Analysis of Polymorphic Residues Reveals Distinct Enzymatic and Cytotoxic Activities of the *Streptococcus pyogenes* NAD\(^+\) Glycohydrolase*\(^*\)\(^*\)

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**Background:** Two phenotypic variants of the *Streptococcus pyogenes* NAD\(^+\) glycohydrolase SPN exist among clinical isolates. One lacks NADase activity.

**Results:** There are 9 polymorphic residues. Three residues are responsible for differences in NADase activity; however, both variants have equivalent cytotoxicity.

**Conclusion:** SPN is a multifunctional toxin.

**Significance:** Learning how evolution has adapted multiple SPN activities is crucial for understanding its contribution to tissue tropism.

The *Streptococcus pyogenes* NAD\(^+\) glycohydrolase (SPN) is secreted from the bacterial cell and translocated into the host cell cytosol where it contributes to cell death. Recent studies suggest that SPN is evolving and has diverged into NAD\(^+\) glycohydrolase-inactive variants that correlate with tissue tropism. However, the role of SPN in both cytotoxicity and niche selection are unknown. To gain insight into the forces driving the adaptation of SPN, a detailed comparison of representative glycohydrolase-proficient and -deficient variants was conducted. Of a total 454 amino acids, the activity-deficient variants differed at only nine highly conserved positions. Exchanging residues between variants revealed that no one single residue could account for the inability of the deficient variants to cleave the glycosidic bond of β-NAD\(^+\) into nicotinamide and ADP-ribose; rather, reciprocal changes at 3 specific residues were required to both abolish activity of the proficient version and restore full activity to the deficient variant. Changing any combination of 1 or 2 residues resulted in intermediate activity. However, a change to any 1 residue resulted in a significant decrease in enzyme efficiency. A similar pattern involving multiple residues was observed for comparison with a second highly conserved activity-deficient variant class. Remarkably, despite differences in glycohydrolase activity, all versions of SPN were equally cytotoxic to cultured epithelial cells. These data indicate that the glycohydrolase activity of SPN may not be the only contribution the toxin has to the pathogenesis of *S. pyogenes* and that both versions of SPN play an important role during infection.

Within a single bacterial species, allelic variation of a specific gene can arise through the process of niche specialization. For bacterial pathogens, this can occur when a generalist population diverges into distinct subpopulations with strong tropism for different hosts or for different tissues within the same host. Diversity emerges as continued selection pressure results in variants of virulence genes that function to increase fitness for infection of a particular niche. Understanding the functional consequences of these changes can provide important insights into how a specific virulence factor contributes to exploitation of a niche and its role in pathogenesis.

Variation in the *Streptococcus pyogenes* (group A streptococcus) NAD\(^+\) glycohydrolase (SPN\(^3\); also known as NGA) toxin has been associated with niche specialization. This Gram-positive pathogen is one of the most versatile pathogens of humans capable of causing both superficial and invasive diseases, including pharyngitis, impetigo, and necrotizing fasciitis, as well as postinfection sequelae such as rheumatic fever and acute glomerulonephritis. Part of this versatility can be attributed to its ability to secrete a multitude of proteins that affect host cell function in numerous ways (1). One of these is SPN, which was originally identified by its ability to cleave the nicotinamide-riboyl bond of β-NAD\(^+\) to produce nicotinamide and adenosine diphosphoribose. All *S. pyogenes* strains examined to date possess the gene encoding SPN (2). However, more recent studies have shown that these *spn* alleles exhibit diversity. Furthermore, SPN is evolving under positive selection and is diverging into multiple subtypes, including subtypes that lack its signature NAD\(^+\) glycohydrolase (NADase) activity. Neither the function of the NADase-inactive subtypes nor the molecular basis of their loss of activity is well understood.

However, it is understood that although there is not a clear consensus as to whether SPN subtypes have any association with isolates that can cause invasive disease there is a strong association between SPN subtype and tissue tropism. Most

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\(^\text{3}\) The abbreviations used are: SPN, *S. pyogenes* NAD\(^+\) glycohydrolase; NADase, NAD\(^+\) glycohydrolase; IFS, immunity factor for SPN; CMT, cytolyisin-mediated translocation.
cases of *S. pyogenes* infection are superficial and occur at one of two tissue sites: the throat (pharyngitis) or the skin (impetigo). Substantial epidemiological evidence indicates that there are distinct subpopulations of strains that are specialized for infection of just one of these two tissues (specialists). There is also a distinct subpopulation (generalists) that readily infects either tissue. Analysis of a collection of 113 strains that was assembled to maximize diversity revealed that intact alleles of *spn* were found in all strains and that NADase-active and -inactive haplotypes were equally prevalent (2). Of interest, tissue and throat specialist strains correlated with NADase-inactive SPN, whereas generalist strains correlated with NADase-active SPN. The reason underlying these associations is unknown. Furthermore, because there is evidence that the NADase activity of SPN can contribute to pathogenesis, the prevalence of NADase-inactive SPN is unclear.

The observation that NADase-inactive SPN remains under positive selection suggests that it does contribute to pathogenesis. Support for this idea comes through analysis of an endogenous competitive inhibitor of the NADase activity of SPN known as immunity factor for SPN (IFS). The gene encoding IFS is located immediately adjacent to *spn*, and the ability of *S. pyogenes* to produce NADase-active SPN is absolutely dependent on IFS, which acts to inhibit self-toxicity that may arise from any SPN molecules that inadvertently fold prior to their export from the streptococcal cell (3). As expected for an essential activity, *ifs* has very little sequence divergence in these strains. However, NADase-inactive SPN haplotypes are associated with nonfunctional truncated alleles of *ifs* that are undergoing a pattern of random nucleotide change characteristic of a pseudogene. Thus, although *ifs* loses selective constraint and becomes non-functional, the same degradation does not occur for NADase-inactive *spn*, which remains under positive selection.

During infection, SPN undergoes complicated interactions with host cells that provide some clues to its role. Although *S. pyogenes* is an extracellular pathogen, SPN is delivered into the host cell cytoplasmic compartment by a process termed cytolyisin-mediated translocation (CMT). In this process, *S. pyogenes* first attaches to a host cell and exports SPN via the general secretory pathway. SPN is then translocated across the host cell membrane by an unknown mechanism that requires a second streptococcal protein, the cholesterol-dependent cytolsin streptolysin O (SLO) (4). Analyses of CMT have revealed that SPN has multiple domains, including an N-terminal domain required for translocation and a C-terminal enzymatic domain (5). Both active and inactive SPN haplotypes undergo CMT, and *S. pyogenes* mutants defective for expression of either SPN or SLO have a reduced cytotoxic effect for host epithelial cells. How these activities may contribute to niche specialization is not known.

Multiple enzymatic activities have been attributed to NADase-active SPN that could contribute to cytotoxicity, including ADP-ribosyltransferase, ADP-ribosyl cyclase, and NADase activities (6, 7). The structure of the enzymatic domain of SPN has recently been solved and shows that it is related to the ADP-ribosyltransferase family of bacterial toxins (8). However, a re-evaluation of the enzymatic activity of SPN using highly purified recombinant protein has shown that SPN functions strictly as an NADase (9). Interestingly, comparison of NADase-active and -inactive subtypes reveals that the inactive haplotypes segregate into only two Bayesian clusters, and these differ only minutely from the active haplotypes, varying at only 9–10 amino acid residues of 454 total. Of these, only 5–6 polymorphic residues are located in the enzymatic domain, and all were identified as sites that are evolving under positive selection (2).

Although minor, these polymorphisms contribute to a significant difference in enzymatic activity and may affect pathogenesis and niche specialization. Thus, to gain greater insight into the function of SPN, its two phenotypic subtypes, and the forces that are driving their evolution, we conducted a detailed analysis of the effect of polymorphism on enzymatic activity and the contribution of enzymatic activity to cytotoxicity. These studies revealed that differences in enzymatic activity were the result of polymorphism at not one but rather multiple residues. Furthermore, this analysis revealed an unexpected NADase-independent cytotoxic activity that was retained by the enzymatically inactive haplotypes. Together, these data reveal that SPN has multiple activities and provide insight into its molecular evolution.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—The studies with *S. pyogenes* utilized the M serotype 6 strain JRS4 (10) and derivatives, including SPN1 (Δspn; Ref. 4) and Joy1, which expresses SPN<sub>14</sub> with a C-terminal influenza hemagglutinin (HA) epitope tag. Chromosomal DNA from strain HSC5 (11) was used a template in PCRs for molecular cloning of SPN<sub>14</sub>. Other molecular cloning and protein expression studies utilized *Escherichia coli* TOP10 (Invitrogen). Routine culture of *S. pyogenes* and *E. coli* was conducted using Todd Hewitt yeast extract and Luria-Bertani media, respectively, as described (9). Where appropriate, antibiotics were used at the following concentrations: chloramphenicol, 7.5 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes*; erythromycin, 500 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*; and carbenicillin, 50 μg/ml for *E. coli*.

**Manipulation of DNA**—*E. coli* was transformed using the method of Kushner (12), and *S. pyogenes* was transformed by electroporation (13). Plasmid DNA was isolated by standard techniques, and all enzymes, including restriction endonucleases, ligases (New England Biolabs), and polymerases (Pfx, Invitrogen), were used according to the manufacturers’ recommendations. All site-specific mutations described in the text were generated by PCR with the mutagenic oligonucleotide primers listed in supplemental Tables S1 and S2 and the DpnI digestion method to degrade template DNA using a commercial kit (QuikChange XL kit, Stratagene). Fidelity of all DNA sequences generated by PCR was verified by DNA sequence analyses performed by commercial vendors (SeqWright, Galveston, TX; GeneWiz, South Plainfield, NJ).

**Expression of SPN derivatives in *S. pyogenes***—Plasmids for expression of SPN in *S. pyogenes* were based on pJOY3 (5) and include pJOY7, which expresses SPN<sub>14</sub> with a C-terminal HA epitope tag. A fragment containing SPN<sub>14</sub> was amplified from HSC5 chromosomal DNA using primers oJOY5 and oJOY6.
(supplemental Table S1) and introduced between the EcoRI and BstEII sites of pJOY7. The resulting plasmid (pJOY39) expresses SPNJ45 with a C-terminal HA tag (supplemental Table S1). The various site-specific mutations were then introduced into pJOY7 and pJOY39 by PCR using the primers listed in supplemental Table S1. For expression and analysis, each plasmid was used to transform the SPN deletion strain SPN1, and the resulting strains were analyzed by the assays described below. Plasmid constructions are summarized in supplemental Table S1.

Expression and Purification of Recombinant SPNs—PCR was used to introduce the various site-specific mutations of interest into a derivative of the E. coli expression plasmid pBAD (Invitrogen) that encodes a His6-tagged SPNJ4 (pMAM3.18; Ref. 3). Mutagenic primers and the relevant features of this panel of expression plasmids are summarized in supplemental Table S2. Expression and purification of the His6-tagged recombinant proteins was performed as described previously (9). As a final step, the purified proteins were dialyzed at 4 °C against a buffer consisting of 50 mM potassium phosphate and 100 mM sodium chloride. Purity of the 48.6-kDa proteins was routinely assessed by SDS-PAGE and staining with Coomassie Brilliant Blue, and protein concentrations were determined using a BCA assay (Pierce) with a BSA standard.

Single Copy S. pyogenes SPN Expression Strains—SPNJ4 and SPNJ16 were amplified by PCR from templates pJOY7 and pJOY39, respectively, using primers oJOY180 (5′-CGGTG-GTTTACTCGAGAAACAAAAAAGTAACATTAGC-3′) and oJOY6 (see supplemental Table S1). The resulting fragments were inserted between the XhoI and PstI sites of pBRs233 (14) to produce pJOY109 and pJOY110. Site-directed mutagenesis of these two plasmids was performed to generate spn mutants described in supplemental Table S3. These plasmids were then used to replace the native spn allele in JRS4 by a standard method (15) to generate the designated strains in supplemental Table S3. Chromosomal sequences were verified by DNA sequence analysis of PCR products generated using appropriate primers.

Tests for ifs Essentiality—The requirement for ifs to support viability of strains expressing SPN of differing NADase activities was assessed by two complementary mutational strategies. In the first, an attempt was made to replace ifs with the ada9 spectinomycin resistance gene. In the second, an attempt was made to insertionally inactivate ifs using an integrational plasmid. Both strategies were based on established methods using plasmids with conditional temperature-sensitive replication (14). The construction of the two mutagenic plasmids and steps involved in mutagenesis are described in detail in the supplemental Experimental Procedures. Supplemental Fig. S3, and Tables S4 and S5. It was concluded that ifs was essential in a given strain if both strategies failed to generate an ifs-deficient mutant. In successful ifs mutants, DNA sequence analyses of spn and its promoter were conducted to confirm that each mutant retained the expected spn allele.

β-NAD+ Glycohydrolase Activity of SPN from S. pyogenes—A fluorometric assay was used to analyze the β-NAD+ glycohydrolase activity in culture supernatants from S. pyogenes strains expressing the various SPN constructs (9). Concentrations of SPN in culture supernatants were normalized by immunoblotting to detect an HA epitope tag as described previously (9). Specific activities of each SPN mutant are reported relative to wild type JRS4 as described (5). Data presented are derived from three independent experiments, each performed in triplicate.

β-NAD+ Glycohydrolase Activities of Recombinant Enzymes—Rates of β-NAD+ cleavage were determined by analytical HPLC as described (supplemental Fig. S2) (9). In initial trials, it was found that inconsistencies in results obtained from several of the low activity enzymes could be eliminated by the inclusion of NaCl in the reaction mixture (100 mM). Because this had no effect on the K_m of SPNJ4, all analyses were conducted using this modification. Briefly, the molar concentrations of SPN indicated in the text were incubated with 2.5 mM β-NAD+ in 100 μl of reaction buffer (50 mM potassium phosphate, 100 mM sodium chloride, pH 7.4, 0.5 mg/ml BSA) at 37 °C. Reactions were quenched by the addition of 100 μl of 20% ice-cold perchloric acid and incubated on ice for 30 min to precipitate protein and BSA. Precipitates were removed by centrifugation, supernatants were diluted into 980 μl of deionized water, and the mixture was subjected to reverse-phase HPLC (SunFire; C18 column, Waters; 5 μm, 4.6 × 250 mm) developed isocratically with 1% (v/v) acetonitrile in buffer A (10 mM diammom phosphate buffer, pH 6.4) with a flow rate of 1.5 ml/min over 22 min. Quantitation of reaction products and determination of initial reaction rates and other kinetic parameters were performed as described in detail elsewhere (9). Data presented are derived from three independent experiments, each performed in triplicate.

End Point β-NAD+ Glycohydrolase Activity Assay—A single end point assay to determine the amount of uncleaved β-NAD+ remaining following reaction with various SPN derivatives was conducted as a follows. Enzyme (20 pmol) was incubated in reaction buffer (50 mM potassium phosphate, 100 mM sodium chloride, pH 7.4, 0.5 mg/ml BSA) with 1 mM β-NAD+ for 1, 5, 7, and 20 h at 37 °C. Reactions were quenched by chromatography using a centrifugal column (Millipore; 3-kDa exclusion). The flow-through fraction was diluted into deionized water and analyzed by reverse-phase HPLC as described above. Data presented are derived from three independent experiments, each performed in triplicate.

Infection of Epithelial Cells—Analysis of CMT and cytotoxicity were assessed following infection of A549 epithelial cells conducted as described in detail elsewhere (4). Briefly, various S. pyogenes strains were cultured overnight, back-diluted, grown to midlogarithmic phase, and used to infect confluent monolayers of A549 cells. Following incubation for 285 min, cells were lysed by the addition of saponin to a final concentration of 0.1%, the lysate was fractionated by ultracentrifugation, and the concentration of SPN in the cytosolic fraction was determined by immunoblotting to detect the HA epitope tag. Efficiencies of CMT were quantitated relative to a strain expressing SPNJ4 as described (5). Cytotoxicity of various SPN-expressing S. pyogenes strains was assessed by the ability of infected cells to exclude the membrane-impermeable fluorescent probe ethidium homodimer-1 (LIVE/DEAD®; catalog number L3224, Invitrogen) as described previously (4). Data
its native strain JRS4 (SPN1) engineered to contain an internal deletion of SPNJ4 (from strain JRS4) and NADase activity-deficient SPNH5 (SPN12). A direct comparison between NADase-active and -inactive Bayesian clusters (clusters 2 and 3, respectively) is shown in Figure 1, where indicated, differences between observed experimental mean values were tested for significance using the Tukey-Kramer multiple comparisons test. The null hypothesis was rejected for p < 0.05.

RESULTS

SPN Diversity—As discussed above, all S. pyogenes genomes analyzed to date possess the gene encoding SPN. However, it has been recognized that this population has distinct functional heterogeneity, including haplotypes that lack the signature NADase activity of SPN. Our prior analysis of 113 genomes from a strain collection assembled to reflect diversity indicated that these SPN haplotypes grouped into four Bayesian clusters, two of which lacked detectable NADase activity (2). For the present study, we chose two strains (JRS4 and HSC5) whose SPN haplotypes were representative of the most common NADase-active and -inactive Bayesian clusters (clusters 2 and 3, respectively). A direct comparison between NADase-active SPNJ4 (from strain JRS4) and NADase activity-deficient SPNH5 (from strain HSC5) revealed the presence of 9 polymorphic residues, each of which has previously been shown to be under positive selection in the activity-deficient haplotypes (2). Of these, three were located in the translocation domain with the remaining residues distributed throughout the enzymatic domain (Fig. 1). As is typical, the gene encoding the NADase-active enzyme (SPNJ4) is associated with an intact gene for IFS, the endogenous inhibitor of SPN, whereas that of the activity-deficient protein (SPNH5) is associated with an intact (full-length) or degraded (truncated) gene encoding IFS, the endogenous inhibitor of SPN.

Multiple Polymorphic Residues at the C-terminal End of SPN Contribute to Enzymatic Activity—To determine which polymorphic residues may affect NADase activity, mutations were made to switch residues in NADase-active SPNJ4 to their counterparts from activity-deficient SPNH5. To provide a uniform background for comparison, the mutant genes were expressed from a plasmid vector that was introduced into a derivative of strain JRS4 (SPN1) engineered to contain an internal deletion of its native spn chromosomal locus (4). All proteins were found to be expressed at equivalent levels following analysis of cell-free culture supernatants by Western blotting (data not shown). These supernatants were then subjected to a standard end point titer assay to determine the relative levels of NADase activity produced by each protein. Individual swaps of the residues located in the translocation domain had no significant effect on the ability of the proteins to cleave a β-NAD" glycohydrolase activity substrate (R103H, G136R, and M195I; Fig. 2) as compared with the native SPNJ4 (Fig. 2, Unmod). Similarly, swaps of the 3 C-terminal residues of the enzymatic domain (L199I, Q253H, and L280V; Fig. 2) also did not affect activity. In contrast, swaps of the 7th and 9th polymorphic residues (R289K and I374V) resulted in a significant reduction in NADase activity (p < 0.01; Fig. 2). Consistent with prior reports (2, 16), a swap of the 8th polymorphic residue (G330D) resulted in an undetectable level of NADase activity in these supernatants (Fig. 2). These data indicate that the activity of SPNH5 is influenced by varying degrees by 3 different C-terminal polymorphic residues.

Conversion to an NADase-active Enzyme Requires 3 Residues—Reciprocal exchanges of the 3 C-terminal residues affecting activity of SPNJ4 were then made in NADase-inactive SPNH5. Starting with the most N-terminal amino acids, a swap at residue 289 of SPNJ4 (K289R) did not increase the NADase activity of the resulting protein above background levels (Fig. 2). Also, despite its dramatic effect on reduction of SPNJ4 activity, the reciprocal exchange at residue 330 of SPNH5 (D330G) resulted in only a very modest increase in NADase activity to levels less than 3.0% of SPNJ4 (Fig. 2, K289R/D330G). An exchange of both of these C-terminal residues resulted in a protein with a significant increase in activity over the single swap proteins (p < 0.01; Fig. 2). Multiple swaps of the enzymatic domain (L199I, Q253H, and L280V; Fig. 2) did not increase the NADase activity of the resulting protein above background levels (Fig. 2). Also, despite its dramatic effect on reduction of SPNJ4 activity, the reciprocal exchange at residue 330 of SPNH5 was obtained by the reciprocal swap of all 3 C-terminal residues (Fig. 2, K289R + D330G + V374I). Thus, all of these residues make a critical contribution to the functional difference between these two naturally occurring variants of SPN.

SPNJ4 Single and Double Residue Swap Proteins Have Detectable Activity—In our prior analysis of 113 clinical isolates, all alleles from NADase-active Bayesian clusters 1 and 2 contained the identical C-terminal residues at the positions analyzed above for SPNJ4 (2). Similarly, all activity-deficient cluster 3 proteins had the identical polymorphisms at these positions.
found in SPNJ4 (2). Thus, no naturally occurring intermediates containing just one or any combination of two of these polymorphisms were observed. To gain greater insight into this distribution, the properties of the engineered intermediate proteins were analyzed further. Recombinant versions of the intermediates were purified, and their reaction products following incubation with $\beta$-NAD$^+$ were analyzed by a more sensitive HPLC-based assay. After a 1-h reaction under these conditions, essentially all $\beta$-NAD$^+$ was converted to nicotinamide and ADP-ribose by SPNJ4, whereas all $\beta$-NAD$^+$ remained uncleaved following reaction with SPNH5 (Fig. 3, A and B). In contrast to the assay described above, the R289K protein did not appear different from unmodified SPNJ4, and the G330D protein readily consumed substrate although to a much lower extent than SPNJ4 (Fig. 3, A and B). The ability of a double swap protein with these substitutions (R289K/G330D) to consume substrate was further reduced than by either single swap, although it was still more active than SPNH5 (Fig. 3, A and B). Similarly, the introduction of the I374V along with G330D resulted in a more inactive enzyme than the G330D protein alone (Fig. 3, A and B). Only when all three polymorphisms were combined in the same protein did activity become indistinguishable from SPNJ4 (Fig. 3, A and B). When combined with the results described above, these data suggest that the single and double swap proteins retain at least partial activity.

**The NADase-deficient Bayesian Cluster 4 Protein Has Partial Activity**—As discussed above, Bayesian cluster 4 proteins represent a second distinct cluster of NADase-deficient SPN in clinical isolates with a truncated immunity factor (supplemental Fig. S1). These cluster 4 proteins differ from the activity-proficient enzymes at 2 important residues. First, they share the G330D polymorphism with the cluster 3 SPNH5 (Fig. 4A). Second, instead of the lysine for arginine substitution found at residue 289 in SPNH5, the cluster 4 proteins substitute an asparagine (Fig. 4A). Finally, unlike the cluster 3 proteins, the cluster 4 proteins do not differ from the activity-proficient (SPNJ4) proteins at residue 374 (Fig. 4A). Single and double swaps of these polymorphic residues were introduced into SPNJ4 followed by purification and analysis utilizing an HPLC-based assay as describe above. Similar to the cluster 3 R289K polymorphism, the cluster 4 R289N single swap protein retained an appreciable ability to consume substrate (Fig. 4B). However, when combined with G330D, the resulting double swap protein failed to consume substrate and was indistinguishable from SPNH5 (Fig. 4B). Nevertheless, upon an extended period of incubation (up to 20 h), the cluster 4 double swap protein did demonstrate some activity, whereas both SPNH5 and its corresponding triple swap protein (R289K/G330D/I374V) failed to consume any $\beta$-NAD$^+$ (Fig. 4C). Taken together, these data indicate that polymorphism at two positions contribute to the NADase-deficient property of the cluster 4 protein, although it is more active than the cluster 3 protein.

**Each C-terminal Polymorphism Reduces the Rate of Enzymatic Activity**—To gain greater insight into the effect of each polymorphic residue on enzymatic activity, the kinetic properties of the various swap proteins were analyzed. Analysis of the SPNJ4 derivative modified at residue 289 revealed a modest (<2-fold) decrease in $k_{cat}$ (Table 1, R289K and R289N). However, despite being able to cleave substrate at a fast rate, alterations at this position did have a marked effect on catalytic efficiency for the R289K protein, resulting in over a 10-fold increase in $K_m$ and an ~20-fold decrease in efficiency ($k_{cat}/K_m$; Table 2). The modification at residue 330 was more inhibitory, resulting in over a 5,000-fold decrease in $k_{cat}$ (Table 1, G330D), and in combination with other cluster 3 polymorphisms resulted in up to an 8-fold further decrease in $k_{cat}$ (Table 1). In the enzyme modified with both of the polymorphic residues of the cluster 4 protein, $k_{cat}$ was reduced nearly 60,000-fold, whereas for the enzyme with all 3 of the cluster 3 residues, $k_{cat}$ was below the limit of the assay (reduced over $2 \times 10^8$-fold versus SPNJ4; Table 1). For all proteins containing the G330D polymorphism, it was not possible to determine $K_m$ at the concentrations of $\beta$-NAD$^+$ obtained in the assay. Furthermore, attempts to measure NAD$^+$ binding to the NADase-inactive
SPN were unsuccessful in part due to a high $K_m$. Taken together, these data implicate a role for these 3 polymorphic residues in influencing substrate affinity.

**IFS Is Not Essential in Strains Encoding Minimally Active SPN**—To protect its own pool of intracellular $\beta$-NAD$^+$, *S. pyogenes* encodes IFS, an inhibitor of the NADase activity of SPN (3). However, in strains with an NADase-negative allele, the gene encoding IFS has accumulated mutations and has become a pseudogene (2, 3). Thus, to gain insight into the co-evolution of IFS and SPN, it was of interest to determine the requirement for IFS in strains of intermediate NADase activity. A test for essentiality was conducted using two different mutational strategies to determine whether the IFS gene could be replaced or insertionally inactivated in strains expressing various *spn* alleles. Consistent with prior results (3), it was not possible to replace or inactivate *ifs* in a strain expressing SPNJ4 (JRS4; Table 3 and supplemental Table S5). However, when *spn* was deleted from this strain, mutations in *ifs* were readily obtained ($\Delta$SPN; Table 3 and supplemental Table S5). Similarly, *ifs* was not essential for expression of the activity-deficient triple swap protein or any of the intermediate strains expressing a low activity SPN (Table 3 and supplemental Table S5). The exception was the intermediate strain of highest NADase activity (R289K) for which no *ifs* insertion or replacement mutants could be recovered (Table 3 and supplemental Table S5). These results show that IFS is not absolutely required for expression of NADase-active SPN but is required when a certain threshold of activity is exceeded.

**NADase-deficient SPN Is Cytotoxic to Eukaryotic Cells**—The observation that both clusters of NADase activity-deficient SPN are maintained under positive selection whereas the gene encoding IFS degrades into a pseudogene (2) suggests that the cluster 3 and cluster 4 proteins make an important contribution to pathogenesis that is independent of NADase activity. One documented function for NADase-proficient SPN is that it is cytotoxic for mammalian cells when translocated into their cytosolic compartment via CMT (17, 18). To gain insight into the role of NADase activity in this context, the native *spn* allele of JRS4 (Fig. 5A) was modified so that various SPN proteins containing an influenza HA epitope tag (Fig. 5B) would be expressed from an identical host background for comparison with a derivative with an in-frame deletion (SPN1; Fig. 5A and

![Figure 3. SPN J4 single and double swap proteins have NADase activity.](image-url)

The various polymorphic residues from SPNH5 were introduced into SPNJ4 as indicated in the figure. The NADase activity of purified proteins was then analyzed using an HPLC-based assay. A, representative HPLC chromatograms showing the products of enzymatic cleavage of $\beta$-NAD$^+$ following a 1-h reaction at 37 °C. Also evaluated was a reaction mixture lacking protein (NAD control). Identities of the various products, including $\beta$-NAD$^+$ (NAD), nicotinamide, and ADP-ribose (ADPr) are indicated at the top of the figure. B, relative activity of each of the proteins shown in A quantitated as the percentage of the $\beta$-NAD$^+$ substrate remaining uncleaved following a 1-h reaction. BL indicates that values were below the limit of detection (<0.05%), and an asterisk indicates that significantly more substrate was consumed when compared with SPNH5 ($p < 0.05$). Data presented are the mean and S.E. (error bars) derived from at least three independent experiments.
Ref. 4). These included a triple mutant of SPNJ4 (R289K/G330D/I374V) and SPNH5 (Fig. 5B), which were as efficiently translocated into A549 cell cytosols following infection as the SPNJ4 derivative (Fig. 5C, inset). Surprisingly, although the deletion strain was minimally cytotoxic as expected (SPN1; Fig. 5C), the strains expressing both NADase activity-deficient proteins were as cytotoxic as the strain expressing the SPNJ4 protein with nearly all A549 cells showing evidence of membrane compromise following 7 h of infection (Fig. 5C). These data suggested that NADase activity could be uncoupled from cyto-

FIGURE 4. Cluster 4 swaps have low but detectable NADase activity. A, residues polymorphic between the Bayesian cluster 2 (SPNJ4) and cluster 4 proteins are shown. An asterisk indicates that the cluster 4 residue corresponds to the residue in the cluster 3 NADase activity-deficient (SPNH5) protein (compare with Fig. 1A). B, polymorphic residues from NADase activity-deficient cluster 4 were introduced into SPNJ4 as indicated and analyzed as described in Fig. 3. Shown are representative HPLC chromatograms indicating the products of enzymatic cleavage of β-NAD⁺ following a 1-h reaction. The β-NAD⁺ control is a reaction that does not include enzyme. C, relative activity of selected proteins following the extended incubation periods shown in the figure. Data presented are the mean and S.E. (error bars) derived from at least three independent experiments.
TABLE 1

| Enzymatic properties of SPN_{4A} variants | Bayesian cluster$^c$ | k_{cat} (min^{-1})$^d$ | Fold difference$^c$ |
|-----------------------------------------|----------------------|------------------------|---------------------|
| SPNJ4                                   | 2                    | 2.21 ± 0.25 × 10^3     |                     |
| R289K                                   | 3                    | 1.40 ± 0.12 × 10^3     | 1.58                |
| R289N                                   | 4                    | 1.26 ± 0.08 × 10^3     | 1.75                |
| G330D                                   | 3, 4                 | 3.96 ± 1.5             | 5.580               |
| G330D/1374V                             | 3                    | 11.5 ± 0.7             | 1.9 × 10^11         |
| R289K/G330D                             | 3                    | 6.3 ± 0.2              | 3.5 × 10^4          |
| R289N/G330D                             | 4                    | 3.8 ± 0.4              | 5.8 × 10^4          |
| R289K/G330D/1374V                       | 3                    | <1.0 × 10^{-3}         | >2.6 × 10^10        |

$^a$ Variants were derived from SPNJ4. Recombinant proteins were purified from E. coli as described under “Experimental Procedures.”

$^b$ Based on Riddle et al. (2). The number indicates the cluster(s) from which the polymorphism is derived.

$^c$ Determined using the HPLC-based assay modified as described in the text. Data represent mean ± S.E. from at least three independent experiments.

$^d$ Relative to SPNJ4.

TABLE 2

| Catalytic efficiency of SPNJ_{4A} and R289K | k_{cat}$^a$ | k_{cat}/K_{m} | 1/k_{cat} | Fold change$^b$ |
|-------------------------------------------|------------|--------------|--------|-----------------|
| SPNJ_{4A}                                  | 175 ± 30   | 1.263        | 0.4    | 5.8             |
| R289K                                     | 2,140 ± 290| 65           | 1263   | 1               |

$^a$ Variants and enzyme assays are described in Table 1.

$^b$ Determined using the HPLC-based assay modified as described in the text. Data represent mean ± S.E. from at least three independent experiments.

$^c$ k_{cat} from Table 1.

TABLE 3

| Essentiality of IFS in strains encoding various SPN variants | SPN variant | NADase activity$^b$ | IFS essentiality$^c$ |
|-------------------------------------------------------------|-------------|---------------------|---------------------|
| Joy1                                         | SPNJ_{4A}   | ++ +                | Yes                 |
| Suki3                                        | SPN_{R289K} | ++ +                | Yes                 |
| Suki4                                        | SPN_{G330D} | +                  | No                  |
| Suki5                                        | SPN_{R289K/G330D} | +                  | No                  |
| Suki2                                        | SPN_{IFN}   | –                  | No                  |
| Joy10                                        | SPNJ_{3A}   | –                  | No                  |
| HSC5                                       | SPNJ_{6A}   | –                  | No                  |

$^a$ Recipient of IFS-inactivating plasmids. Strains were generated as described in supplemental Table S3.

$^b$ Defined as described in Table 1 as -fold change in k_{cat} relative to SPNJ_{4A} as follows: ++ ++, equal to SPNJ_{4A}; ++, 1–2-fold lower; +, 10^3-fold lower; −, 10^6-fold lower.

$^c$ Defined by the ability to recover a viable mutant with a deletion or insertion mutation in ifs as described under “Experimental Procedures.” Yes, IFS is essential; No, IFS is dispensable.

SPN Enzymatic Variants Contribute to Cell Death

Understanding the functional consequences of polymorphism can provide insight into how a specific virulence factor has been adapted in disparately evolving lineages, including those that are diverging with regard to niche selection. The present analysis of SPN has revealed that differences in enzymatic activity, revealing that NADase activity is not the sole function of SPN that contributes to cytotoxicity. To confirm this, the biglutamic acid motif previously shown to be essential for catalysis (9) was exchanged for glycines in all three of these proteins, and cytotoxicity was assessed. Again, all derivatives demonstrated an ability to damage A549 cells as compared with the deletion strain (Fig. 6). The strains expressing the proteins derived from either the triple mutant or SPNJ_{15} were not as cytotoxic as the SPNJ_{4} derivate, but this likely reflects a lower level of translocation into the host cell cytosols (Fig. 6, inset).

DISCUSSION

The evolution of IFS is likely intimately linked with that of SPN. Consistent with the scheme described above, IFS is no longer essential when paired with an SPN that has the G330D polymorphism. The development of this variant would then release ifs from selective pressure to begin its transition to a pseudogene. If correct, this model implies several things about SPN-IFS co-evolution. First, the fact that ifs has become a pseudogene in the absence of robust NADase activity indicates that inhibition of this activity is its principal function rather than...
having any additional contributions to virulence, including SPN secretion or the CMT injection process. Second, the fact that \(S.\ pyogenes\) can tolerate a low level of NADase activity in the absence of IFS indicates that alterations to \(ifs\) itself were not a major driver of the continuing evolution of SPN to an activity-deficient phenotype. Instead, the lack of intermediates for the SPN NADase-deficient haplotypes in the \(S.\ pyogenes\) population structure suggests that the near to complete loss of NADase activity in these lineages was driven exclusively by selective pressure imparted by some virulence-related function associated with how SPN interacts with host cells.

Although their origins may not be clear, insight into the mechanistic role of polymorphism in function can be gained from analysis of the structure of SPN. The three-dimensional structure of the enzymatic domain of SPN has recently been determined, revealing that it is structurally related to the broad family of NAD\(^+\)/H\(_{11001}\) ribosyltransferases (8). These proteins share a conserved core structure consisting of seven \(\beta\)-strands arranged in two perpendicular \(\beta\)-sheets that bracket the \(\beta\)-NAD\(^+\) binding pocket. In addition, SPN and the NAD\(^+\) ribosyltransferases share an active site ADP-ribosyl turn-turn motif that contains a catalytically essential glutamate residue (Glu-391 for SPN) that lies at the base of a bowl-like substrate binding pocket (Fig. 7A). The three positions of interest are all situated in the wall of the substrate binding pocket, although two of these are distal to the active site and are located near the lip of the bowl. One of these is Gly-330, which hydrogen bonds with Gln-216 from the opposing surface to form one of the outside walls of the bowl (Fig. 7B). Molecular modeling of the glutamate polymorphism reveals significant steric clashes with Gln-216 that likely lead to a significant distortion of this wall, possibly leading to a reorientation of the two halves of the enzyme relative to each other. Consistent with this, the G330D change had the highest impact on NADase activity as compared with changes at the other two sites and likely radically altered substrate binding as the \(K_m\) was raised to a level that was difficult to determine with precision. On a different face of the wall, Arg-289 interacts with Asp-286 and Lys-288 to form a surface that molecular modeling suggests may be involved in positioning the ribose moiety of the \(\beta\)-NAD\(^+\) substrate in the active site.

**FIGURE 5. NADase-deficient SPN remains cytotoxic to A549 cells.** A, the chromosomal \(spn\) loci of JRS4 and an in-frame deletion mutant of JRS4 (SPN1; Ref. 4) are shown. Adjacent to the gene encoding SPN\(_n\) (\(spn\)), \(ifs\) encodes immunity factor for SPN, and \(slo\) encodes streptolysin O. B, strain Joy1 is a derivative of JRS4 expressing SPN\(_n\) with an HA epitope tag (black box) from the native \(spn\) locus. Derived from Joy1 are strains Suki2 and Joy110, which express the chimeric SPN proteins listed in parentheses at the right of the figure. Regions and residues derived from SPN\(_n\) are shown by shaded boxes and in italics, respectively. C, the strains expressing the proteins listed in parentheses were used to infect A549 cells, and cytotoxicity was evaluated at the indicated times by fluorescence microscopy following LIVE/DEAD staining. SPN1 does not express SPN protein (\(\Delta SPN\)). Refer to supplemental Table S3 for detailed construction of these strains. Data presented represent the mean and S.E. (error bars) derived from at least three independent experiments. BL, below the limit of detection (<0.3%). The inset shows an overlay of a protein ladder with the Western blot analysis of A549 cytosolic fractions prepared after 5 h of infection developed with an HA epitope antiserum. Lane 1, molecular weight standards; lane 2, uninfected; lane 3, Joy1 (SPN\(_n\)); lane 4, SPN1 (\(\Delta SPN\)); lane 5, Suki2 (SPN\(_{TM}\)); lane 6, Joy110 (SPN\(_{HT}\)). The molecular weights of selected standards are indicated at the left of the inset.
the lumen of the bowl, the side chain of Ile-374 is buried in the bottom of the bowl near catalytic Glu-

and yeast that show restricted to specific regions of the toxin and generates a wide range of toxicity from variants that produce extensive vacuolation, to those that only produce vacuolation in a limited range of cultured cells, to variants that fail to cause any detectable cytotoxicity in in vitro assays (24, 25). Also like SPN, variation has a functional consequence, although for VacA this appears to correlate with the risk of developing certain complications of long term colonization like peptic ulcer disease or gastric cancer (26, 27). However, VacA is multifunctional and in addition to cytotoxicity has been reported to alter membrane permeability, to target and damage mitochondria, and to activate numerous host cell signaling pathways (24). Similar to SPN, it is thought that this multifunctional nature likely explains why VacA is found in essentially all H. pylori strains that infect humans despite extensive variation in vacuolating ability (27).

A common property of multifunctional toxins exhibited by VacA is the ability to cause distinct cellular effects by acting at different cellular locations (24). Whether SPN acts at different cellular locations is not clear. Translocated SPN is found exclusively in the cytoplasmatic compartment (4, 28), although the presence or absence of SPN in defined mutants has been reported to affect the invasiveness of S. pyogenes during infection of cultured epithelial cells (18), a property that could contribute to niche selection. SPN itself is a multidomain protein and in addition to its NADase domain has an N-terminal domain that is essential for its translocation (5). Although the structure of this domain has not been solved, homology modeling indicates that it adopts a “jelly roll” fold common to many carbohydrate-binding proteins, which suggests that it may promote interaction at the cell surface (5). The role of this domain in cytotoxicity remains to be determined. Prior reports suggested that SPN may have multiple enzymatic activities that could produce differential cellular effects, including ADP-ribosyltransferase and cyclase activities (6, 7). However, more refined analyses of highly purified recombinantly produced SPN have failed to confirm these additional activities (9). Removing the NADase activity does not affect cytotoxicity, calling into question the contribution of the NADase activity in cell death. However, support for a role for NADase activity in cytotoxicity comes from studies in E. coli and yeast that show...
that expression of SPN in the absence of IFS, the endogenous inhibitor of its NADase activity, is lethal (9, 17). In contrast, expression in yeast of an activity-deficient haplotype is not lethal (9). Yeast may lack a target required for the NADase-independent activity, or this may be due to the observation that full penetrance of the cytotoxic effect on epithelial cells requires a synergistic interaction with the pore-forming activity of the streptococcal streptolysin O protein (29). Taken together, these data suggest two possible modes of SPN toxicity.

The studies described here have expanded our understanding of SPN structure and function and the molecular basis for its evolution. However, several important questions remain, including the structural and molecular basis of its NADase-independent cytotoxic activity and how its various activities contribute to tissue tropism. Several studies have suggested that the number of \textit{S. pyogenes} isolates that demonstrate NADase activity has been increasing since the late 1980s and that the activity-proficient SPN haplotypes have entered into some lineages that are now more frequently isolated from invasive disease (7, 16, 30). Thus, a detailed understanding of SPN function and evolution will further our understanding of how \textit{S. pyogenes} populations continue to evolve.

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**SPN Enzymatic Variants Contribute to Cell Death**