Rassf Family of Tumor Suppressor Polypeptides*

Published, JBC Papers in Press, December 17, 2008, DOI 10.1074/jbc.R800073200

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The Rassf1–6 polypeptides each contain a Ras/Rap association domain, which enables binding to several GTP-charged Ras-like GTPases, at least in vitro or when overexpressed. The Ras/Rap association domains are followed by SARAH domains, which mediate Rassf heterodimerization with the Mst1/2 protein kinases. Rassf1A is unequivocally a tumor suppressor, and all Rassf proteins behave like tumor suppressors, exhibiting epigenetic silencing of expression in many human cancers and pro-apoptotic and/or anti-proliferative effects when re-expressed in tumor cell lines. Herein, we review the binding of the Rassf polypeptides to Ras-like GTPases and the Mst1/2 kinases and their role in Rassf function.

The Rassf (Ras association domain family) polypeptides, a family of non-catalytic proteins encoded by six genes, each expressed as multiple splice variants, were found with the description of Nore1 (now designated Rassf5) as a Ras-GTP-binding protein (1). The description of the Rassf1 polypeptides followed shortly thereafter (2) and elicited considerable attention because of their identification as the products of a tumor suppressor gene (3, 4). Thus, the gene encoding Rassf1 is silenced tumor suppressor gene in a wide variety of cancers unmethylated. Rassf1A is firmly established as an epigenetically silenced Rassf1A expression inhibits proliferation and tumor growth in nude mice. Most persuasively, specific knock-outs of the exon encoding the unique N terminus of Rassf1A result in increased numbers of tumors in older mice, specifically lymphomas, lung tumors, and gastrointestinal tumors (7, 8); increased numbers of tumors have also been reported in Rassf1A heterozygotes (7).

This review will emphasize Nore1/Rassf5 and Rassf1, the most extensively characterized members of the Rassf1–6 polypeptide family, focusing on their structure and binding to Ras-like GTPases and on the Mst1/2 protein kinases as likely physiologic effectors. Excellent recent reviews of the entire Rassf family in human cancers (6) and of Rassf1A (5) are recommended.

Rassf Sequence Features and Domain Organization

The Rassf polypeptides align into two groups; Rassf1, Rassf3, and Rassf5 (Nore1) exhibit ~50% amino acid sequence identity, whereas Rassf2 and Rassf4 are nearly 60% identical to each other and ~40% identical to Rassf6. Identity between the two subfamilies is ~25% overall and largely confined to the RA2 domain and the unique motif called the SARAH domain (9) located immediately C-terminal to the RA domain; together, these domains occupy the C-terminal 150 amino acids of all major isoforms of the Rassf1–6 polypeptides. The polypeptides designated Rassf7 and Rassf8 have an N-terminal RA domain but lack the SARAH domain and any other conserved motifs seen in Rassf1–6; their functional relationship to Rassf1–6 is unknown.

The human (all numbering refers to the human sequences) Rassf1 and Nore1/Rassf5 polypeptides are expressed as two major isoforms. The longer isoforms, Nore1A (418 aa) and Rassf1A (340 aa), have non-homologous N termini (~120 and 40 aa, respectively), upstream of homologous C1-type zinc fingers (Nore1A, aa 123–170), central linker regions (aa 194–250), and RA domains (aa 274–364), followed immediately by the SARAH domains (aa 366–413). The shorter isoforms, Nore1B (265 aa) and Rassf1C (270 aa), are ~50% identical to each other and to the single major Rassf3 polypeptide and are homologous along their entire sequence. Compared with Nore1A, Nore1B has a unique 40-aa N-terminal segment appended to the shared linker and RA and SARAH domains. Caenorhabditis elegans has a single Rassf-related gene, T24F1.3, encoding proteins of 554 and 615 amino acids, whose central region contains a C1 zinc finger and RA and SARAH domains and is ~40% identical to the C-terminal 300 aa of Nore1A/Rassf1A. Rassf2 and Rassf4 are expressed predominantly as single polypeptides of 326 and 321 aa, respectively, whereas Rassf6 occurs as 337- and 369-aa proteins, the longer encoding one additional N-terminal exon.

Drosophila melanogaster has a single Rassf gene, CG4656, encoding an 806-aa polypeptide with an N-terminal LIM domain and C-terminal RA/SARAH domains that are nearly

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* This work was supported, in whole or in part, by National Institutes of Health Grants DK17776 and P30 DK57521 (to J. A.), HL08297 and DK043351 (to R. X.), and T32 DK007028 (to D. Z.). This is the second article of three in the Thematic Minireview Series on Novel Ras Effectors. This minireview will be reprinted in the 2009 Minireview Compendium, which will be available in January, 2010.

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2 The abbreviations used are: RA, Ras/Rap association; aa, amino acid(s); GppNHP, guanyl-5′-yl imidodiphosphate; RBD, Ras-binding domain; PLC, phospholipase C.
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40% identical to those in Rassf2, Rassf4, and Rassf6 but less similar to those of Rassf1, Rassf3, and Rassf5.

Rassf Interactions though the RA Domain

The non-catalytic nature of the Rassf proteins and the presence of an RA domain lead to the inference that they serve as adapters, recruiting to activated Ras-like GTPases proteins that modulate GTPase activity and/or mediate certain of the GTPase downstream actions. However, apart from the Nore1/Rassf5 polypeptides, which are clearly established as effectors of Ras-like GTPases, physiologic regulation of the other Rassf proteins by endogenous wild-type or mutant Ras family proteins remains unproven. Nore1, the founding member, was retrieved in a two-hybrid screen with Ha-Ras(G12V) and binds \textit{in vitro} with strong preference for Ras-GTP (1). In a quantitative two-hybrid assay, Nore1 interacted with the activated forms of other Ras-like GTPases in the order M-Ras/R-Ras3 > Rap2A ≈ Ki-Ras ≈ R-Ras > R-Ras2/TC21 > Ha-Ras > Rap1 but not with RaLA (10). An extensive \textit{in vitro} analysis using purified polypeptides observed that the $K_d$ between Nore1 and GppNHp-loaded Ha-Ras or M-Ras, determined by isothermal calorimetry, is nearly identical (0.31 μM versus 0.32 μM) and much lower than for Rap1 (2.8 μM) (11); by comparison, the values for the c-Raf RBD are 0.080 μM for Ha-Ras and 0.67 μM for Rap1. Kinetic measurements give a much lower $K_d$ for the Nore1/Ha-Ras interaction (0.02 μM) compared with that calculated from calorimetric measurements, whereas the estimate for c-Raf (0.066 μM) is similar in both methods. The high affinity of Nore1 for Ha-Ras is attributable to a very low off rate ($k_{\text{off}} = 0.18$ s$^{-1}$ versus 4.34 s$^{-1}$ for c-Raf).

A Nore1 fragment encompassing the canonical RA domain (aa 254–363) is unable to bind Ras, but an N-terminally extended fragment (aa 203–363, identical in Nore1A and Nore1B) binds Ras much like full-length Nore1; the structure of a co-crystal of Nore1-(203–363) with Ha-Ras-GppNHp was recently described (12). Residues 230–358 of Nore1 exhibit the ubiquitin-like folding pattern ($\beta$-$\beta$-$\beta$-$\beta$-$\beta$) seen with other Ras partners, with five residues of the $\beta$ strand of Nore1A interacting in an antiparallel manner with 10 residues of the Ras switch 1 loop. The segment (aa 203–230) N-terminal to the canonical Nore1 RA domain forms a helix and a short $\beta$ strand connected by a reverse turn and packs tightly against the ubiquitin fold, a structure not seen thus far in any other Ras partner. Cys$^{225}$ and Leu$^{226}$ of the turn form a hydrophobic interface with Ras switch 2 residues Met$^{67}$ and Tyr$^{68}$. The importance of this Nore1/switch 2 interaction and its specificity for the Nore1/Ras interaction are illustrated by Ras mutation Y64A, which reduces the interaction with Nore1 by nearly 100-fold but has little effect on binding to the c-Raf RBD. Moreover, the Y64A mutation causes a 150-fold increase in the unusually low $k_{\text{off}}$ between Ras and Nore1. Thus, by virtue of a larger than usual RA domain and interaction surface with Ras, Nore1 exhibits a much slower dissociation, and the Nore1-Ras complex has an estimated lifetime (~10 s) that is radically longer than that of Raf, phosphatidylinositol 3-kinase, or RafGDS (60–250 ms). This property is likely to bear significantly on the function of Nore1 as an adapter.

The Nore1A RA domain can also bind directly to the Nore1 zinc finger (13), an intramolecular interaction that recalls the ability of the c-Raf zinc finger to bind to a c-Raf segment immediately N-terminal to the catalytic domain (14). Binding of Ras-GTP to Nore1A displaces the zinc finger from the RA domain, presumably freeing the zinc finger for intermolecular interactions. The isolated Nore1A zinc finger shows highly selective binding to phosphatidylinositol 3-phosphate and to sulfatide (13), which may contribute to Nore1A membrane targeting. Whether, as with Raf, the Nore1A zinc finger participates in the interaction with prenylated Ras (14) has not been tested.

Surprisingly, the Rassf1 polypeptides are greatly inferior to Nore1 in their ability to bind Ras-GTP. In a two-hybrid assay, Rassf1A showed no interaction above background levels with any of the activated Ras-like GTPases that bind Nore1A (10). Indirect calorimetry provides an estimated $K_d$ for the Rassf1C/ Ha-Ras-GppNHp interaction of 39 μM, <1% that of Nore1, with binding to Rap1 being undetectable; using another method, the interaction of Ha-Ras with Rassf1A was 33-fold weaker than that with Nore1A (11). Nevertheless, transiently overexpressed Rassf1C can be co-precipitated with coexpressed Ha-Ras(G12V) (15). Moreover, massive overexpression of Rassf1C in 293T cells promotes apoptosis, a response enhanced by coexpression of Ha-Ras(G12V) and opposed by the inhibitory Ha-Ras mutant Q61L/C186S, indicating that Rassf1C-induced apoptosis requires an interaction through its RA domain (15). A similar synergistic pro-apoptotic effect has been reported for Ki-Ras(G12V), Rassf2/4/6, and Nore1/Rassf5 (reviewed in Refs. 5 and 6). Such functional collaboration does not, however, necessarily result from a direct physical interaction between the Rassf and Ras-GTP polypeptides. In fact, the physical interaction between Rassf1A and Ras in transient transfections appears to be largely indirect (10). Thus, both Rassf1A and Nore1A are capable of homodimerizing through their unique N termini as well as heterodimerizing with each other, whereas Rassf1C shows little or no ability to homo- or heterodimerize. Coexpression of Nore1A with Rassf1A strongly enhances the association of Rassf1A with coexpressed Ha-Ras(G12V), whereas coexpression with Nore1A (1–267), which lacks an RA domain, abolishes the association of Rassf1A (but not full-length Nore1A) with coexpressed Ha-Ras(G12V), indicating that the ability of Rassf1A to associate with activated Ras is attributable to its heterodimerization with Nore1A (10). The ability of other Rassf polypeptides to engage in homo- or heterodimerization is unexplored, and whether heterodimerization of endogenous Nore1A/Rassf1A occurs is uncertain because the reliability of available antibodies remains to be verified.

Regarding the interactions of other Rassf polypeptides with Ras-like GTPases, recombinant Rassf2, Rassf4, and Rassf6 are capable of binding specifically to GTP-charged Ki-Ras \textit{in vitro} and during transient transfection (5, 6), whereas the ability of Rassf3 to bind Ras-GTP is undocumented as yet. Nevertheless, as with Rassf1, evidence supporting a physiologic interaction of Rassf2/4/6 is scant; when expressed transiently at levels comparable with Nore1A, Rassf6 does not co-precipitate coexpressed activated mutants of Ras-like GTPases, whereas Nore1 exhibits strong binding (16). A quantitative analysis of Rassf2/
4/6 binding to Ras-GTP in vitro relative to Nore1 and Rassf1 has not been reported, nor has the association of endogenous Rassf1–4 or Rassf6 with an endogenous Ras-like GTPase been observed. Thus, the role of Ras-like GTPases as physiologic binding partners of Rassf polypeptides other than Nore1A/B must be considered unproven. Although it is premature to conclude that GTP-charged Ras subfamily GTPases do not interact with endogenous Rassf1–4/6 polypeptides under “physiologic” conditions, the inability of polypeptides encoding an “RA” domain to bind to a Ras-like GTPase is not unprecedented, as, for example, that in Myr5 or the RA1 domain of PLCγ (11). The ubiquitin fold (βαββαββ) of the RA domain and other RBDs also forms the backbone of the UBQ and B41/ERM domains; the Rassf1–4/6 RA domains may bind protein regions that resemble those selected by the UBQ and/or B41/ERM domains (17).

**SARAH Domain**

Each of the six Rassf polypeptides contains at its C terminus this novel domain of ~50 amino acids. Only three other polypeptides encoded in the human genome contain this motif, i.e. WW45/SAV1, the ortholog of the Drosophila Salvador/Shar-pei gene product, and the Mst1 and Mst2 protein kinases, orthologs of the Drosophila protein kinase Hippo (9). An NMR analysis (18) of the isolated, 13C,15N-labeled Mst1 SARAH domain (aa 432–480) indicates that it adopts a hairpin-like structure, with a short N-terminal helix (aa 433–437) folding back at a ~45° angle with respect to the longer C-terminal helix (aa 441–480); the latter mediates the formation of a homodimer through an antiparallel, head-to-tail association stabilized primarily by hydrophobic residues. The addition of a slight molar excess of the Nore1 SARAH domain (aa 366–413, as a glutathione S-transferase fusion) to the Mst1 SARAH homodimer results in the stoichiometric displacement of the Mst1 SARAH domain into a 1:1 Mst1/Nore1 heterodimer, utilizing the same Mst1 surface as in the Mst1 homodimer, with an estimated dissociation constant of the heterodimer in the nanomolar range. The isolated WW45/SAV1 SARAH domain (aa 321–373) also associates with the Mst1 SARAH polypeptide through its long helix; however, different, predominantly hydrophobic Mst1 residues exhibit signal broadening compared with the addition of the Nore1 SARAH domain. Nevertheless, the addition of an excess of the WW45 SARAH domain to the Mst1/Nore1 SARAH heterodimer does not produce any alterations in the Mst1 signals, nor is there evidence of a trimer upon gel filtration. These results imply that the Mst1 (or Mst2) association with Nore1 will be as a constitutive heterodimer, a conclusion supported by the detection of Nore1A and Nore1B (as well as Rassf1A) in immunoprecipitates of endogenous Mst1 from a variety of cells, regardless of stimulation (19, 20). Moreover, they suggest that the complexes of Mst1 with Nore1 and with WW45/SAV1 will be mutually exclusive. Consistent with this is the finding that immunoprecipitates of either endogenous Salvador or dRASSF from Drosophila Kc cells contain Hippo, but not each other, and that a trimeric complex cannot be detected when all three polypeptides are transiently coexpressed (21). Conversely, Guo et al. (22) detected cross-immunoprecipitation of endogenous WW45 and Rassf1A from HeLa cells, apparently mediated via their SARAH domains. Thus, whether or not the complexes of the Mst1/2 kinases with the Rassf polypeptides and WW45 are physically independent is unresolved; although a Nore1/WW45/Mst1 trimer does not occur, perhaps the Rassf1A SARAH domain is capable of engaging in a trimer with Mst1 or Mst2 and WW45.

**Physiologic Regulation and Actions of Nore1**

The strong interaction of Nore1 with Ras-GTP observed in vitro, similar in affinity to that seen with c-Raf, as well as the ability of epidermal growth factor to cause the association of endogenous Nore1A with endogenous Ras (1), strongly supports the designation of Nore1A/B as a physiologic partner of Ras-GTP. Nevertheless, a requirement for Nore1A or Nore1B in the execution of a physiologic Ras output has not been conclusively identified. Overexpression of Ki-Ras(G12V) or the Ha-Ras mutant G12V/E37G, which can bind Nore1 but not phosphatidylinositol 3-kinase, induces apoptosis (23). Mst1, which interacts through its SARAH domain with all human Rassf polypeptides as well as with the T24F1.3 polypeptide, is known to induce apoptosis when transiently expressed (24). The finding that Ras-induced apoptosis is inhibited by coexpression with the Nore1 or Mst1 SARAH domain (23) strongly supports the conclusion that a Nore1-Mst1/2 complex mediates this phenotype. Nevertheless, a physiologic counterpart to this model of Ras-induced apoptosis is not known.

In contrast, a compelling body of evidence indicates that Rap1-GTP is a physiologic regulator of Nore1B in T-cells. Rap1 mediates the antigen- and chemokine-induced “inside-out” activation of T-cell integrins (25, 26). As with receptor stimulation, Rap1(G12V) enhances T-cell affinity for ICAM (intercellular adhesion molecule), an LFA-1 ligand, and also promotes LFA-1 clustering at the leading edge. T-cell antigen receptor cross-linking activates Rap1 in a sustained manner through PLCγ, whereas chemokines activate Rap1 through a pertussis-sensitive pathway to PLCβ. Kinashi (25, 26) retrieved Nore1B (called RAPL) in a two-hybrid screen using a Rap1(G12V) bait and showed that overexpression of Nore1B in primary T-cells per se is sufficient to strongly activate adhesion to ICAM. Activation of the T-cell antigen or chemokine receptors induces association of endogenous Nore1B/RAPL with both Rap1 and LFA-1 and promotes recruitment of the Nore1B-LFA-1 complex to the T-cell leading edge. The requirement for Nore1B in the consequent activation of LFA-1 was initially shown by the ability of a Nore1B-(101–265) fragment to block Rap1-GTP recruitment of Nore1B and LFA-1 to the leading edge and to inhibit T-cell receptor and chemokine (but not phorbol 12-myristate 13-acetate) activation of LFA-1. Definitive proof for the importance of Nore1B to integrin activation is the finding that Nore1B-deficient mice exhibit diminished numbers of mature T-cells in peripheral lymphoid organs, attributed to impaired thymic egress, as well as defects in multiple integrin-controlled functions of peripheral T- and B-cells (25, 26); independent contributions of Nore1B-Rap1 and Nore1B-Rap2 are documented (27).

Notably, the dominant inhibitory fragment Nore1B-(101–265) is unable to bind Rap1 as it lacks the N-terminal segment of the extended Nore1 RA domain; it does, however, contain an
Stable re-expression of Rasf1A in tumor cell lines lacking this polypeptide results in slowed proliferation, diminished colony size in soft agar, and impaired tumorigenesis upon implantation in vivo (Fig. 1) (2, 5). The major slowing is in the G₁/S transition accompanied by diminished cyclin D₁ accumulation (30). In addition, Rasf1A and Nore1 (as well as the Mst1 and Mst2 kinases) are visualized predominantly on microtubules in interphase and on the centrosome, spindle, and cytokinetic furrow during mitosis. The Rasf1A polypeptide contributes to microtubular stability, and Rasf1A deficiency is accompanied by chromosomal instability (8, 31, 32). Although transient Rasf overexpression generates marked pro-apoptotic effects (5, 6), the relevance of apoptosis to the tumor suppressor function of the endogenous Rasf proteins is unclear. A large number of candidate effectors for Rasf1A have been proposed (5, 6), however, the following discussion will be limited to the Mst1 and Mst2 kinases as candidate Rasf effectors.

Mst1 and Mst2 are class 2 GC (germinal center) kinases (86% identical); they are best known for their ability to initiate apoptosis when overexpressed (24), through a combination of p53- and JNK (c-Jun N-terminal kinase)-mediated pathways (33, 34). Reciprocally, apoptosis induced by a variety of stimuli is accompanied by activation of Mst1/2, followed by caspase cleavage, yielding highly active catalytic fragments with unrestricted nuclear entry and altered substrate specificity (35). In addition, Mst1 and Mst2 are activated in mitosis by unknown mechanisms and delay mitotic exit and progression through G₁ to S, in part through the phosphorylation of Mob1 (36). Loss of function of the Mst1/2 Drosophila ortholog Hippo causes marked overproliferation because of accelerated cell cycle progression coupled with a failure of developmental apoptosis, a phenotype rescued by human Mst2. Hippo lies at the center of a crucial growth regulatory pathway (37, 38); downstream are its substrates MATS (Mob1), a non-catalytic regulatory protein, and the protein kinase Warts/Lats. Hippo and Lats bind the scaffold Salvador, which facilitates Hippo-catalyzed Lats phosphorylation; after binding to phospho-MATS, phospho-Lats autoactivates, phosphorylates, and inhibits the transcriptional co-activator Yorkie, a pro-proliferative, anti-apoptotic effector. Upstream regulation of Hippo is less well characterized; activation is probably initiated by cell-cell contact and mediated by atypical cadherins and involves FERM domain proteins (Merlin, Expanded). Salvador may also facilitate Hippo activation. The pathway is well conserved, and its importance to mammalian tumorigenesis and organ size control is demonstrated by the overgrowth and tumor predisposition phenotypes of mice with mutations in orthologous genes and by genomic alterations in human cancers, e.g. NF2/Merlin, Lats, and Yap/Yorkie are established tumor suppressors and oncogenes.

Mst1 and Mst2 are activated by autophosphorylation in their activation loop; when transiently expressed, they exist as a slightly active (although pro-apoptotic) homodimer that can be fully activated upon the addition of protein phosphatase inhibitors. Coexpression with excess Nore1/Rasf1 displaces Mst1/2 into a heterodimer and inhibits kinase activity (19), although activation of Mst2 by coexpressed Rasf1A has also been reported (22, 39). Membrane recruitment of the complex by Ras- or Rap1-GTP results in activation, which persists only while the kinase is at the membrane (19). This contrasts with c-Raf-1, whose association with Ras-GTP initiates a series of modifications that result in stable activation independent of continued Ras association (14). Although the physiologic relevance of the Nore1B-Mst1 complex is securely established, the role of the Mst1/2 kinases in the tumor suppressor function of Rasf1A and Nore1A, although highly plausible, is uncon-
firmed. Whether Rassf1A operates within the mammalian “Hippo” tumor suppressor pathway or in an independently regulated tumor suppressor pathway, through Mst1/2 and/or other effectors, remains to be established. Illustrative of these uncertainties, Nore1-(1–250), lacking the RA and SARAH domains, suppresses colony formation by A549 cells nearly as well as full-length Nore1 (40). Although it is not known whether Nore1A and Nore1A-(1–250) inhibit proliferation by the same mechanism, it is possible and perhaps probable that the ability of the Rassf proteins to serve as tumor suppressors reflects, wholly or in part, their participation in pathways that are entirely independent of Ras-like GTPases and Mst1/2 kinases.

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