Enhancement of Streptolysin O Activity and Intrinsic Cytotoxic Effects of the Group A Streptococcal Toxin, NAD-Glycohydrolase*

Streptolysin O (SLO) is a cholesterol-dependent cytolysin produced by the important human pathogen, group A Streptococcus (Streptococcus pyogenes or GAS). In addition to its cytolytic activity, SLO mediates the translocation of GAS NAD-glycohydrolase (NADase) into human epithelial cells in vitro. Production of both NADase and SLO is associated with augmented host cell injury beyond that produced by SLO alone, but the mechanism of enhanced cytotoxicity is not known. We have now shown that expression of NADase together with SLO dramatically enhanced the lytic activity of GAS culture supernatants for erythrocytes but had no effect on SLO-mediated poration of synthetic cholesterol-rich liposomes. This result revealed a previously unknown contribution of NADase to the cytolytic activity associated with GAS production of SLO. Purified recombinant SLO bound NADase in vitro, supporting a specific, physical interaction of the two proteins. Exposure of human keratinocytes to wild-type GAS, but not to a NADase-deficient mutant strain, resulted in profound depletion of cellular NAD+ and ATP. Furthermore, expression of recombinant GAS NADase in yeast, in the absence of SLO, induced growth arrest, depletion of NAD+ and ATP, and cell death. These findings have provided evidence that the augmentation of SLO-mediated cytotoxicity by NADase is a consequence of depletion of host cell energy stores through the enzymatic action of NADase. Together, the results have provided mechanistic insight into the cytotoxic effects of a unique bipartite bacterial toxin.

Group A Streptococcus (Streptococcus pyogenes or GAS),2 the causative agent of streptococcal pharyngitis, skin and soft tissue infections, and severe invasive infections, produces more than 40 secreted proteins, many of which have been implicated in disease pathogenesis (1–3). Among the secreted products of GAS are streptolysin O (SLO) and the NAD-glycohydrolase (NADase). SLO is a member of the pore-forming cytolytic family known as cholesterol-dependent cytolysins due to their ability to bind cholesterol in eukaryotic cell membranes (4, 5). Individual SLO molecules oligomerize and insert into the cell membrane to form transmembrane pores up to 30 nm in diameter. Sufficient doses of SLO result in cell lysis (4, 6, 7). Purified SLO is a potent toxin that is lethal within minutes of intravenous injection in animal models, primarily because of cardiotoxicity (8, 9).

NAD-glycohydrolases are enzymes that catalyze the hydrolysis of the nicotinamide-ribose bond of NAD+ to yield nicotinamide and adenosine diphosphoribose. These enzymes have been described in several bacterial species and in a variety of eukaryotes (10, 11). The GAS NADase differs from most other known bacterial NAD-glycohydrolases in that it is secreted instead of being cell-associated and it possesses ADP ribosyl cyclase activity (12–14). Thus, in addition to producing adenosine diphosphoribose, the GAS NADase can convert NAD+ to cyclic adenosine diphosphoribose as a side reaction. Cyclic adenosine diphosphoribose is an important second messenger and signaling molecule for the regulation and mobilization of intracellular calcium stores (14–16).

The GAS genes encoding SLO (slo) and NADase (nga) are expressed from an operon that is transcribed under the control of a promoter upstream of nga. Some studies have identified downstream of nga a second weak promoter for slo (17, 18). The two proteins are functionally linked in that SLO is required for the translocation of NADase from GAS bound to the surface of epithelial cells into the host cell cytoplasm (19). Studies using mutant strains of GAS deficient in SLO or NADase indicated that NADase can enhance the extracellular survival of GAS and induce apoptosis after infection of keratinocytes in vitro (20). However, the biological targets and the precise function of NADase inside the host cytoplasm are not fully understood. In particular, the mechanism through which NADase augments the cytotoxic activity of SLO has not been defined.

We have now reported evidence that NADase contributes not only to cytotoxic injury of nucleated cells but also to hemolytic activity associated with GAS production of SLO. We found that the capacity of NADase to enhance SLO-mediated cytotoxicity is not due to a direct effect on pore formation but rather due to depletion of cellular NAD+ and ATP. The results have provided new insights into the intrinsic and cooperative functions of NADase and SLO during GAS infection.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—M-type 3 GAS strain 950771 (referred to hereafter as 771) and its isogenic mutants 771nga— and 771slo— have been described (20). GAS was grown at 37°C in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco). For cloning experiments, Escherichia coli DH5α or TOP10 (Invitrogen) was grown in Luria-Bertani medium (21). When appropriate, antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml.
Keratinocyte Cell Culture—OKP7 primary human soft palate keratinocytes (22) were cultured in serum-free keratinocyte medium (Invitrogen) supplemented with 50 μg/ml bovine pituitary extract, 0.1 ng/ml epidermal growth factor (Invitrogen), and 0.3 mM calcium chloride. Keratinocytes were seeded into 75-cm² flasks (Costar) and cultured for 3–5 days at 37 °C in 5% CO₂ until confluent.

Keratinocyte Infection with GAS and Isolation of Keratinocyte Cell Fractions—GAS was resuspended in unsupplemented, serum-free medium to the desired multiplicity of infection and inoculated onto the surface of the keratinocyte monolayer. The infected keratinocytes were incubated at 37 °C in 5% CO₂ for the required length of time. GAS cytotoxicity for keratinocytes in vitro was assessed using the LDH cytotoxicity detection kit (Roche Applied Science). Values were expressed as the percentage of lactate dehydrogenase (LDH) release compared with Triton X-100-treated cells (100% cytotoxicity, maximum LDH release) minus background LDH release from uninfected cells. In experiments to measure intracellular NAD⁺ and ATP, after exposure to GAS, the keratinocytes were incubated with 10 μg/ml clindamycin (to which strain 771 and derivatives are susceptible) for 15 min before harvesting to prevent contamination of the cell fractions with NADase produced by associated bacteria during processing of the samples. Cell culture supernatants were collected and cleared of bacteria and cell debris by centrifugation at 20,800 × g for 5 min. Cell fractions for measuring NAD⁺ and ATP concentration were prepared as described (19, 23). Adequacy of lysis and fractionation were verified using the LDH assay (Roche Applied Science) for cytoplasmic fractions and alkaline phosphatase assay (Sigma) for membrane fractions.

Measurement of SLO Hemolytic Activity and NADase Activity—SLO activity was assayed as described (24). Activity was quantified in hemolytic units as the reciprocal of the highest dilution of culture supernatant that resulted in erythrocyte lysis equivalent to 50% of the value obtained with 50% lysis reagent (Sigma). The infected keratinocytes were harvested and washed twice in PBS before lysing (10 min at 4 °C, 14,000 × g). The lysate was clarified by centrifugation at 20,800 × g for 15 min. NADase activity was assayed as described (13). NADase activity was quantified as the reciprocal of the highest supernatant dilution that hydrolyzed half of the amount of added NAD⁺.

Measurement of SLO Lytic Activity with Liposomes—Liposomes containing calcein were prepared with egg phosphatidylcholine and cholesterol at a ratio of 2:1 (total lipid concentration, 3.5 mg/ml) by extrusion containing calcein were prepared with egg phosphatidylcholine and cholesterol at a ratio of 2:1 (total lipid concentration, 3.5 mg/ml) by extrusion treatment. The extruded liposomes were resuspended in PBS and transferred to a 96-well plate, and the percentage of calcein release was determined fluorometrically (HTS 7000, Bioassay Reader, PerkinElmer Life Sciences, excitation, 490 nm; emission, 520 nm). PBS was used to determine background fluorescence, and a sample of liposomes treated with 1% Triton X-100 was used to determine the value for complete lysis. Relative fluorescence values for test samples were calculated from the equation: 100 × (sample − background)/ (complete lysis − background).

Measurement of NAD⁺ and ATP—NAD⁺ was assayed by direct measurement of the reduction of NAD⁺ to NADH at 340 nm in the presence of graded concentrations of NADH oxidase from bovine brain (25). ATP was measured using an ATP bioluminescence assay (Sigma). The ATP assay mixture was diluted 10 times, and the amount of light produced was measured with a luminometer (HTS 7000, bioassay reader, PerkinElmer Life Sciences).

TaQMan Real-time Reverse Transcription (RT) PCR Analysis—RNA was extracted from six independent cultures at late exponential phase for each GAS strain using the RNeasy mini kit (Qiagen) followed by DNase I treatment. Fifty ng of RNA samples was analyzed in duplicate using the TaqMan one-step RT-PCR master mix reagents (Applied Biosystems) as described (27) in an ABI 5700 sequence detection system (Applied Biosystems). Oligonucleotide primers and FAM-labeled probes (Table I) were designed with Primer Express 1.0 software (ABI Prism; PE Biosystems) and initially evaluated in serial dilution experiments. Results were normalized to the values of the constitutively transcribed control gene recA, expressed as mean arbitrary relative units. Statistical analysis was performed by one-way analysis of variance using the StatView statistical software program (SAS, Cary, NC).

Expression of Recombinant SLO (SLOr) and a C-terminal Fragment of NADase—The slo gene lacking the first 31 codons predicted to encode the signal peptide (Signal IP V2.0 software) was amplified (28) using primers slo2F(PshAI) and sloR(EcoRI) (Table I) and chromosomal DNA of strain 771 as template. The PCR product was cloned into the pGEM-T vector (Promega). The cloned insert was released from the recombinant plasmid with PshAI/EcoRI digestion and subcloned into PshAI/EcoRI-digested pET-41 (Novagen) as an in-frame translational fusion with glutathione S-transferase (GST) and an N-terminal His6 tag. The plasmid construct was verified by sequencing. E. coli strain BL21(DE3) carrying the recombinant plasmid was grown to A600 nm of 0.4, and then expression of SLOr was induced with isopropyl-1-thio-D-galactopyranoside (1 mM final concentration) for 2 h. The cells were collected by centrifugation (8000 × g, 20 min, 4 °C), lysed in a French pressure cell (3 × 8000 p.s.i.), and clarified by centrifugation (18,000 × g, 20 min, 4 °C). Purification of GST–His6-SLO or was done by Ni-NTA affinity chromatography (Qiagen) at 4 °C according to the manufacturer's instructions. Column fractions were concentrated and desalted using a Centricon column YM-30 (Millipore) and diluted with 0.5 units of enterokinase (Novagen), and the cleaved GST–His6-SLO or was removed by adsorption to Ni-NTA resin. Purified SLO or was stored in 25% glycerol at −80 °C.

The 3’ portion of the nga gene corresponding to nucleotides 718–1356 was amplified using primers nga2F(PshAI) and ngaR(EcoRI) (Table I). Cloning, expression, and purification of the truncated NADase protein (tNADase) were performed as described above for SLOr.

Preparation of Antisera and Purification of IgG—The purified SLOr and tNADase proteins were used to immunize rabbits (Lampire Biolog-

TABLE 1
Oligonucleotide primers and probes used in this study

| Primer/probe name          | Description                        |
|----------------------------|------------------------------------|
| slo2F(PshAI)               | Forward primer for SLOr            |
| sloR(EcoRI)                | Reverse primer for SLOr            |
| nga2F(PshAI)               | Forward primer for nga              |
| ngaR(EcoRI)                | Reverse primer for nga              |
| slo-M1 TaqMan probe        | TaqMan probe for SLOr              |
| nga-F Taq                  | TaqMan probe for nga               |
| nga-R Taq                  | TaqMan probe for nga               |
| nga TaqMan probe           | TaqMan probe for nga               |
| recA-F Taq                 | TaqMan probe for recA              |
| recA-R Taq                 | TaqMan probe for recA              |
| recA-TaqMan probe          | TaqMan probe for recA              |

| Oligonucleotide primers and probes used in this study | Description                        |
|-------------------------------------------------------|------------------------------------|
| slo2F(PshAI)                                          | Forward primer for SLOr            |
| sloR(EcoRI)                                           | Reverse primer for SLOr            |
| nga2F(PshAI)                                          | Forward primer for nga              |
| ngaR(EcoRI)                                           | Reverse primer for nga              |
| slo-M1 TaqMan probe                                   | TaqMan probe for SLOr              |
| nga-F Taq                                             | TaqMan probe for nga               |
| nga-R Taq                                             | TaqMan probe for nga               |
| nga TaqMan probe                                      | TaqMan probe for nga               |
| recA-F Taq                                            | TaqMan probe for recA              |
| recA-R Taq                                            | TaqMan probe for recA              |
| recA-TaqMan probe                                     | TaqMan probe for recA              |

| Oligonucleotide primers and probes used in this study | Description                        |
|-------------------------------------------------------|------------------------------------|
| slo2F(PshAI)                                          | Forward primer for SLOr            |
| sloR(EcoRI)                                           | Reverse primer for SLOr            |
| nga2F(PshAI)                                          | Forward primer for nga              |
| ngaR(EcoRI)                                           | Reverse primer for nga              |
| slo-M1 TaqMan probe                                   | TaqMan probe for SLOr              |
| nga-F Taq                                             | TaqMan probe for nga               |
| nga-R Taq                                             | TaqMan probe for nga               |
| nga TaqMan probe                                      | TaqMan probe for nga               |
| recA-F Taq                                            | TaqMan probe for recA              |
| recA-R Taq                                            | TaqMan probe for recA              |
| recA-TaqMan probe                                     | TaqMan probe for recA              |
| tetaacctgatgctatggcatacata                             | TaqMan probe for recA              |
| ttaactgacctgctatggcataca                               | TaqMan probe for recA              |
| 6FAM-ccatgtcggattgcaagcacg                             | TaqMan probe for recA              |

Preparation of Antisera and Purification of IgG—The purified SLOr and tNADase proteins were used to immunize rabbits (Lampire Biolog-

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ical Laboratories, Inc.) Immune sera were evaluated by Western blot for reactivity with protein bands corresponding to SLO or NADase in culture supernatant samples from GAS strains 771, 771slo−, or 771nga−.

IgG was purified from immune sera by protein A affinity chromatography (Immunopure Protein A IgG purification kit, Pierce) for use in subsequent experiments.

Enzyme-linked Immunosorbent Assay for SLO—Microtiter plates (Nalge Nunc) were coated with IgG antibodies to SLOr at 2 μg/ml in 0.05 M carbonate buffer, pH 9.6. The plates were blocked with 1% bovine serum albumin in PBS, pH 7.4. Serial dilutions of SLOs or GAS culture supernatant in PBS, 0.05% Tween 20, 1% bovine serum albumin were added. Bound SLO was detected with anti-SLO IgG conjugated to biotin (EZ-Link Sulfo-NHS-LC-Biotinylation Kit, Pierce) diluted 1:2000 followed by ImmunoPure streptavidin-horseradish peroxidase conjugate (Pierce) at 1 μg/ml and peroxidase substrate (Sure Blue TMB Microwell peroxidase substrate, Kirkegaard & Perry Laboratories), 100 μl/well for 30 min. The reaction was stopped with the addition of 2 N H2SO4, and absorbance was read at 450 nm in a microplate reader (HTS 7000, bioassay reader, PerkinElmer Life Sciences). Results were quantified by comparison with optical absorbance values obtained from a standard curve generated using purified SLOr.

Affinity Chromatography for Interaction of NADase with SLO—E. coli cell lysate containing recombinant GST-His6-SLOr was clarified by centrifugation and then loaded on a Ni-NTA resin column. After washing, 20 ml of filtered supernatant from a culture of 771slo− grown to late exponential phase was loaded onto the column. After washing, GST-His6-SLOr and proteins bound to it were eluted with 250 mM imidazole, pH 8.0. Column fractions were assayed for NADase activity and tested for the presence of NADase by Western blot probed with IgG purified from anti-tNADase rabbit serum. To test for the specificity of NADase-SLO interaction, we performed parallel experiments using, in place of GST-His6-SLOr, a GST-His6 fusion to a 12-kDa peptide representing a portion of the predicted extracellular domain of the GAS CsrS protein (molecular mass ~43 kDa). CsrS is the sensor component of the CsrR-CsrS (CovR-CovS) two-component regulatory system (29, 30).

SDS-PAGE and Western Blot—SDS-PAGE and Western blot were performed according to standard techniques (31). Primary antibodies were used at a concentration of 2–6 mg/ml and a dilution of 1:5000 and secondary antibodies at a dilution of 1:10000. Development and detection of hybridization bands were done using the ECL Western blotting detection system (Amersham Biosciences).

Expression of NADase in Saccharomyces cerevisiae—For expression of NADase in S. cerevisiae INVSc1 strain, we used the pYES2/NT/C inducible expression vector (Invitrogen). This vector carries the GAL1 promoter (32) that is repressed in the presence of glucose and induced by galactose. To amplify nga from chromosomal DNA of GAS strain 771, we used primers ngaF(BamHI) and ngaR(EcoRI) (Table I). The nga sequence was introduced into pYES2/NT/C to produce recombinant plasmid pYES-nga. Plasmids pYES (pYES2/NT/C), pYES-lacZ (control plasmid carrying lacZ, encoding β-galactosidase), and pYES-nga were transformed separately into S. cerevisiae following the manufacturer’s recommendations. Transformants were selected on SC-U (SC minimal medium lacking uracil) agar plates. The selected colonies were inoculated into 100 ml of SC-U to an initial A600 nm of 0.05 and incubated for 24 h at 30 °C with shaking. Expression was induced for 24 h at 30 °C by adding galactose to a final concentration of 2%. Samples were taken before and 2, 4, 8, 18, and 24 h after the addition of galactose.

Measurement of Cytotoxicity in S. cerevisiae—The FUN1 viability-cytotoxicity stain (Molecular Probes) was used to assess the cytotoxic effects of NADase expression in S. cerevisiae. Dead yeast cells exhibit diffuse green-yellow fluorescence, whereas metabolically active cells are marked with red fluorescent cylindrical intravacuolar structures (33). For measuring cytotoxicity, 100 μl of yeast culture was washed once in 1 ml of sterile water containing 2% d(-)-glucose and 10 mM Na-HEPES, pH 7.2 (glucose-HEPES solution), concentrated by centrifugation for 5 min at 10,000 × g, and suspended again in 1 ml of glucose-HEPES solution. FUN1 stain was added to the yeast cell suspension at a final concentration of 15 μl, mixed thoroughly, and incubated at 30 °C in the dark for 30 min. 200 μl of the mixture was transferred to a 96-well flat bottom plate (Nalge Nunc) and read in a fluorescence microplate reader (HTS 7000, bioassay reader, PerkinElmer Life Sciences) using two filter combinations: ~485 nm excitation/~530 nm emission (green) and ~485 nm excitation/~620 nm emission (red). Dye conversion was expressed as a change in the ratio of red/green for each well after subtracting the value for a blank well containing glucose-HEPES solution and dye. Samples from each time point were applied to poly-l-lysine-coated slides (Sigma) and examined by confocal microscopy.

RESULTS

SLO Hemolytic Activity Is Unexpectedly Low in the NADase Mutant Strain 771nga−—Previous studies in two independent strains of GAS reported that SLO hemolytic activity was apparently unaffected by the introduction of an inactivating deletion mutation in the upstream gene nga encoding NADase (19, 20). By performing more detailed testing, we found that the NADase mutant strain 771nga− produces similar amounts of SLO hemolytic activity as wild-type strain 771 during early exponential phase but substantially less than strain 771 during later phases of growth (Fig. 1A). These differences in SLO hemolytic activity were observed both during growth of GAS in THY and during growth in tissue culture medium in contact with OKP7 human oropharyngeal keratinocytes (Fig. 1B). In addition, exposure of keratinocytes to wild-type strain 771 resulted in release of approximately twice as much LDH, a marker of cell membrane damage, as did exposure to 771nga− (Fig. 1C). These results raised the possibility that the attenuated cytotoxic effects of 771nga− could be attributable, at least in part, to reduced SLO cytolytic activity in this mutant strain.

Transcription of slo Is Not Significantly Reduced in 771nga−—When Compared with Wild-type Strain 771—Although the non-polar deletion mutation in the nga locus in strain 771nga− was not expected to affect transcription of the downstream slo locus, we used real-time RT-PCR to evaluate this possibility. The amount of slo mRNA in 771nga− and 771 was measured in GAS cells grown to mid-exponential phase. The results of real-time RT-PCR revealed only a modest and not statistically significant reduction in the relative slo transcript level in 771nga− when compared with that in 771 (771:3.87 ± 1.72, 771nga−: 2.62 ± 1.58, mean relative units ± S.E., p = 0.22) that does not explain the 80–90% reduction in SLO hemolytic activity of 771nga− relative to that of 771.

Development of Specific Antiserum against Recombinant SLO and NADase—Previous assessments of SLO protein production have used antisera that recognized not only SLO but also NADase (19, 20). To develop a more specific antiserum for use in SLO and NADase immunoassays, we expressed separately SLOr and tNADase. The proteins were expressed as GST-His6 fusions in E. coli. SLOr had the expected molecular mass of ~61 kDa on SDS-PAGE and produced hemolytic activity of 250,000 units/mg of protein. Antiserum raised in rabbits to

2I. Gryllios and M. R. Wessels, unpublished results.
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SLO reacted strongly and specifically with a 61-kDa band in culture supernatants of wild-type strain 771 but not with culture supernatants from 771slo (Fig. 2A).

Multiple attempts to express the complete NADase in a variety of vectors and inducible expression systems failed. Recombinant plasmids recovered from transformants were invariably found to contain one or more mutations in the nga sequence that produced an enzymatically inactive product, an observation that suggests that NADase is toxic to E. coli. A recent study by Meehl et al. (34) suggests that NADase is maintained in an inactive state within GAS by an endogenous inhibitor that must be co-expressed for expression of NADase in E. coli. However, we were able to clone and express an enzymatically inactive 22-kDa peptide (termed tNADase) corresponding to the C terminus of the native NADase protein. Rabbit antiserum to tNADase reacted specifically with a 49-kDa band in culture supernatants of strains 771 and 771slo but not 771nga (Fig. 2B).

A Quantitative Immunoassay Reveals Similar Amounts of SLO Protein in Culture Supernatants of 771nga and Wild-type Strain 771—Because slo transcript levels were similar in 771nga and wild-type strain 771, we considered whether the lower level of SLO activity in 771nga might reflect a reduction in translation of slo message or more rapid degradation of SLO. To determine the concentration of SLO in culture supernatants, we developed a quantitative immunoassay using purified rabbit anti-SLO IgG. Results were quantified by comparison with values obtained from a standard curve generated using purified SLO. SLO protein was measured in supernatants of GAS cultures grown to late exponential phase, at which point SLO hemolytic activity of 771nga was ~10% of that of 771. Similar to the results of RT-PCR experiments, this quantitative assessment revealed only a slight and not statistically significant reduction (p = 0.56) in the amount of SLO protein in culture supernatants of 771nga (0.72 ± 0.11 μg/ml) when compared with those of wild-type 771 (0.80 ± 0.21 μg/ml). The fact that 771nga produces similar amounts of SLO protein but markedly reduced SLO hemolytic activity suggests that NADase enhances cytotoxicity of SLO not only for nucleated cells, but also for erythrocytes.

NADase Binds to Recombinant SLO—The discrepancy between the amount of immunoreactive SLO protein and SLO hemolytic activity in 771nga raised the possibility that association of NADase with SLO might serve to stabilize or enhance the activity of SLO as a pore-forming toxin. We used affinity chromatography to test whether NADase could bind specifically to recombinant SLO. First, GST-HisS-SLOr was bound to Ni-NTA-agarose. Culture supernatant of 771slo was then loaded onto the column, and NADase activity was assayed in the column flow-through and in the eluate after releasing GST-HisS-SLOr with imidazole. By Western blot using NADase-specific antiserum, NADase was readily detected in the 771slo—culture supernatant loaded onto the column, was absent in the column flow-through and wash fractions, and then reappeared upon elution of bound GST-HisS-SLOr. Measurement of NADase enzymatic activity in the same fractions confirmed retention of ~80% of NADase activity on the column and essentially quantitative recovery of enzyme activity upon elution (Fig. 3A). To rule out nonspecific binding of NADase to the affinity matrix, a control resin was prepared using a GST-HisS fusion to an irrelevant GAS peptide representing part of the predicted extracellular domain of CarS (CovS) (29, 30). NADase failed to bind to the control resin, a finding that supported a specific interaction between NADase and SLO (Fig. 3B).

NADase Does Not Enhance SLO Pore-forming Activity in a Cell-free System—Because NADase appears to bind to and enhance the hemolytic activity of SLO, we wondered whether the previously reported cytolytic effects of NADase could simply reflect a stabilizing effect of NADase on SLO, independent of the enzymatic activity of NADase. To
determine whether the synergistic lytic effect of the two toxins is a direct result of their physical interaction, we used cholesterol-rich liposomes containing the fluorescent marker calcein as a cell-free system to assay SLO lytic activity of GAS culture supernatants. In this model system, the release of calcein from liposomes is detected fluorometrically as a measure of pore formation. Exposure of liposomes to supernatant from wild-type strain 771 or the NADase-deficient mutant 771nga/H11002 produced identical levels of calcein release (Fig. 4), in striking contrast to the markedly greater hemolytic activity of wild-type when compared with NADase-deficient culture supernatants. The inability of NADase to augment SLO lytic activity in a cell-free system provided strong evidence that the synergistic toxicity of the two toxins for eukaryotic cells depends on NADase effects on cellular metabolism rather than a direct enhancement of the pore-forming activity of SLO.

GAS Infection of Keratinocytes Results in NADase-mediated Depletion of Cellular NAD⁺—One consequence of the enzymatic activity of NADase in the host cell could be the consumption of intracellular NAD⁺. We measured NAD⁺ levels in keratinocytes exposed to GAS wild-type strain 771 or the NADase-deficient mutant 771nga—. We found marked depletion of intracellular NAD⁺ in keratinocytes exposed to strain 771 (Fig. 5A). There was a slight reduction in NAD⁺ in cells exposed to 771nga— that was largely accounted for by a shift of NAD⁺ from the cytoplasmic fraction to the membrane fraction, perhaps as a consequence of SLO poration of the cell membrane. We also measured ATP levels and found a similar pattern of depletion as that observed for NAD⁺ in keratinocytes exposed to 771 (Fig. 5B). The latter result is an expected secondary effect since depletion of cellular NAD⁺ is a stimulus for NAD⁺ synthesis, a reaction that consumes ATP. These results indicated that the enzymatic activity of NADase produces profound depletion of target cell energy stores that may enhance the cytolytic effects of SLO by crippling the ability of the cell to repair membrane damage.

Expression of NADase Independently of SLO Is Cytotoxic in Yeast—The results presented above have provided evidence that the enzymatic action of NADase can markedly derange cellular metabolism during infection. However, since the delivery of NADase to GAS-infected host cells depends on SLO-mediated translocation, it has been difficult to completely separate the effects of the two toxins on eukaryotic cell biology. To further investigate the intrinsic cytotoxic effects of NADase, we expressed NADase under the control of a galactose-inducible promoter in S. cerevisiae as a model system for eukaryotic cell toxicity. After induction of NADase expression, we observed marked inhibition of growth in yeast cells containing pYES-nga but not in cells containing the pYES plasmid alone (Fig. 6A). After induction of NADase expression, we found time-dependent depletion of cellular NAD⁺ and ATP, similar to that observed in GAS-infected keratinocytes (Fig. 6B and C). At the same time points after induction, we also stained yeast cells using a dual color fluorescent viability probe. These experiments revealed progressive loss of viability in cells expressing NADase but not in control cells expressing β-galactosidase or carrying the pYES vector alone (Fig. 6D). Confocal microscopy confirmed the presence of red fluorescent cylin-
Cytotoxicity Mechanisms of Group A Streptococcal NADase

That NADase enhanced SLO lytic activity for keratinocytes and erythrocytes, but not for synthetic liposomes, suggests that the effect of NADase depends on interaction with cellular metabolism. This conclusion was supported by the finding that NADase translocated from bound GAS into human keratinocytes catalyzed the consumption of NAD$^+$ and produced marked depletion of cellular stores of both NAD$^+$ and ATP. Such depletion was much less profound in cells exposed to GAS that produce SLO but not NADase, so NADase itself appears to mediate NAD$^+$ and ATP consumption, consistent with its known enzymatic activity. These intrinsic toxic effects of NADase were observed also upon expression of the enzyme, in the absence of SLO, in S. cerevisiae. By placing the gene under the control of an inducible promoter, we could show that expression of NADase resulted in growth arrest, depletion of intracellular NAD$^+$ and ATP, and cell death. Therefore, in this model eukaryotic cell system, NADase consumed cellular NAD$^+$ and led to cell death within hours. Although the enzymatic activity of NADase has been thought to contribute to cytotoxicity, these results clearly distinguished the toxic effects of NADase on eukaryotic cells.
assays to measure SLO actually detect the combined effects of both toxins.

Regardless of whether or not NADase contributes to virulence in human invasive infection, the evolutionary pressure for its expression is more likely to reflect a selective advantage in colonization and survival of GAS in the human pharynx. Human pharyngeal cells can internalize and kill GAS in vitro and may help control bacterial proliferation on the pharyngeal mucosa as a result (39). Certain GAS products have been shown to interfere with this process, thereby enhancing GAS survival on the surface of the pharyngeal epithelium. Internalization is inhibited by the hyaluronic acid capsule polysaccharide (40). SLO also serves to inhibit internalization and to prevent trafficking of internalized GAS into lysosomes, thereby prolonging intracellular survival (39). The present investigation suggests that NADase also contributes to GAS survival in this setting by depriving the cell of metabolic energy sources that power bacterial internalization and intracellular killing.

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