Nonsel self Recognition Through Intermolecular Disulfide Bond Formation of Ribonucleotide Reductase in *Neurospora*

Robert P. Smith,^{*,1,2,3} Kenji Wellman,^{*,1} Leila Haidari,^{*} Hirohisa Masuda,[†] and Myron L. Smith^{*}

^{*}Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada, and [†]Laboratory of Cell Regulation, Cancer Research UK London Research Institute, Lincoln's Inn Fields Laboratories, London, WC2A 3LY, United Kingdom

ABSTRACT Type I ribonucleotide reductases (RNRs) are conserved across diverse taxa and are essential for the conversion of RNA into DNA precursors. In *Neurospora crassa*, the large subunit of RNR (UN-24) is unusual in that it also has a nonself recognition function, whereby coexpression of Oak Ridge (OR) and Panama (PA) alleles of *un-24* in the same cell leads to growth inhibition and cell death. We show that coexpressing these incompatible alleles of *un-24* in *N. crassa* results in a high molecular weight UN-24 protein complex. A 63-amino-acid portion of the C terminus was sufficient for *un-24*^{PA} incompatibility activity. Redox active cysteines that are conserved in type I RNRs and essential for their catalytic function were found to be required for incompatibility activity of both UN-24^{OR} and UN-24^{PA}. Our results suggest a plausible model of *un-24* incompatibility activity in which the formation of a complex between the incompatible RNR proteins is potentiated by intermolecular disulfide bond formation.

HYPHAL fusion between conspecific fungi can result in the formation of heterokaryons: cells with more than one nuclear type. Growth of heterokaryotic cells is governed by nonself recognition loci (Glass and Kaneko 2003; Smith and Lafontaine 2013) that function to restrict transfer of parasitic genetic elements (Biella *et al.* 2002). *Neurospora crassa* has 11 heterokaryon incompatibility loci that regulate nonself recognition during vegetative growth (Perkins 1988). Allelic differences at one or more of these loci triggers incompatibility activity leading to cell death (Jacobson *et al.* 1998).

The *N. crassa* incompatibility locus *un-24* contains two genes, *un-24* and *het-6*, that function together as an incompatibility gene complex (Lafontaine and Smith 2012). Heteroallelism at *un-24* results in an incompatibility reaction in strains in which *het-6* is deleted, indicating that *un-24* can autonomously mediate nonself recognition. *un-24* also encodes the

large subunit of a type I ribonucleotide reductase (RNR) and is therefore the only described example of an RNR large subunit having nonself recognition function (Smith *et al.* 2000b). RNR is an essential enzyme that reduces ribonucleotides to their corresponding deoxyribonucleotides and is necessary for DNA synthesis and repair (Elledge *et al.* 1993). Type I RNRs are found in all eukaryotic species, as well as selected viruses and prokaryotes. The holoenzyme functions as a tetramer composed of two large subunits (R1) and two small subunits (R2) (Reichard 1993). R1 contains the catalytic site and two allosteric effector sites (Eriksson *et al.* 1997; Reichard 2002). The R2 subunit contains a dinuclear iron center that generates a tyrosyl radical (Y122 in *Escherichia coli*) (Nordlund and Eklund 1993). Based on studies done in *E. coli*, the reaction mechanism of RNR involves five conserved cysteines (C225, C439, C462, C754, and C759) and a radical intermediate (Aberg *et al.* 1989; Mao *et al.* 1992). The cysteine residues located in the catalytic site (C225, C439, and C462) mediate reduction of the nucleotide via disulfide bond formation. Subsequently, the active site disulfide bond is transferred to C754 and C759 of the C terminus region, which is then reduced by glutaredoxin or thioredoxin to regenerate the enzyme (Aberg *et al.* 1989; Mao *et al.* 1992). Although the mechanism by which this disulfide bond transfer occurs is unknown, it has been demonstrated that, in yeast, the labile structure of the C

Copyright © 2013 by the Genetics Society of America

doi: 10.1534/genetics.112.147405

Manuscript received November 5, 2012; accepted for publication January 14, 2013
Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.147405/-/DC1>.

¹These authors contributed equally to this work.

²Present address: Division of Math, Science and Technology, Nova Southeastern University, Fort Lauderdale, FL 33314.

³Corresponding author: Division of Math, Science and Technology, Nova Southeastern University, Fort Lauderdale, FL 33314. E-mail: rsmith@nova.edu

terminus allows for the C-terminal cysteine residues of one R1 subunit to act in *trans* to reduce the active site of the neighboring R1 subunit (Zhang *et al.* 2007).

The tertiary structure of the large RNR subunit from *Saccharomyces cerevisiae* (Rnr1p) is similar to the *E. coli* ortholog (Xu *et al.* 2006), while the predicted protein sequence of UN-24 in *N. crassa* is ~85% similar to Rnr1p. A major departure from the similarity of these proteins occurs in the C terminus (Smith *et al.* 2000b). Relative to the *E. coli* form, Rnr1p and UN-24 have dissimilar insertions of ~128 and ~160 amino acids, respectively, located inside a conserved C terminus domain. This variable region also differs between the two allelic forms of UN-24, designated Oak Ridge (OR) and Panama (PA). The OR and PA alleles differ by 30 of 106 amino acid positions in the C terminus (relative to the PA allelic form) (Smith *et al.* 2000b), whereas the N terminus is identical in both variants (Lafontaine and Smith 2012).

While it has been proposed that reactions mediated by different incompatibility loci share a common downstream cell death pathway (Hutchison *et al.* 2009), the mechanism(s) by which nonself recognition processes are initiated remains unclear. One possibility is that incompatibility involves protein–protein interactions that result in “toxic” complexes (Coustou-Linares *et al.* 2001; Sarkar *et al.* 2002). We sought to determine whether such a protein complex coincided with *un-24*-associated incompatibility and examine whether there is a functional correlation between the catalytic and incompatibility activities of UN-24 in *N. crassa*. We report that *un-24*-associated incompatibility is correlated with the formation of a high molecular weight protein complex that is likely composed of the two allelic forms of the UN-24 protein. Residues involved in both catalytic and incompatibility activities are also identified. Finally, we propose a model of how allelic differences in RNR trigger nonself recognition in *N. crassa*.

Materials and Methods

Manipulation of *N. crassa* strains and molecular genetic methods

N. crassa strains used (with OR alleles at all undesignated *het* loci) were: C2(2)-1 *un-24*^{PA} *het-6*^{PA} *thr-2 a*, C9-2 *un-24*^{OR} *het-6*^{OR} *het-c*^{PA} *thr-2 a*, and C8c-164 *un-24*^{ts} *het-6*^{OR} *trp-1*; *inl a*. DNA cloning was performed with plasmids pCB1004 (Carroll *et al.* 1994), which contains *hph* conferring resistance to hygromycin B, and pCR2.1 (Invitrogen, Carlsbad, CA). PCR reactions were performed with iProof DNA polymerase (BioRad, Mississauga, ON, Canada). DNA transformation and spheroplast preparation of *N. crassa* strains were done as previously reported (Smith *et al.* 2000b). Transformants were selected on medium containing 200 µg/ml of hygromycin B (Roche, Mississauga, ON, Canada). Primer sequences are available upon request.

Protein isolation and Western blotting

Protein was extracted from C9-2 that was transformed with *un-24*^{OR} (self-compatible), or with *un-24*^{PA} (self-incompatible),

and from C2(2)-1 transformed with *un-24*^{PA} (self-compatible). Self-incompatible transformants escape to a near-wild-type phenotype ~4–10 days after subculture (Micali and Smith 2006). Self-incompatible transformants were grown to ~3 mm diameter on agar medium, cut into small pieces, and transferred to 250-ml flasks containing 50 ml liquid medium. The flasks were incubated at 30° for ~6 days, at which time cultures that had not escaped were harvested by filtration. Self-compatible transformants and escaped self-incompatible colonies were harvested for protein extractions after ~4 days incubation at 30°, prior to conidiation. Mycelia were sonicated with four, 20-sec pulses at 120 W, separated by 40-sec intervals. Cell debris was removed by centrifugation at 16,000 × *g* for 15 min at 4° and proteins in the supernatant were quantified by a Bradford protein assay prior to storage as aliquots at –20°. Approximately 30 µg of protein (from the supernatant) from each strain was boiled in an equal volume of 2× Laemmli buffer (containing 0.2 M DTT) for 5 min prior to SDS–PAGE (4% stacking gel, 10% running gel). Duplicate gels were used; one was stained with Coomassie brilliant blue to insure even protein loading and the second gel was used for Western blot analysis. Proteins were transferred overnight at 30 volts to a nitrocellulose membrane. Chemiluminescent detection was carried out with anti-mouse-RNR-N polyclonal antibodies, which bind to the 13-amino-acid N terminus epitope KRDRGRQERVQMFDFK, and have been observed to recognize diverse eukaryotic RNRs (Takada *et al.* 2000). The predicted *N. crassa* sequence for this region is KRDRGRQERVQMFDFK, which differs by the single italicized amino acid from the mouse sequence. Detection of antimouse-RNR-N was with goat anti-rabbit IgG/horseradish peroxidase. Fibroblast 3T3 mouse protein was used as a positive control for RNR detection. Molecular weight was determined using a Broad Range Pre-Stained Protein Marker (New England Biolabs). Similar results were observed in five replicates.

For detection of the HA epitope, mycelia of pre- and post-escape self-incompatible C9-2::hygunPA(788-923)-HA and C2(2)-1::hygunPA(788-923)-HA (self-compatible control) were frozen in liquid nitrogen, lysed using a modified bead beater method (Adams *et al.* 1997), and resuspended in modified extraction buffer [50 mM HEPES (pH 7.5), 2 mM EDTA, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor (Complete Mini-Protean, Roche)] (Pandey *et al.* 2004). Cell debris was removed by centrifugation at 16,000 × *g* for 1 hr at 4°. We refer to the supernatant and the cell debris from this centrifugation as the lysate (*i.e.*, soluble) fraction and the pellet (*i.e.*, insoluble) fraction, respectively. Protein loading and Western blotting were performed as described above with the exception that 2× Laemmli buffer also contained 0.7 M β-mercaptoethanol. To extract proteins from the pellet fraction, the pellet was boiled in 2× Laemmli buffer for 10 min. The HA epitope was detected using rat anti-HA primary antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rat

Figure 1 Localization of distinct PA and OR UN-24 incompatibility domains. Regions of *un-24^{PA}* and *un-24^{OR}* were fused to the hygromycin B resistance gene (*hph*) (with or without an HA epitope) and tested for incompatibility activity by transformations of PA [C2(2)-1] and OR (C9-2) strains. The cross-hatched (PA) and shaded (OR) areas at the right represent the allele specific C terminus regions. At the far right of each construct, +* indicates PA-like activity, - represents no incompatibility activity, + designates strong OR-like activity, and +/- indicates weak OR-like activity. Superscript 1 indicates that the construct was characterized previously (R. P. Smith, K. Wellman, and M. L. Smith, unpublished data). Each interval on the bottom bar represents a length of 100 amino acid residues.

secondary antibody (1:2000) (Promega, Madison, WI). Bound antibodies were visualized using a Chemiluminescent Peroxidase Substrate-3 kit (Sigma-Aldrich, Oakville, ON, Canada).

un-24 constructs used in incompatibility and complementation assays

A series of fusion constructs was made by ligating an *un-24^{OR}* or *un-24^{PA}* fragment in-frame to the 3' end of *hph*. The amino acids of *un-24* (either PA or OR) incorporated into these constructs is given in parentheses. After positively identifying incompatibility activity of hygunPA(788-923) (Figure 1; see below for criteria used to assess incompatibility activity), deletion or base substitution mutations were introduced into the *un-24^{PA}* segment by PCR and amplicons were cloned into pCR2.1.

Each *un-24* construct was tested for incompatibility activity using three independent transformation assays with recipient strains C9-2 and C2(2)-1. After ~4 days at 30°, transformed colonies on each plate were scored as compatible or incompatible using the following criteria. When the *un-24^{OR}* DNA is transformed into an *un-24^{PA}* strain there is a ~95% decrease in the number of transformed colonies recovered compared to transformations with pCB1004 (control). When *un-24^{PA}* is transformed into OR-background cells there is a reduction in transformation efficiency (~20%, as compared to pCB1004) and the majority of transformants form small, morphologically distinct “star-like” colonies. When subcultured, these self-incompatible transformants grow slowly with little or no aerial hyphae until about day 6 when they routinely “escape” to a near-wild-type growth rate and morphology (Smith and Lafontaine 2013).

RNR catalytic activity was determined by a complementation assay using the temperature-sensitive (*un-24^{ts}*) form of the OR allele (Smith *et al.* 2000a). Complementation was

assayed by cotransformation of strain C8c-164 with 900 ng of a given *un-24* construct in pCR2.1 along with 100 ng of pCB1004 (*hph*) DNA. At least five separate transformants generated on hygromycin-containing medium at 30° were transferred to 39° and monitored for growth over a period of 7 days. Continuous growth beyond 3 days incubation at 39° by C8c-164 transformants indicated complementation of the temperature-sensitive *un-24^{ts}* mutation. Complementation of *un-24^{ts}* with *un-24^{PA}* results in self-incompatible colonies that exhibit escape at restrictive temperatures.

Results

Incompatibility activity correlates with an aberrant UN-24 protein complex

We sought to determine whether coexpression of the full-length UN-24 variants in *N. crassa* resulted in formation of a protein complex. We performed Western blotting using an anti-R1 antibody (Takada *et al.* 2000). We extracted proteins from self-incompatible *un-24^{OR}::un-24^{PA}* strains, an escaped *un-24^{OR}::un-24^{PA}* strain, and, as controls, the OR-background C9-2 strain transformed with *un-24^{OR}* and the PA-background C2(2)-1 strain transformed with *un-24^{PA}*. Western analyses showed that UN-24 protein from self-incompatible strains migrates to the stacking gel/running gel interface (Figure 2A). We hypothesize that this band corresponds to a non-reducible (as we used the reducing agent DTT in our extraction), high molecular weight protein complex consisting of UN-24^{OR} and UN-24^{PA} subunits. This complex may also include the HET-6^{OR} protein, since it is known that *het-6^{OR}* and *un-24^{PA}* exhibit non-allelic incompatibility (Lafontaine and Smith 2012). Furthermore, the absence of monomeric UN-24 protein suggests that all of the UN-24^{OR} and UN-24^{PA} subunits are covalently linked. The localization

Figure 2 Anti-R1 antibody binds to a high molecular weight protein complex in self-incompatible *un-24^{OR}::un-24^{PA}* transformants. (A) Protein extracts are from mouse (lane 1), self-incompatible *N. crassa* (strain C9-2 *un-24^{OR}* with ectopic copy of *un-24^{PA}*, lane 2), a post-escape self-incompatible colony (lane 3), and control C9-2 (OR, lane 4) and C2(2)-1 (PA, lane 5) strains. Size standards in kilodaltons and the location of the stacking gel (SG) and running gel (RG) are shown at the left. Opened arrowhead indicates the putative UN-24^{OR}-UN-24^{PA} protein complex in self-incompatible colonies, the open arrowhead indicates the ~103-kDa predicted size of UN-24 and the solid arrowhead indicates the ~90-kDa size of

mouse R1. Loading controls below each lane represent a Coomassie blue stained protein of ~48 kDa from a replicate gel. (B) Close-up images of compatible (top) and self-incompatible (bottom left) transformants. The bottom right shows an escape sector (E) emerging from a self-incompatible colony (SI) after 4 days incubation.

of this putative UN-24 protein complex in SDS-PAGE is consistent with the localization of other large protein complexes (Allen *et al.* 2005). A band of ~103 kDa, the expected molecular weight of the UN-24 protein, is evident in proteins from the self-incompatible colony that had undergone escape. This suggests that, upon escape, the UN-24^{OR}-UN-24^{PA} complex resolves into soluble UN-24 monomers, which permits a return to wild-type morphology and growth rate. Proteins extracted from either control strain have a band of ~103 kDa. To confirm the specificity of the anti-R1 antibody, we used proteins from mouse 3T3 fibroblasts, which produced a band of ~90 kDa, the correct molecular weight of the mouse R1 monomer. In Figure 2B, we present representative images of self-incompatible and self-compatible transformants, along with a self-incompatible colony that has undergone escape.

The incompatibility domain of UN-24^{PA} localizes to the cell-debris pellet

We next sought to determine whether, similar to the full-length variants, coexpression of the UN-24^{PA} C terminus and UN-24^{OR} would result in formation of a complex in *N. crassa*. For this we used a fusion protein that causes incompatibility when expressed in OR-background strains. This construct consists of *hph* fused in-frame to the C-terminal PA incompatibility domain [hygunPA(788–923), Figure 1]. In this manuscript, we use a naming scheme for constructs in which the range of UN-24 amino acid residues included in the fusion gene product is given in parentheses. For example, the hygunPA(788–923) construct used here contained the *un-24^{PA}* region from residue 788 to residue 923.

Previous studies have indicated that large protein complexes often localize to the pellet fraction and not in the lysate fraction (Coustou-Linares *et al.* 2001). When these large protein complexes are treated with reductants, some monomeric proteins are liberated and detected on Western blots (Allen *et al.* 2005). We hypothesized that if a complex consisting of hygunPA(788–923) and UN-24^{OR} is formed in self-incompatible colonies, then the hygunPA(788–923) monomer would not be present in the lysate fraction, but would be observed when the pellet fraction was treated with

reductants. To detect the hygunPA(788–923) protein, we inserted an HA epitope at the 3' end creating hygunPA(788–923)-HA. We extracted proteins from self-incompatible *un-24^{OR}::hygunPA(788–923)*-HA colonies and performed Western blotting. When proteins extracted from these pre-escape transformants were probed with anti-HA antibodies, the ~60 kDa hygunPA(788–923)-HA protein was not detected in the lysate fraction but was detected in the running gel when the pellet was boiled with reductants (DTT and β -mercaptoethanol, Figure 3). In contrast, the hygunPA(788–923)-HA protein monomer was detected in both the lysate and pellet fractions in proteins extracted from post-escape *un-24^{OR}::hygunPA(788–923)*-HA and in self-compatible *un-24^{PA}::hygunPA(788–923)*-HA transformants (control). Our results suggest that the UN-24^{PA} C terminus can form a complex with UN-24^{OR}, the localization and behavior of which is consistent with previous findings regarding large protein complexes (Coustou-Linares *et al.* 2001; Mathur *et al.* 2012). We could not observe colocalization of hygunPA(788–923)-HA and UN-24^{OR} in our Western blots despite using FLAG and HA epitopes placed separately at various positions within the hygunPA(788–923) construct, and with various extraction conditions and size exclusion chromatography (not shown). We did not detect hygunPA(788–923)-HA in the stacking gel in the lysate fraction from *un-24^{OR}::hygunPA(788–923)*-HA pre-escape strains. This may suggest that the PA C terminus is buried within UN-24^{OR} and is not accessible to antibodies despite our use of reducing agents.

Genetic analysis of UN-24 incompatibility domains

We sought to further explore the incompatibility domains of each UN-24 allele by testing whether the OR and PA C terminus regions alone were sufficient to trigger incompatibility activity (Figure 1). We made constructs consisting of *hph* fused in-frame with various portions of either UN-24^{OR} or UN-24^{PA} and tested these constructs for incompatibility activity using transformation assays. *un-24^{PA}* incompatibility activity was conferred by the final 63 C-terminal residues [hygunPA(861–923)] and when three residues were removed from the C terminus end [hygunPA(788–920)].

Figure 3 The UN-24^{PA} C terminus localizes to the pellet fraction in *un-24^{OR}::hygunPA(788–923)-HA* self-incompatible colonies. Protein fraction (lysate or pellet fractions) is indicated across the top. The HA-tagged C terminus of UN-24^{PA} is not evident in the lysate fraction of a pre-escape self-incompatible strain but is pronounced in proteins extracted from a post-escape strain and from the control *un-24^{PA}::hygunPA(788–923)-HA* self-compatible strain. Size standards in kilodaltons are shown at right.

Removal of six residues from the C terminus end [hygunPA(788–917)], including the putative redox-active and catalytically required C terminus cysteine pair, resulted in a loss of incompatibility activity. These residues lie in the putative flexible C terminus arm of R1 as defined in yeast (Xu *et al.* 2006; Zhang *et al.* 2007).

A larger region of *un-24^{OR}* is required for incompatibility activity since *hygunOR(788–929)* does not cause incompatibility, whereas *hygunOR(335–929)* does (Figure 1). Amino acids 335–788 encompass portions of the N-terminal catalytic pocket. Replacing C444 with a serine resulted in a loss of catalytic activity for both OR and PA forms, but interfered with incompatibility of the OR form only (Supporting Information, Figure S1). The C444 residue is homologous to one of the redox reactive cysteines (C462) within the *E. coli* R1 catalytic pocket. These results indicate that catalytically important cysteine residues are required for incompatibility activity for both UN-24 allelic variants.

C-terminal cysteines are involved in incompatibility activity

Constructs containing mutations at cysteines in the C terminus of UN-24 were assayed for incompatibility and/or catalytic activity. In addition to the conserved cysteine pair on the C terminus of all R1 subunits, we investigated a third cysteine at position 913 from UN-24^{OR} and 907 from UN-24^{PA} that has no corresponding cysteine in the *E. coli* R1. This third cysteine is found in the R1 of several eukaryotic species; for example, it is in 17 of 18 sequences from a diverse selection of eukaryotic species at a distance of 7–12 residues from the penultimate cysteine (Figure S2). The function of this third cysteine has yet to be investigated, although its prevalence in eukaryotic forms of R1 and close proximity to the reactive C-terminal cysteine pair intimate potential involvement in protein function. Furthermore, this third C-terminal cysteine (C907), along with C918, is present in our construct *hygunPA(788–920)*, which retains incompatibility activity.

We created six constructs from *hygunPA(788–923)*, each with one or two of the three terminal cysteines replaced with glycine and an additional construct having each cysteine replaced with serine (Figure 4A). We parenthetically designated these constructs to denote which of the three cysteines had been substituted. For example, *hygunPA(CCG)*, had the 3' cysteine codon altered to encode glycine. An additional construct was created from *hygunPA(788–923)* and designated

hygunPA(-PGA) because proline, glycine, and alanine at positions 911–913 found in UN-24^{PA} were deleted (Figure 4A). Sequence alignments indicate that this PGA triad is restricted to *Neurospora* (Figure S2), suggesting a possible role in incompatibility activity. Every construct in which C907 was substituted lacked incompatibility activity [e.g. *hygunPA(GCC)*]. In contrast, when either C918 or C921 was substituted, [*hygunPA(CGC)* and *hygunPA(CCG)*] incompatibility activity was maintained. Loss of incompatibility activity was observed when both C918 and C921 were substituted [*hygunPA(CGG)*], which is consistent with our earlier experiments (Figure 1) demonstrating that at least one of the two C-terminal cysteines is required for incompatibility activity. The *hygunPA(SSS)* construct was examined in case glycine substitutions altered protein structure, rather than function. This serine-substituted protein exhibited no incompatibility activity. Finally, the *hygunPA(-PGA)* construct retained incompatibility activity. Analysis of these constructs showed that redox-reactive cysteines that function in the C terminus to regenerate the catalytic pocket are involved in incompatibility.

To test similar mutations in the OR allele, we made a set of mutants derived from the full-length *un-24^{OR}* (Figure 4B). In addition to testing incompatibility activity, the ability of these constructs to complement *un-24^{ts}* was used to assess RNR catalytic activity of each. In our complementation assays we cotransformed the C8c-164 strain, which carries *un-24^{ts}*, with the pCB1004 vector conferring hygromycin B resistance and each of the *un-24^{OR}* cDNA-derivative constructs. After selection of transformed colonies on hygromycin-containing medium, subcultures of individual transformants were monitored for growth at 39°. Continuous growth by these subcultures for more than 72 hr at 39° indicated that the construct complemented *un-24^{ts}* and therefore encoded RNR activity. Colonies lacking complementation attained a colony diameter of ~4.5 cm but stopped growing by day 2 after transfer to 39° (Smith *et al.* 2000a). Substitutions of any one of the C-terminal cysteines (C913, C924, or C927 in UN-24^{OR}) with glycine resulted in a loss of catalytic and incompatibility functions. This is unlike the situation with PA constructs in which one, but not both terminal cysteines, is required for incompatibility activity. The construct *un-24^{OR}(-PGA)* maintained incompatibility, and it also complemented the *un-24^{ts}* allele however, these colonies grew significantly slower than the wild-type rates normally observed when complementation occurs. Therefore, the PGA triad is needed for efficient catalytic

Figure 4 Mutational analysis of cysteine residues involved in RNR catalysis reveal their importance in incompatibility activity. (A) PA-specific incompatibility activity of mutant forms of hygunPA(788–923). For each construct, C terminus cysteines were replaced with glycines or serines as underlined. An additional construct with the PGA amino acid triad deleted was found to have incompatibility activity. (B) *un-24^{OR}*-derived constructs were tested for RNR catalytic and incompatibility activities. Catalytic activity was based on complementation of *un-24^{ts}* and is indicated as wild-type-like growth (+), slow growth (+/-), or no growth (–) at restrictive temperatures. The constructs have a C terminus cysteine substituted with a glycine or the PGA triad deleted, as shown with underlines. In both panels, incompatibility activity is either present (+) or absent (–).

activity of UN-24^{OR}. Analysis of these amino acid substitutions implicates all three C-terminal cysteines in *un-24^{OR}*-associated incompatibility activity and in catalytic activity.

Discussion

We demonstrate that UN-24 forms a non-reducible, high molecular weight protein in self-incompatible *un-24^{OR}::un-24^{PA}* strains (consisting of UN-24^{PA} and UN-24^{OR} subunits) and that during escape, this complex resolves into monomeric UN-24 proteins. Furthermore, the PA C terminus incompatibility domain localizes to the pellet fraction in self-incompatible colonies, whereas it is found in the lysate fraction after escape. These results are similar to studies that demonstrate that high molecular weight, insoluble protein complexes resolve to the stacking gel in SDS–PAGE (Kryndushkin *et al.*

2003; Lee and Eisenberg 2003; Allen *et al.* 2005) or the pellet fraction during protein extraction (Coustou-Linares *et al.* 2001). Denaturing conditions that are similar to those used in our study fail to resolve such complexes (Coustou-Linares *et al.* 2001; Allen *et al.* 2005). The presence of the hygunPA(788–923)-HA protein in the pellet fraction (although less than observed in the self-incompatible colony) from the self-compatible colony may be due to our use of a *trpC* promoter to drive fusion protein expression. Previous studies have observed that expression from strong, constitutive promoters causes some overexpressed protein to become insoluble (Yi *et al.* 2009; Tegel *et al.* 2011), including studies that examine fungal protein complexes involved in nonself recognition (Mathur *et al.* 2012).

Our mutational analyses show that redox-active cysteine residues involved in normal RNR catalysis are required for

Figure 5 Model of incompatibility mechanism by large subunit of RNR in *N. crassa*. (A) During normal catalytic activity, the conserved redox-active cysteine pair on the C-terminal flexible arm transfers reducing equivalents from glutaredoxin (GR) or thioredoxin (TR) to the cysteine pair in the catalytic pocket. The flexible arm of one monomer moves into the catalytic site of the other monomer in the homodimer and disulfide interchange occurs. This leads to the regeneration of the catalytic cysteine pair and another round of product turnover. (B) We infer that *un-24* incompatibility proceeds following the point when the cysteine pair in the catalytic pocket of the OR protein (shaded) interacts with the

cysteine pair on the flexible arm of the PA protein (solid). We propose that an aberrant disulfide bond subsequently occurs that results in the conversion of the holoenzyme into an inactive form. This could then potentiate the formation of a higher order protein complex or protein aggregate. In both panels the small subunit dimer of holoenzyme is not shown.

incompatibility activity. In addition to a requirement for the catalytic domain for OR but not PA incompatibility activity, there are other functional differences between the two forms of UN-24. Substituting any of the three C-terminal cysteines with glycines abolishes UN-24^{OR} incompatibility activity, whereas, for PA-associated incompatibility activity, the terminal two cysteines are essential in an apparently redundant manner since the hygunPA(CGG) double mutant, but neither single mutant (CCG and CGC), showed a loss of activity. The third cysteine anterior to the C-terminal pair is critical for both OR- and PA-associated incompatibility activity and for RNR function of UN-24^{OR}. Although loss of incompatibility activity caused by the cysteine-to-glycine/serine substitutions in UN-24^{OR} could be due to changes in protein structure, our observations that replacement of the C-terminal cysteines or C444, one member of the cysteine pair that undergoes disulfide bond formation in the catalytic site, results in loss of catalytic activity suggest that the cysteine redox reactions are also necessary for incompatibility activity. Given the strong correlation between the presence of the high molecular weight complex (Figure 2A) and the presence of incompatibility, we suspect that mutation of cysteine residues that lead to the abolishment of incompatibility activity would also impair the formation of this complex, which warrants future study.

We propose a model of how allelic differences at *un-24* trigger a nonself recognition reaction (Figure 5). We propose that an aberrant disulfide bond between cysteines in the UN-24^{PA} C terminus (C907 and C918/C921) and the UN-24^{OR} catalytic site (e.g., C444) leads to an aberrant heterodimeric complex, which in turn may lead to a toxic higher order complex. This is consistent with evidence from yeast that the C-terminal domain of one R1 subunit acts in *trans* to reduce the active site of the adjoining R1 subunit (Zhang *et al.* 2007). These intermolecular disulfide bonds could align the PA and OR UN-24 proteins and facilitate new covalent or noncovalent intermolecular interactions that

may lead to a complex or aggregate that is resistant to the denaturants used in this study (*i.e.*, DTT), which have been used previously to reduce disulfide bonds (Scigelova *et al.* 2001). The formation of intermolecular disulfide bonds has been observed to potentiate the formation of protein aggregates, which are ultimately linked together via domain swapping (Lee and Eisenberg 2003). Similar to our study, these aggregates are found in the insoluble cell pellet and localize to the stacking gel in SDS-PAGE (Lee and Eisenberg 2003). It is currently unclear whether domain swapping holds a higher order UN-24 complex together or whether additional intermolecular bonds unaccounted for in our model contribute to potential oligomerization. Localization of the UN-24^{PA} C terminus to the pellet fraction in self-incompatible transformants suggests that the C terminus of UN-24^{PA} alone is sufficient to cause the hypothetical formation of a higher order protein complex. However, the ability of reductants to separate monomeric hygunPA(788–923)-HA from the pellet suggests that, in comparison to the full-length variants, the UN-24^{OR}-hygunPA(788–923)-HA complex is not as stable. This suggests a potential role for the UN-24^{PA} N terminus in maintaining the increased stability of a UN-24^{OR}–UN-24^{PA} higher order complex. To our knowledge the R2 subunit of RNR is not involved in this complex formation aside from its role in contributing the tyrosyl radical required for RNR catalytic activation.

There are several structural similarities between UN-24 and prion proteins in yeast and *Podospora anserina*. In the fungal prion proteins, Sup35p, Ure2p, and HET-s, a structured domain adjoining a flexible domain is a common feature (Balguerie *et al.* 2003; Chiti and Dobson 2006) that is also found in UN-24. Our results showed that the *un-24* region necessary for incompatibility activity encompasses the flexible C-terminal domain and the highly structured UN-24^{OR} catalytic domain. This is reminiscent of the *het-s* system, where the structured domain determines higher order aggregate organization and cytotoxicity (Balguerie *et al.* 2004), while

a truncated version encompassing the last 133 residues of the C terminus of HET-s retains prion propagating and incompatibility activities (Balguerie *et al.* 2004). The essential RNR function of UN-24 likely precludes it as a prion that propagates during normal cell growth, due to the proclivity of prions to produce loss-of-function phenotypes. However, an insoluble, and possibly prion-like, UN-24 heterocomplex presents a plausible mechanism of incompatibility activity since this could lead to growth inhibition and cell death by decreasing the amount of functional RNR and limiting the availability of DNA precursors. Incompatibility activity through disruption of an essential cellular function would be an anomaly.

Due to its essential function in the cell, RNR presents an attractive drug target. The development of peptide inhibitors that disrupt the quaternary structure of RNR is a field that may present an efficacious chemotherapeutic strategy (Cerqueira *et al.* 2005; Cooperman *et al.* 2005). Inherent within the *N. crassa* sequence of R1 already lies the potential for antibiotic-like activity, as manifested by the growth inhibition of cells resulting from nonself fusions in *N. crassa*.

Acknowledgments

We thank S. Hepworth for her comments on this manuscript. This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant to M.L.S. and NSERC CGS to R.P.S.

Literature Cited

- Aberg, A., S. Hahne, M. Karlsson, A. Larsson, M. Ormo *et al.*, 1989 Evidence for two different classes of redox-active cysteines in ribonucleotide reductase of *Escherichia coli*. *J. Biol. Chem.* 264: 12249–12252.
- Adams, A., D. Gottschling, C. Kaiser, and T. Steans, 1997 *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Allen, K. D., R. D. Wegrzyn, T. A. Chernova, S. Muller, G. P. Newnam *et al.*, 2005 Hsp70 chaperones as modulators of prion life cycle: novel effects of Ssa and Ssb on the *Saccharomyces cerevisiae* prion [PSI⁺]. *Genetics* 169: 1227–1242.
- Balguerie, A., S. Dos Reis, C. Ritter, S. Chaignepain, B. Coulary-Salin *et al.*, 2003 Domain organization and structure-function relationship of the HET-s prion protein of *Podospora anserina*. *EMBO J.* 22: 2071–2081.
- Balguerie, A., S. Dos Reis, B. Coulary-Salin, S. Chaignepain, M. Sabourin *et al.*, 2004 The sequences appended to the amyloid core region of the HET-s prion protein determine higher-order aggregate organization in vivo. *J. Cell Sci.* 117: 2599–2610.
- Biella, S., M. L. Smith, J. R. Aist, P. Cortesi, and M. G. Milgroom, 2002 Programmed cell death correlates with virus transmission in a filamentous fungus. *Proc. R. Soc. Lond. B Biol. Sci.* 269: 2269–2276.
- Carroll, A., J. Sweigard, and B. Valent, 1994 Improved vectors for selecting resistance to hygromycin. *Fungal Genet. Newsl.* 41: 22.
- Cerqueira, N., S. Pereira, P. Fernandes, and M. Ramos, 2005 Overview of ribonucleotide reductase inhibitors: an appealing target in anti-tumour therapy. *Curr. Med. Chem.* 12: 1283–1294.
- Chiti, F., and C. M. Dobson, 2006 Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* 75: 333–366.
- Cooperman, B. S., Y. Gao, C. Tan, O. B. Kashlan, and J. Kaur, 2005 Peptide inhibitors of mammalian ribonucleotide reductase. *Adv. Enzyme Regul.* 45: 112–125.
- Coustou-Linares, V., M.-L. Maddelein, J. Bégueret, and S. J. Saupe, 2001 In vivo aggregation of the HET-s prion protein of the fungus *Podospora anserina*. *Mol. Microbiol.* 42: 1325–1335.
- Elledge, S. J., Z. Zhou, J. B. Allen, and T. A. Navas, 1993 DNA damage and cell cycle regulation of ribonucleotide reductase. *Bioessays* 15: 333–339.
- Eriksson, M., U. Uhlin, S. Ramaswamy, M. Ekberg, K. Regnstrom *et al.*, 1997 Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure* 5: 1077–1092.
- Glass, N. L., and I. Kaneko, 2003 Fatal attraction: nonself recognition and heterokaryon incompatibility in filamentous fungi. *Eukaryot. Cell* 2: 1–8.
- Hutchison, E., S. Brown, C. Tian, and N. L. Glass, 2009 Transcriptional profiling and functional analysis of heterokaryon incompatibility in *Neurospora crassa* reveals that reactive oxygen species, but not metacaspases, are associated with programmed cell death. *Microbiology* 155: 3957–3970.
- Jacobson, D., K. Beurkens, and K. Klomparens, 1998 Microscopic and ultrastructural examination of vegetative incompatibility in partial diploids heterozygous at het loci in *Neurospora crassa*. *Fungal Genet. Biol.* 23: 45–56.
- Kryndushkin, D. S., I. M. Alexandrov, M. D. Ter-Avanesyan, and V. V. Kushnirov, 2003 Yeast [PSI⁺] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *J. Biol. Chem.* 278: 49636–49643.
- Lafontaine, D. L., and M. L. Smith, 2012 Diverse interactions mediate asymmetric incompatibility by the het-6 supergene complex in *Neurospora crassa*. *Fungal Genet. Biol.* 49: 65–73.
- Lee, S., and D. Eisenberg, 2003 Seeded conversion of recombinant prion protein to a disulfide-bonded oligomer by a reduction-oxidation process. *Nat. Struct. Mol. Biol.* 10: 725–730.
- Mao, S. S., T. P. Holler, G. X. Yu, J. M. Bollinger, S. Booker *et al.*, 1992 A model for the role of multiple cysteine residues involved in ribonucleotide reduction: amazing and still confusing. *Biochemistry* 31: 9733–9743.
- Mathur, V., C. Seuring, R. Riek, S. J. Saupe, and S. W. Liebman, 2012 Localization of HET-S to the cell periphery, not to [Het-s] aggregates, is associated with [Het-s]-HET-S toxicity. *Mol. Cell Biol.* 32: 139–153.
- Micali, C. O., and M. L. Smith, 2006 A nonself recognition gene complex in *Neurospora crassa*. *Genetics* 173: 1991–2004.
- Nordlund, P. R., and H. Eklund, 1993 Structure and function of the *Escherichia coli* ribonucleotide reductase protein R2. *J. Mol. Biol.* 232: 123–164.
- Pandey, A., M. G. Roca, N. D. Read, and N. L. Glass, 2004 Role of a mitogen-activated protein kinase pathway during conidial germination and hyphal fusion in *Neurospora crassa*. *Eukaryot. Cell* 3: 348–358.
- Perkins, D., 1988 Main features of vegetative incompatibility in *Neurospora*. *Fungal Genet. Newsl.* 35: 44–46.
- Reichard, P., 1993 From RNA to DNA, why so many ribonucleotide reductases? *Science* 260: 1773–1777.
- Reichard, P., 2002 Ribonucleotide reductases: the evolution of allosteric regulation. *Arch. Biochem. Biophys.* 397: 149–155.
- Sarkar, S., G. Iyer, J. Wu, and N. L. Glass, 2002 Nonself recognition is mediated by HET-C heterocomplex formation during vegetative incompatibility. *EMBO J.* 21: 4841–4850.
- Scigelova, M., P. Green, A. Giannakopoulos, A. Rodger, D. Crout *et al.*, 2001 A practical protocol for the reduction of disulfide bonds in proteins prior to analysis by mass spectrometry. *Eur. J. Mass Spectrom.* 7: 29–34.

- Smith, M., and D. Lafontaine, 2013 The fungal sense of nonself, *Neurospora: Genomics and Molecular Biology*, edited by D. Kasbekar, and K. McCluskey. Horizon Scientific Press, Norfolk, UK.
- Smith, M. L., S. P. Hubbard, D. J. Jacobson, O. C. Micali, and N. L. Glass, 2000a An osmotic-remedial, temperature-sensitive mutation in the allosteric activity site of ribonucleotide reductase in *Neurospora crassa*. *Mol. Gen. Genet.* 262: 1022–1035.
- Smith, M. L., O. C. Micali, S. P. Hubbard, N. Mir-Rashed, D. J. Jacobson *et al.*, 2000b Vegetative incompatibility in the het-6 region of *Neurospora crassa* is mediated by two linked genes. *Genetics* 155: 1095–1104.
- Takada, S., T. Shibata, Y. Hiraoka, and H. Masuda, 2000 Identification of ribonucleotide reductase protein R1 as an activator of microtubule nucleation in *Xenopus* egg mitotic extracts. *Mol. Biol. Cell* 11: 4173–4187.
- Tegel, H., J. Ottosson, and S. Hober, 2011 Enhancing the protein production levels in *Escherichia coli* with a strong promoter. *FEBS J.* 278: 729–739.
- Xu, H., C. Faber, T. Uchiki, J. W. Fairman, J. Racca *et al.*, 2006 Structures of eukaryotic ribonucleotide reductase I provide insights into dNTP regulation. *Proc. Natl. Acad. Sci. USA* 103: 4022–4027.
- Yi, M., M. Chi, C. Khang, S. Park, S. Kang *et al.*, 2009 The ER chaperone LHS1 is involved in asexual development and rice infection by the blast fungus *Magnaporthe oryzae*. *Plant Cell* 21: 681–695.
- Zhang, Z., K. Yang, C.-C. Chen, J. Feser, and M. Huang, 2007 Role of the C-terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. *Proc. Natl. Acad. Sci. USA* 104: 2217–2222.

Communicating editor: E. Selker

GENETICS

Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.147405/-/DC1>

Nonself Recognition Through Intermolecular Disulfide Bond Formation of Ribonucleotide Reductase in *Neurospora*

Robert P. Smith, Kenji Wellman, Leila Haidari, Hirohisa Masuda, and Myron L. Smith

Construct	Activity	
	Incompatibility	RNR
<i>un-24^{PA}</i> (wild-type)	PA	+
<i>un-24^{OR}</i> (wild-type)	OR	+
<i>un-24^{PA}</i> (C444>S)	PA	-
<i>un-24^{OR}</i> (C444>S)	-	-

Figure S1 The redox active cysteine, C444, is required for RNR catalytic activity, but is only required for incompatibility activity in UN-24^{OR}. Site directed mutagenesis was used to replace the C444 residue with serine. The light grey (PA) and dark grey (OR) areas at the right represent the allele specific C-terminus regions. Incompatibility activity is designated as PA or OR specific, or absent (-), and RNR catalytic function is designated as present (+) or absent (-).

Species	Accession	C-terminus sequence and aa size	Group
<i>Plasmodium falciparum</i>	U01323	TESTVT-----QNVPLRRN---NDEQCLM--SG 806	Alveolate
<i>Cryptosporidium parvum</i>	AF043243	---LIA-----PLRPTNMK---DDEECMM--SG 803	Alveolate
<i>Homo sapiens</i>	X59543	KERNTAA-----MVSL---NRDECLM--GS 792	Animal
<i>Mus musculus</i>	K02927	KERNTAA-----MVSL---NRDECLM--GS 792	Animal
<i>Danio rerio</i>	U57964	KERNKAA-----MVSL---NRDECLM--GS 794	Animal
<i>Drosophila melanogaster</i>	AAD33590	RERKMAD-----MVSL---NKDACMS--GS 812	Animal
<i>Caenorhabditis elegans</i>	Q03604	---QQA-----ETPATVAES---QDEECMLM--SG 788	Animal
<i>Glycine max</i>	AAN87548	-NTKMAQ-----MVSLT---NRDECLA--GS 808	Plant
<i>Musa acuminata</i>	AY484588	VEAKMAQ-----VVSLE---NREECMA--GS 810	Plant
<i>Aspergillus fumigatus</i>	EAL90119	RESDIYS-----QKVLCSIE---NKEACLM--QG 859	Fungus
<i>Schizosaccharomyces pombe</i>	X65116	EEIDIYN-----EKVLAISK---NPEACEM--SA 811	Fungus
<i>Yarrowia lipolytica</i>	XM_506109	SEYDIYA-----DKVLAISLA---NPESCEM--SG 892	Fungus
<i>Candida albicans</i>	CAB77640	LENDIYS-----AKVIAAID---NPESCMTM--SG 854	Fungus
<i>Saccharomyces cerevisiae</i>	NP_010993	TEFDIYN-----SKVIAAID---NPEACEM--SG 888	Fungus
<i>Gibberella zeae</i>	XM_385350	RERDIYS-----EAVLCSIE---DPESCIM--SG 913	Fungus
<i>Podospora anserina</i>	Pa_6_4460	REVDIYS-----EAVLAISIE---NPEACVM--SG 907	Fungus
<i>Neurospora crassa (OR)</i>	AF171697	RDENIYSNAPLSEQQVAAAWNPGADPSSCEM--SG 929	Fungus
<i>Neurospora crassa (PA)</i>	DQ525966	REGDIYS-----DEVVSPPYVPGADPSSCVM--SG 923	Fungus
<i>Escherichia coli</i>	X06999	-----DDCESGACKI 761	Prokaryote

Figure S2 Alignment of RNR large subunit C-terminus regions from multiple eukaryotes and *E. coli* demonstrates conservation of the C-terminal cysteine residues and the unique PGA triad in *N. crassa* UN-24. The two most C-terminal cysteines (highlighted) are conserved in all the eukaryotes shown and in *E. coli*. The third, left-most cysteine shown (also highlighted) is present in 17 of the 18 eukaryotic sequences shown. The highlighted PGA triad is unique to UN-24 but does not apparently influence incompatibility function.