination in differentiating normal Ras signaling, which will lead to better understanding and options for treatment of Ras-driven cancers

Acknowledgements

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