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Glycosylation of onconase increases its conformational stability and toxicity for cancer cells

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Abstract

Onconase (ONC) is currently in Phase III clinical trials as a cancer chemotherapeutic agent. Despite the finding that ONC contains an N-linked glycosylation site (-N69-V70-T71-), only the non-glycosylated form of the protein has been identified to date. We employed the *Pichia pastoris* expression system to produce recombinant glycosylated ONC (gONC) protein. Approximately 10 mg of ONC protein was secreted per liter of culture media, of which about 80% was glycosylated at N69. CD spectroscopic analyses revealed that the secondary structure of gONC is identical to that of ONC. We found that gONC contains a high-mannose core structure. Importantly, glycosylation of ONC at N69 greatly increased its toxicity for K-562 cancer cells. Specifically, the IC_{50} value of gONC was 50-fold lower than that of ONC. Glycosylation increased both the T_m of ONC and its resistance to proteinase K, suggesting that the elevated cytotoxicity of gONC is related to higher conformational stability.

Keywords: Onconase; Cancer chemotherapeutic agent; Glycosylation; Conformational stability; Cytotoxicity; *Pichia pastoris* expression system

Onconase (ONC) isolated from oocytes or early embryos of *Rana pipiens* is selectively cytotoxic to cancer cells, both in vitro and in vivo [1,2]. The amino acid sequence of ONC displays 30% identity to that of ribonuclease A (RNase A) [3]. Although the active site and tertiary structure of ONC are similar to those of RNase A, ONC has a more compact structure. ONC is 20 residues shorter than RNase A, whereby all the deletions are positioned in surface loops and at the N-terminus [4]. The protein has been evaluated in Phase I and II human clinical trials for the treatment of numerous solid tumors [5,6] and is currently in Phase III human clinical trials for malignant mesothelioma therapy. ONC-treated cells display apoptosis associated with cell blebbing, nuclear pyknosis, DNA fragmentation, PARP (polyADP-ribose polymerase) cleavage, and activation of caspase 3-like activity [7]. Ribonucleolytic activity is required for ONC cytotoxicity [7–10]. In addition to anti-cancer activity, the protein has anti-viral properties. At 10^{-8} M, ONC inhibits HIV-1 replication in chronically infected human cells by degrading viral RNA [11,12]. Pancreatic ribonucleases, such as RNase A, are inhibited by ribonuclease inhibitor (RI), a 50-kDa protein that binds to ribonucleases with 1:1 stoichiometry and dissociation constants below 1 pM [13–15]. Since ONC is not inhibited by RI [16], it can catalyze RNA degradation within the cell.

ONC contains a disulfide bond between Cys87 and Cys104, which links the C-terminal Cys104 residue to a central β-strand. This Cys87–Cys104 disulfide bond is unique to the amphibian homologs of RNase A. Removal of the bond results in a significant decrease in conformational stability and cytotoxic activity [17–19]. The conformational stability of ribonucleases correlates directly with cytotoxicity and resistance to proteolysis [20,21], and could thus be a key determinant of ONC cytotoxicity by facilitating escape from proteolysis. The non-native Cys4–Cys118 disulfide bond of pancreatic

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ribonucleases, such as RNase A and RNase 1, enhances their cytotoxicity while preserving their structure and ribonucleolytic activity in the cytosol [17,20].

Although a putative N-linked glycosylation site has been identified at Asn69 in a solvent-exposed surface loop of ONC, only the non-glycosylated form has been identified from the frog *R. pipiens* [1]. The dynamic properties and flexibility of the oligosaccharide chain around the N-glycosidic linkage could facilitate protection of extensive regions of the protein surface [22]. To determine the mechanism by which glycosylation affects the properties of ONC, recombinant protein was produced in its glycosylated form. To do so, we employed *Pichia pastoris*, a methylotrophic yeast capable of metabolizing methanol as a carbon source [23,24]. We established a production system in which glycosylated ONC (gONC) was secreted into the medium in high yield. The gONC protein displays greatly increased toxicity for K-562 cancer cells. In addition, ONC glycosylation is associated with enhanced conformational stability, suggesting that increased stability is responsible for the prodigious cytotoxicity.

Materials and methods

Materials. K-562 cells derived from a human erythroleukemia cell line were obtained from the Korean Collection for Type Culture. The *P. pastoris* expression system was purchased from Invitrogen. Oligonucleotides were from Korea Genotech, while RI (human) was from Promega. Enzymes used for DNA manipulation were acquired from New England Biolabs. *Endo*-β-*N*-acetylglucosaminidase H (EndoH), ribosomal RNA (rRNA; 16S and 23S), and RNase A were from Boehringer–Mannheim. [*methyl*-^{3}H]Thymidine and SP-Sepharose were obtained from DuPont/NEN and Pharmacia Biotech, respectively. Concanavalin A–agarose, polycytidylic acid[poly(C)], methyl-α-D-mannopyranoside, and $(NH_4)_2SO_4$ were from Sigma. Bacto peptone, Bacto yeast extract, and Yeast nitrogen base were purchased from Difco.

Plasmid construction and yeast transformation. A DNA fragment encoding ONC was amplified by PCR using pONC [19] as a template and 5′-CCGCTCGAGAAAAGACAGGACTGGCTGACTTTC-3′ and 5′-CGGGATCCTAGCAAGAACCAACACCAAC-3′ as primers. The amplified fragment was digested using *Xho*I and *Bam*HI and ligated into pHIL-S1 to generate pHIL205. The pHIL205 plasmid was mutated at the *Xho*I site with the Quick-Change Mutagenesis kit (Stratagene), using the mutagenic oligomers, 5′-TTGCAATCTGTCT TCGCTCAGGACTGGCTGACTTTC-3′ and 5′-GAAAGTCAGCC AGTCCTGAGCGAAGACAGATTGCAA-3′. This led to the generation of pHP205, whereby the N-terminus of ONC was fused to the C-terminus of the *PHO1* signal sequence. pHP205Q encoding for the mutant N69Q ONC protein was additionally generated with the Quick-Change Mutagenesis kit, using the oligomers, 5′-CTGTCTG ACTGCCAAGTTACTAGTCGTCCGTGC-3′ and 5′-GCACGGA CGACTAGTAACTTGGCAGTCAGACAG-3′. All plasmid constructs were confirmed by DNA sequencing. *P. pastoris* SMD1168 was transformed with *Bgl*II-cleaved plasmid DNA using the lithium chloride method, as described in the manufacturer's manual (Invitrogen). Integration of ONC genes into the *aox1* locus of the chromosome was confirmed by PCR.

Protein expression and purification. Recombinant proteins were expressed in *P. pastoris* cells according to the manufacturer's instructions (Invitrogen). The culture was centrifuged at 7000*g* and the supernatant was further used for ONC purification. Proteins were precipitated by adding ammonium sulfate to the culture medium at a final concentration of 40%. The protein pellet was resuspended in 50 mM sodium acetate buffer (pH 5.5) and subjected to SP-Sepharose cation exchange chromatography. The eluted ONC fraction was applied to a Mono S cation exchange FPLC column (Pharmacia Biotech) equilibrated with 50 mM sodium acetate buffer (pH 5.5). ONC was eluted with a linear gradient of NaCl (0.1–0.4 M) in 50 mM sodium acetate buffer (pH 5.5). ONC concentrations were determined by UV spectroscopy, using $\Sigma_{280} = 0.87$ ml/mg/cm [25]. The ONC protein mixture comprising glycosylated and non-glycosylated forms was added to a concanavalin A–agarose column equilibrated with buffer S (0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02% w/v NaN_3, and 50 mM potassium phosphate, pH 6.0). Elution was performed with buffer S containing 0.5 M methyl-α-D-mannopyranoside. ONC fractions were further purified by Mono S cation exchange chromatography to remove residual concanavalin A.

N-terminal sequence analysis. For N-terminal sequencing, the purified protein was separated by SDS–polyacrylamide gel electrophoresis and transferred onto an Immobilon P membrane (Millipore). Edman degradation of the protein was performed on an Applied Biosystems 494 Sequencing System (Perkin–Elmer).

Analysis of N-linked oligosaccharides. gONC was denatured by boiling for 10 min in 0.5% (w/v) SDS and 1% (v/v) of 2-mercaptoethanol. The denatured protein was deglycosyated with endoglycosidase H (Endo H) (4 U/ml) for 30 h at 37 °C in buffer containing 0.1 M sodium acetate (pH 5.2), 0.15 M NaCl, 1% (v/v) Triton X-100, 1% (v/v) of 2-mercaptoethanol, and 0.5% (w/v) SDS.

CD spectroscopy. CD spectra of ONC (0.2 mg/ml in PBS) at 200–260 nm were measured using a 2 mm optical path at 25 °C on a Model J715 CD spectrometer (Jasco).

Molecular weight measurements. The molecular weight of ONC was measured by MALDI-TOF spectrometry with a Voyager-DE STR mass spectrometer (PE Biosystems).

Conformational stability assays. The melting temperature (T_m) of ONC was determined by recording the change in molar ellipticity at 204 nm on a Model J715 CD spectrometer (Jasco) at increasing temperatures [19]. The temperature of the ONC solution (0.2 mg/ml) in PBS was increased gradually from 75 to 100 °C at 0.5 °C/min. The decrease in molar ellipticity at 204 nm (θ_{204}) was recorded every minute. CD data were fitted to a two-state model for denaturation [19].

Ribonucleolytic activity assays and its inhibition by RI. ONC catalysis of poly(C) cleavage was measured by an increase in UV absorption [26]. Assays were performed at 25 °C in 100 mM Mes buffer (pH 6.0)containing NaCl (100 mM), poly(C) (5 μM to 1.5 mM), and enzyme (2.5 nM RNase A, 1.5 μM ONC). The values of k_{cat}, K_m, and k_{cat}/K_m were determined from initial velocity data.

To assess the binding of ONC to RI, we employed an agarose gel-based assay [16,17]. Various amounts of ONC were added to PBS containing dithiothreitol (10 mM) and RI (100 U). After a 30 min incubation on ice, rRNA (4 μg) was added to the mixture. The assay mixture (10 μl) was incubated at 37 °C for 60 min. Finally, samples were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.4 μg/ml). The intensity and position of the rRNA bands were correlated with the degree of ribonucleolytic activity.

Assays of proteolytic susceptibility. ONC (1 mg/ml) was incubated with proteinase K (10, 1, 0.1, and 0.01 μg/ml) in 50 mM Tris–HCl buffer (pH 8.0) containing $CaCl_2$ (1.0 mM) at 37 °C for 30 min. To terminate the reaction, PMSF was added at a final concentration of 1 mM. Poly(C) cleavage by proteinase K-treated ONC was measured by an increase in A_{260}. A gel shift assay was additionally employed to determine the susceptibility of ONC to proteinase K. ONC was treated with proteinase K as described above, except that the reaction time was 1 h. Products were analyzed by SDS–PAGE.

Determination of cytotoxic activity. K-562 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum,

penicillin (100 U/ml), and streptomycin (100 μg/ml). The effect of ONC on K-562 cell proliferation was measured as described previously [16].

Results

Expression and purification of recombinant ONC proteins

The ONC coding sequence was fused to the *PHO1* signal sequence in the *P. pastoris* expression system utilizing a strong alcohol oxidase promoter that is highly active in the presence of methanol. Soluble ONC was expressed from *P. pastoris* cells and secreted into the medium (Fig. 1A, lane 1). Approximately 10 mg of soluble protein was secreted per liter of medium. Two distinct bands (12 and 14 kDa) were identified. The size of the smaller protein correlates with that of frog ONC. To determine whether the larger protein was a glycosylated derivative, we mutated asparagine of the putative glycosylation site at position 69 (N69) to glutamine (69Q). The mutant construct (N69Q) generated only one protein band of 12 kDa (Fig. 1A, lane 2). Our results suggest that the 14-kDa band represents the ONC protein glycosylated at N69 (gONC). A 40% ammonium sulfate precipitate fraction of the culture medium was subjected to SP-Sepharose chromatography. Collected protein samples were further purified by Mono S cation exchange chromatography with NaCl gradiemt of 100–400 mM. Non-glycosylated ONC and N69Q derivatives were eluted at 200 mM NaCl. In contrast, gONC was eluted at 180 mM NaCl. Therefore, it is likely that the oligosaccharide attached to gONC weakens binding between protein and the Mono S resin. Pooled fractions containing both ONC and gONC proteins (Fig. 1A, lane 3) were subjected to chromatography on a concanavalin A–agarose affinity column. The ONC protein was detected in flow-through fractions (Fig. 1A, lane 5), while gONC was eluted with 0.5 M methyl-α-D-mannopyranoside (Fig. 1A, lane 4). To further characterize the properties of the oligosaccharide moiety of gONC, we treated the glycosylated protein with *endo*-β-*N*-acetylglucosamidase H (Endo H), which hydrolyzes a high-mannose core structure [24]. Treatment of gONC with Endo H generated a product that migrated with a similar pattern to ONC on a SDS–PAGE gel (Fig. 1A, lane 6), indicating that the glycosylated protein contains a high-mannose core structure.

N-terminal sequencing

Recombinant ONC proteins were subjected to an N-terminal sequence analysis by Edman Degradation. However, we were not able to obtain any N-terminal sequence information for individual proteins. This negative result suggests that the N-terminal residue of the proteins is modified, similar to that of frog ONC

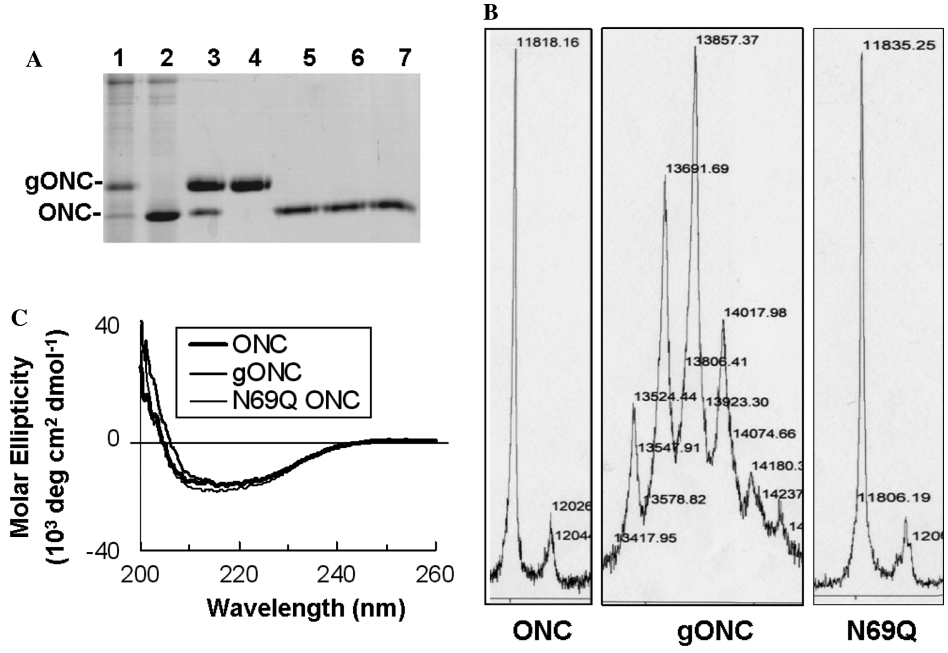

Fig. 1. Purification and characterization of recombinant ONC proteins. (A) Purification. Protein fractions were analyzed on a 15% SDS–polyacrylamide gel. Lane 1, culture medium of *P. pastoris* expressing ONC; lane 2, culture medium of *P. pastoris* expressing N69Q ONC; lane 3, pooled fraction of ONC from a Mono S column; lane 4, the ONC fraction eluted with 0.5 M methyl-α-D-mannopyranoside on a Con A–agarose column; lane 5, the flow-through fraction on a Con A–agarose column; lane 6, digests of gONC with Endo H; lane 7, the pooled N69Q ONC fraction from a Mono S column. (B) MALDI-TOF mass spectrometry. Molecular weights of each peak are shown. (C) CD spectra. CD spectra of ONC, gONC, and N69Q ONC were measured in PBS at 25 °C.

containing a pyroglutamyl residue formed by cyclization of the N-terminal glutamine residue [14].

Molecular weights

The molecular weights of ONC proteins were measured by MALDI-TOF mass spectrometry. The specific molecular mass of recombinant ONC corresponding to the 12-kDa band was 11,818 Da (Fig. 1B), identical to that of authentic frog ONC [1]. This finding indicates that the 12-kDa recombinant ONC is a non-glycosylated protein with a pyroglutamyl residue at its N-terminus. A specific structural feature of ONC is the tight anchoring of both its chain termini to the middle of the protein molecule. The pyroglutamyl residue of the N-terminus is H-bonded to the ε-amino group of the Lys9 side chain, and the carbonyl group of Val96 via the NH group of the cyclic amide [27]. Additionally, the C-terminal Cys104 forms a disulfide bridge with Cys87 [19]. A recombinant ONC from *Escherichia coli* with an N-terminal methionine instead of the native pyroglutamyl residue displayed lower cytotoxicity and enzyme activity [14]. Cleavage of Met1 of the recombinant ONC from *E. coli* and cyclization of the resulting Gln1 residue into the pyroglutamyl N-terminus re-establish cytotoxicity and enzymatic activity [14]. Recombinant ONC corresponding to the 14-kDa protein band displayed multiple mass peaks ranging from 13,524 to 14,180 Da (Fig. 1B). On the other hand, the N69Q ONC protein peak appeared at 11,835 Da (Fig. 1B). The difference between each peak of the 14-kDa protein was approximately 162 Da, corresponding to the molecular weight of a mannose unit. N-glycosylation in *P. pastoris* is distinct to that in animal cells, particularly in later stages of the processing pathway [24]. The maturation of oligosaccharides in *P. pastoris* involves the sequential addition of a mannose to the core oligosaccharide containing eight mannose and two *N*-acetylglucosamine residues. Glycoproteins produced in *P. pastoris* contained 8–12 mannose units, and the relative distribution of mannose units varied from one glycoprotein to another. The molecular weights of the oligosaccharide portions of 13,524, 13,691, 13,857, 14,017, and 14,180 Da gONC correspond to 1707, 1874, 2040, 2200, and 2363, respectively. These values agree well with the molecular weights of $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, $Man_{10}GlcNAc_2$, $Man_{11}GlcNAc_2$, and $Man_{12}GlcNAc_2$, respectively.

CD spectroscopy

Recombinant ONC, gONC, and N69Q ONC proteins displayed identical CD spectra (Fig. 1C), which were additionally similar to those of RNase A superfamily proteins [19]. This finding suggests that glycosylation or a single substitution at the glycosylation site causes insignificant variations in global protein structure.

Ribonucleolytic activity of ONC and inhibition by an RNase blocker

Analogous to RNase A, ONC catalyzes cleavage of the P–$O^{5'}$ bond of RNA on the 3′ side of pyrimidine nucleosides. The principal catalytic residues of RNase A are conserved in ONC, implying that the two proteins catalyze RNA cleavage via similar mechanisms [3]. Despite these similarities, the catalytic efficiency of ONC is much lower than that of RNase A. Nevertheless, ribonucleolytic activity is necessary for the cytotoxicity of ONC. The RNase inhibitor protein (RI) protects tissues from digestion by pancreatic-type ribonucleases. RI forms a tight non-covalent complex with RNase A ($K_d = 4 \times 10^{-14}$ M). Consequently, wild-type RNase A exhibits extremely low cytotoxicity [16]. On the other hand, ONC is capable of evading cytosolic RI and is therefore cytotoxic at much lower concentrations than RNase A [16]. ONC displays $>10^8$-fold lower binding affinity for RI than RNase A ([16], Table 1). Accordingly, we examined whether glycosylation affects the ribonucleolytic activity of ONC. Ribonucleolytic activity of recombinant ONC proteins was determined using

Table 1
Comparison of RNase A, ONC, gONC, and N69Q ONC

Enzyme	T_m[a] (°C)	k_{cat}/K_m (10^2 M^{-1} S^{-1})	K_i[b] (pM)	$I_{1/2}$[c] (nM)	IC_{50}[d] (μM)
RNase A	63[b]	45,000 ± 5000	0.067	ND	—[e]
ONC	90	3.0 ± 0.3	$\geqslant 10^6$	3.5	2.0
gONC	94	2.2 ± 0.2	ND	31.0	0.04
N69Q	88	2.8 ± 0.2	ND	2.9	2.2

ND, not determined.
[a] Values of T_m determined by CD spectroscopy (Fig. 3).
[b] The K_i value for RNase A and ONC from Ref. [16] are for inhibition of poly(C) cleavage by RI.
[c] Values of proteinase K concentration are for 50% inhibition of ribonucleolytic activity.
[d] Values of IC_{50} are for toxicity to K-562 cells (Fig. 5).
[e] No toxicity was observed at a concentration of 10 μM.

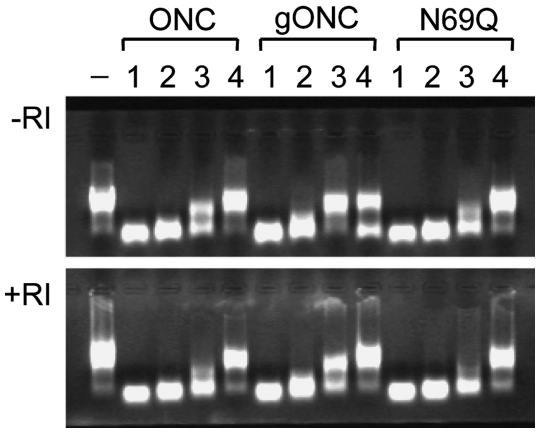

Fig. 2. Agarose gel-based assay for ribonucleolytic activity and inhibition by human RI. Ribonucleolytic activity and inhibition were assessed by visualizing the cleavage of 16S- and 23S-rRNA by ONC, gONC, and N69Q ONC, in the absence or the presence of RI (100 U). The amounts of ONC proteins used in each assay were 1.0 μg (lane 1), 0.1 μg (lane 2), 0.01 μg (lane 3), and 0.001 μg (lane 4). –, no protein.

poly(C) RNA as a substrate. Similar activities were observed with wild-type and mutant N69Q ONC, while gONC exhibited slightly lower activity (Table 1). We conclude that glycosylation renders ONC a somewhat less efficient catalyst for RNA cleavage. Next, we examined the effect of glycosylation on ribonucleolytic activity inhibition of ONC by human RI. Activities of ONC proteins examined were not affected by treatment with human RI (Fig. 2).

Conformational stability

ONC displays unusually high conformational stability due to a C-terminal disulfide bond between Cys87 and Cys104 [19]. We measured the T_m values of recombinant ONC proteins using CD spectroscopy (Fig. 3, Table 1). The T_m values of recombinant ONC and gONC were 90 and 94 °C, respectively. The N69Q

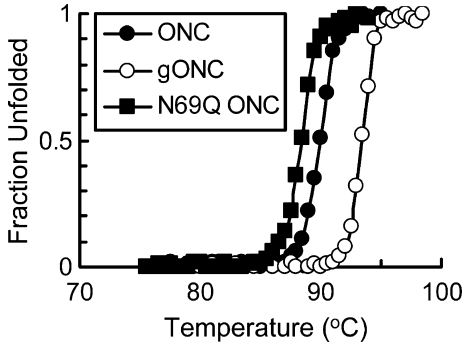

Fig. 3. Melting curves of recombinant ONC proteins. Ellipticity of each ONC protein at 204 nm was measured in PBS by CD spectroscopy. The temperature of the ONC solution was increased continuously from 75 to 100 °C at 0.5 °C/min and the decrease in ellipticity at 204 nm was recorded. CD data were fitted to a two-state model for denaturation.

substitution led to a decrease in the T_m by 2 °C. Our data suggest that the increased thermal stability of gONC is a result of glycosylation.

Proteinase K susceptibility

Analysis of the conformational stability of ONC by limited proteolysis experiments revealed that amino acid residues located within exposed and flexible regions of the protein are subjected to cleavage by protease under controlled conditions [4]. Therefore, glycosylation at these regions may slow down proteolysis by sterically blocking the protease [28]. We assessed the proteolytic susceptibility of recombinant ONC proteins by monitoring their ribonucleolytic activities (Table 1) or via analysis on SDS–polyacrylamide gels (Fig. 4) following proteinase K-treatment. Non-glycosylated ONC and N69Q ONC proteins were particularly susceptible to proteinase K digestion, and thus were severely degraded at low concentrations of proteinase K. However, gONC displayed a 10-fold increase in resistance to proteinase K digestion. These data suggest that the presence of oligosaccharides at Asn69 in the surface loop of ONC is responsible for the increased confirmation stability of gONC.

Cytotoxicity of recombinant proteins

We examined the effects of recombinant ONC on cancer cell viability by measuring the inhibition of K-562 cancer cell proliferation by these proteins (Fig. 5, Table 1). Recombinant ONC proteins repressed cell proliferation in a dose-dependent manner, in contrast to RNase A, which had no effect on cell proliferation within the range of protein concentrations used in our assay (⩽10 μM). The concentration required for 50% inhibition of cell proliferation (IC_{50}) by wild-type ONC was 2.0 μM. Inhibition by the N69Q ONC mutant was similar to that by wild-type protein. In contrast, gONC displayed an IC_{50} value of 0.04 μM, which was 50-fold less than that of non-glycosylated ONC. Thus, gONC is significantly more effective in inhibiting K-562 cell proliferation than its non-glycosylated counterpart.

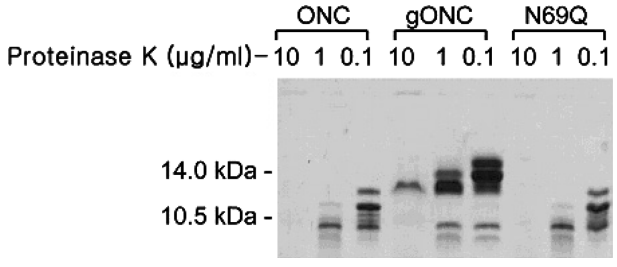

Fig. 4. Proteinase K digestion of recombinant ONC proteins. The ONC protein solution (1 mg/ml) was incubated with the indicated concentrations of proteinase K. Products were analyzed on a SDS polyacrylamide gel (15%) and visualized with Coomassie blue.

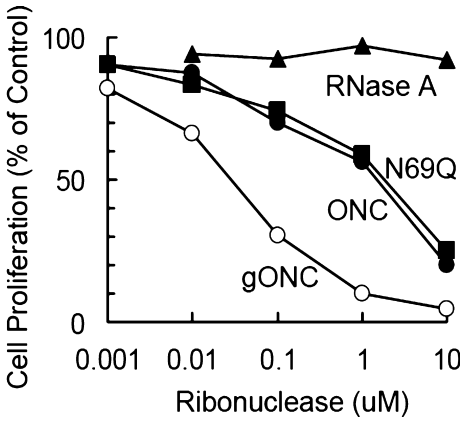

Fig. 5. Inhibition of K-562 cell proliferation by recombinant ONC proteins. K-562 cell proliferation was measured by monitoring the incorporation of [*methyl*-^{3}H]thymidine into cellular DNA after a 44-h incubation period with each protein. All data are presented as mean values from experiments using three different cultures. Cell proliferation of each culture is represented as a percentage of the control culture. The standard error of each value is less than 15%.

Discussion

We report recombinant ONC protein expression and characterization in the yeast *P. pastoris*. The ONC protein, initially isolated from oocytes or early embryos of the frog *R. pipiens* [1], was previously expressed in *E. coli* [14]. However, the recombinant protein from *E. coli* forms inclusion bodies in the cell, which requires solubilization and subsequent refolding. Furthermore, a methionine residue, instead of the native pyroglutamyl residue at the N-terminus, decreases ribonucleolytic activity and cytotoxicity. Cleavage of this N-terminal methionine residue and cyclization of the resulting N-terminal glutamine residue restores enzymatic activity and cytotoxicity [14]. In this study, we generated soluble recombinant ONC proteins using the *P. pastoris* expression system. Recombinant proteins were released as soluble proteins into culture medium and easily purified at large quantities. This is the first report on the purification of ONC as a recombinant protein using a eukaryotic expression system. Although Asn69 is a putative glycosylation site, native ONC was identified as a non-glycosylated protein. Similarly, RNase 1 contains a glycosylation site at Asn88, but the human protein is glycosylated only to a minor extent by *Saccharomyces cerevisiae* [29]. In contrast, our *P. pastoris* expression system predominantly produced gONC, with a glycosylated/non-glycosylated ratio of 4:1. Importantly, gONC displayed 50-fold higher toxicity against K-562 cancer cells.

An N-linked oligosaccharide biosynthetic pathway involving the formation of a $Glc_3Man_9GlcNAc_2$-PP-dolicol lipid-linked precursor is conserved in yeasts and mammals. In both organisms, glycoproteins acquire N-linked oligosaccharides by transferring the $Glc_3Man_9GlcNAc_2$ core sugar to the appropriate asparagine residue of proteins in the ER [30,31]. However, yeast cells do not glycosylate proteins in the same manner as do animal cells. For example, no peripheral sialic acids are added to glucans, and no truncation further than $Man_9GlcNAc_2$ is observed [24], while these modifications occur in mammalian cells. In yeast, the three glucose and one α-1,2-linked mannose residues are removed by glycosidases in the ER, resulting in a $Man_8GlcNAc_2$ core structure that is further modified in the Golgi complex [32]. In *P. pastoris*, this modification includes the addition of a mannose unit that is joined by an α-1,6-linkage to the α-1,3-linked mannose unit in the Manα-1,3Man-β1,4GlcNAc inner core sequence. The α-1,6-linked residue can be elongated up to three α-1,6- and two α-1,2-linked mannose units. The *P. pastoris* expression system has a number of advantages for N-glycosylation of recombinant proteins over other systems. Further glycosylation of recombinant glycoproteins containing the core $Man_8GlcNAc_2$ unit is rather limited due to low activity of the Golgi mannosyl transferase [23]. In addition, hyper-immunogenic terminal α-1,3-linked mannose residues are not generated when the core $Man_8GlcNAc_2$ unit is extended [24]. Very little O-linked glycosylation has been observed in *P. pastoris* [23]. Yeast *S. cerevisiae* containing the RNase A or the RNase 1 genes secrete recombinant proteins into the medium in a variety of forms, due to inefficient processing of the signal sequence, extensive N-glycosylation, and mild O-linked glycosylation [29,33]. We observed five major mass peaks of recombinant gONC proteins, ranging from 13,524 to 14,180 Da. The molecular mass of the oligosaccharide moiety of the smallest gONC is 1707 Da, which is close to 1703 Da of the $Man_8GlcNAc_2$ unit. The difference between neighboring mass peaks is approximately 162 Da, which corresponds to the molecular mass of a mannose unit. We also confirmed the presence of a high-mannose core structure of gONC with treatment by Endo H. These data collectively suggest that the N-linked oligosaccharides of gONC correspond to $Man_{8-12}GlcNAc_2$, with $Man_9GlcNAc_2$ and $Man_{10}GlcNAc_2$ as the major products.

Oligosaccharides of glycoproteins are involved in numerous biological processes, including protein folding, secretion, cell–cell interactions, conformational stability, and proteolytic susceptibility. We are particularly interested in effects of gONC oligosaccharides on conformational stability and proteolytic susceptibility. For example, RNase A is readily degraded by proteases in the presence of denaturants or at high temperatures, but the rate of degradation is reduced upon glycosylation [34]. Glycoproteins display extensive diversity as a result of a variety of carbohydrate structures, differential occupancy of glycosylation sites, and variations in the numbers of attached glycans. Most eukaryotic secretory

proteins are glycosylated. There are functional differences between glycosylated and non-glycosylated forms or between the glycosylated forms. A number of non-glycosylated recombinant proteins generated from *E. coli* display decreased activities, compared to their glycosylated counterparts [24]. gONC displayed lower ribonucleolytic activity than non-glycosylated ONC (Table 1). However, the glycosylated protein exhibited increased conformational stability and resistance to proteolysis. The oligosaccharide portion of gONC constitutes about 20% of the protein moiety with respect to molecular mass. Thus, the large size of the oligosaccharide, together with its dynamic properties, shields the surface loop of ONC from exposure to non-denaturing environment or proteases.

For cytotoxicity against cancer cells, the first necessary step is an interaction between ONC and the plasma membrane of the cancer cell [3]. Other cytotoxic ribonucleases, the ONC homologs from *Rana catesbeiana* and *Rana japonica*, bind specifically to the plasma membranes of cancer cells [35,36]. Oligosaccharides of glycoproteins influence numerous biological processes, including cell–cell and cell–ligand interactions [23]. Accordingly, the oligosaccharide portion of gONC may induce an increase in its ability to bind to the cell surface, resulting in increased internalization of gONC in the cell. ONC is internalized by a dynamin-independent endocytic pathway [37]. Internalized ONC evades RI, an intracellular sentry against secretory ribonucleases [38], due to its low affinity to RI within the cell [16]. Variants of pancreatic ribonucleases, G88R RNase A, and ERDD RNase 1, which evade RI action, also display toxicity for cancer cells [16,17]. However, these proteins still bind RI with significantly higher affinity than does ONC, which may contribute to the differences in cytotoxic activities between pancreatic ribonuclease variants and ONC. ONC molecules that evade RI kill cancer cells, probably by hydrolyzing intracellular RNA. Our data show that gONC evades RI, similar to ONC and N69Q ONC.

Following translocation, ONC encounters cytosolic proteases that limit its cytotoxicity. Protease inhibitors increase the cytotoxicity of a number of other proteins [39]. Similarly, proteolytic degradation signals can decrease protein cytotoxicity [40]. gONC displayed both increased conformational stability and cytotoxicity to K-562 cells, but slightly lower activity than wild-type ONC and N69Q ONC. Recently, Leu et al. [41] showed that minimum catalytic activity is sufficient for cytotoxicity by frog ribonucleases. Therefore, our results suggest that the elevated cytotoxicity of gONC results from increased conformational stability.

ONC is on the verge of approval as a human cancer chemotherapeutic agent. A number of researchers have attempted to increase the cytotoxicity of members of the RNase A superfamily [3]. Nevertheless, ONC is still the most cytotoxic in RNase A superfamily [16,17]. Glycosylation of ONC results in 10-fold increased resistance to proteolysis and 50-fold elevated toxicity for K-562 cells. Our results indicate that gONC may be employed as an improved agent for chemotherapy. Moreover, the *P. pastoris* expression system provides a simple and efficient method for producing large quantities of functional gONC as well as ONC.

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