Biochemical and Biophysical Research Communications 377 (2008) 429–433

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SARS coronavirus: Unusual lability of the nucleocapsid protein

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ARTICLE INFO

Article history:
Received 23 September 2008
Available online 14 October 2008

Keywords:
Coronavirus
Nucleocapsid protein
N-protein
Proteolysis
SARS

ABSTRACT

The severe acute respiratory syndrome (SARS) is a contagious disease that killed hundreds and sickened thousands of people worldwide between November 2002 and July 2003. The nucleocapsid (N) protein of the coronavirus responsible for this disease plays a critical role in viral assembly and maturation and is of particular interest because of its potential as an antiviral target or vaccine candidate. Refolding of SARS N-protein during production and purification showed the presence of two additional protein bands by SDS–PAGE. Mass spectroscopy (MALDI, SELDI, and LC/MS) confirmed that the bands are proteolytic products of N-protein and the cleavage sites are four SR motifs in the serine–arginine-rich region—sites not suggestive of any known protease. Furthermore, results of subsequent testing for contaminating protease(s) were negative: cleavage appears to be due to inherent instability and/or autolysis. The importance of N-protein proteolysis to viral life cycle and thus to possible treatment directions are discussed.

Severe acute respiratory syndrome (SARS) is an atypical pneumonia first described in November 2002 [1]. The causative agent, the SARS coronavirus, belongs to the coronaviradae family of enveloped, positive-sense RNA viruses and posseses four structural proteins. The nucleocapsid (N) protein has been found to bind to a specific packaging-signal motif on the viral RNA; it is the interaction of this protein–RNA complex with the membrane (M), envelope (E) and spike (S) proteins that leads to budding through the cell membrane and virus maturation. The N-protein is of particular interest because of its potential as a vaccine candidate [2,3], as a diagnostic marker for SARS [4,5], and because it appears to play a critical role in the perturbation of several host cell processes during infection [6].

SARS N-protein is a 432 amino acid, 46 kDa protein with the high p*I* (10.1) and high content of basic amino acids characteristic of many DNA- or RNA-binding proteins. Some atypical characteristics of the N-protein include a low percentage of hydrophobic amino acids and an absence of cysteine residues [7]. The peculiar composition of the protein may be important for the RNA-binding properties, but the absence of strong intramolecular interactions also suggests that, in contrast to other viral structural proteins, the structure of SARS N-protein is unstable [8–12].

As with other viral N-proteins, the SARS N-protein exhibits extensive oligimerization that is presumably linked to SARS virus packaging and maturation. Of the potential intermolecular binding sites that have been identified [13–15], the first to be characterized was the highly hydrophilic serine and arginine rich region, ^{184}ssrsssrsrgnsr^{196} [7,16–18]. Deletion of this sequence abrogates N-protein self-association and prevents N-protein localization around the nucleus and thus the RNA-binding and packaging required for SARS virus maturation.

In the process of purifying the N-protein to study its potential in the production of vaccines against the virus, the appearance of two other protein/protein fragment bands on SDS–PAGE was detected. This paper describes the work to identify these proteins/protein fragments and to determine their source. A series of experiments were designed to isolate and characterize these fragments, pinpoint the cleavage site(s), and determine if the cleavage was due to a bacterial protease contaminant or to autolysis. It is anticipated that identification of the source/conditions for cleavage and subsequent inactivation of N-protein and concomitant disabling of the coronavirus' ability to package RNA and/or interfere with host cell function, may lead to development of novel and efficacious treatments for SARS.

Materials and methods

SARS N-protein expression and purification. Escherichia coli M15 and BL21(DE3) cells, transformed with pQE-2/NP (pQE-2 expressing the N-protein), were used to produce protein as previously described [17,19]. Purification was performed under denaturing conditions using a His-trap HP metal affinity column (GE Lifesciences).

The purified N-protein was transferred to dialysis tubing (7500 MWCO) and dialyzed into a urea-supplemented refolding buffer (10 mM Tris, 100 mM sodium phosphate, 150 mM NaCl, and 8 M urea, pH 8.0). Urea was then gradually removed by a stepwise replacement of the buffer with Tris/phosphate buffer (10 mM Tris, 100 mM sodium phosphate, and 150 mM NaCl, pH 8.0) containing

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0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.09.153

decreasing concentrations of urea (8, 4, 2, 1, 0.5, and 0 M). In samples where the N-terminal $(His)_6$-tag was removed, the pH of the buffer was adjusted to 7.0 by dialysis and Qiagen DAPase (dipeptidase) added to the protein solution.

N-protein solutions were analyzed on SDS–poly-acrylamide gel using the 12% cross-link method described by Laemmli [20] or the 20% Tricine method described by Schagger [21]. Gels were stained using Coomassie blue or Sypro Ruby Red.

Mass spectrometric analyses. SELDI-TOF/MS data were generated using a PBS-IIC instrument (Ciphergen, Fremont, CA) that was calibrated using All-in-One peptide standards (Ciphergen) adhered to a normal phase, NP20 protein array. One microgram of the SARS N-protein was applied to each of the remaining sample spots for analysis. SELDI-TOF spectra were generated by laser desorption/ionization using an average 130 laser shots with an intensity of 190–200 (arbitrary units) and detector sensitivity of eight.

MALDI-TOF mass spectrometry was performed on peptides after SDS–PAGE separation and in-gel tryptic digestion of proteins/peptide bands [22]. Peptide fragments were analyzed using a Micromass MALDI-LR instrument (Waters, Mississauga, ON) and analyzed using MassLYNX 3.5 software (Waters). Peptide fingerprint searches were performed using MASCOT (Matrix Science, Boston, MA) and the NCBI protein database.

N-protein fragments were purified on a Thermo Spectra System HPLC using a Vydac C8 reverse phase column. Proteins were eluted using 0.01% trifluoroacetic acid (TFA) and a 10–90% acetonitrile gradient and protein-containing fractions analyzed by LC/MS as previously described [23].

Protease assessment. Quanticleave protease assay using fluorescein isothiocyanate-(FITC)-conjugated casein as described by manufacturer (Pierce). Fluorescence was detected using 485/538 nm excitation/emission wavelengths in a Genios plate reader running XFluor 4 software (Tecan, Durham, NC).

Non-specific protease activity was tested by mixing a 10-fold excess (w/w relative to SARS N-protein) of either ovalbumin or RNAse A and co-refolded with the SARS N-protein. Ovalbumin and RNase A were prepared by cleaving and capping of disulfide bonds with iodoacetamide to prevent disulfide bond formation prior to refolding. The test proteins (N-protein and ovalbumin or RNAase) were then denatured by addition to 6 M guanidine buffer containing SARS N-protein and proteins simultaneously refolded. The presence or absence of cleavage peptides was determined using SDS–PAGE.

Fluorescent resonance energy transfer-labelled peptides (EDANS/DABCYL-conjugated ^{168}LPKGFYAEGSRGGSQASS^{185}- and ^{181}SQASSRSSSRSRGNSRNSTP^{200}-SARS N-protein peptides) were purchased from JPT Peptide Technologies (Acton, MA). EDANS/DABCYL-conjugated peptides were designed to correspond to putative cleavage sites of the SARS N-protein. Cleavage of either peptide would lead to separation of the EDANS reagent from the DABCYL and result in a 40-fold increase in fluorescent signal. An aliquot (100 μl) of each EDANS/DABCYL-conjugated peptide dissolved in PBS at pH 7.2 (0.5 mg/ml final concentration) was placed in a 96-well plate and 100 μl of either N-protein preparation (25 μg total protein) or trypsin (positive control) was added. Fluorescence (relative fluorescent units, RFU) was recorded using 360/465 nm excitation/emission wavelengths over 60 min at 25 °C.

Results and discussion

SARS N-protein production, purification, and refolding

SARS N-protein was produced in both M15 and BL21(DE3) cells. Proteins were expressed as insoluble inclusion bodies and purified using metal affinity resin, then urea-denatured proteins were refolded by stepwise dialysis and the His-tag removed by DAPase digestion. SDS–PAGE confirmed that the recovered N-protein was purified to near homogeneity under denaturing conditions (Fig. 1). However, following the refolding, two additional bands (A and B bands) were observed at approximately 29 kDa (A band) and 25 kDa (B band). The masses were consistent with proteolyed N-protein (approximately 50 kDa) and similar to the SARS N-protein proteolysis products reported in the presence of caspases [24].

To rule out the possibility that the A and B bands could be attributed either to N-protein cleavage by DAPase or a contaminant in DAPase, the purification was repeated using BL21(DE3) cells without DAPase addition. BL21(DE3) cells lack the lon and ompT protease genes and thus reduced recombinant protein proteolysis is expected. However, the A and B bands were still observed, confirming that the SARS N-protein cleavage was not due to the DAPase preparation and was independent of the cell line used (data not shown).

Characterization of protein fragments

To determine the source of the A and B bands, mass spectrometry techniques were used. The experiments used preparations of recombinant N-protein from which the His-tag had not been enzymatically removed; thus, calculation of the resultant protein/

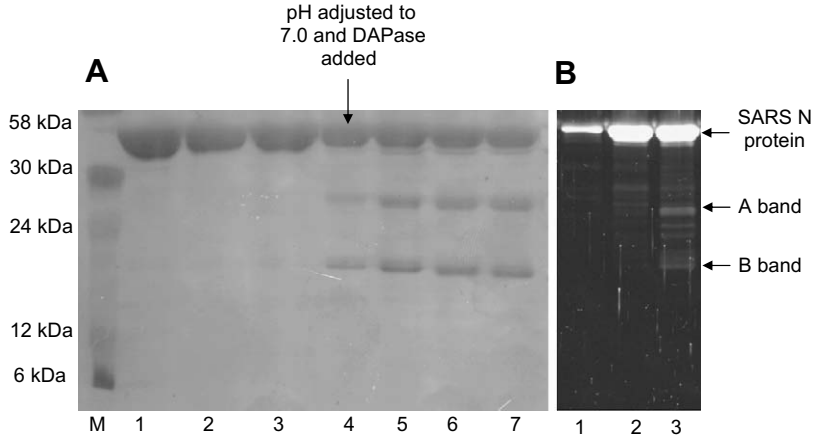

Fig. 1. SARS N-protein refolding and proteolysis. (A) SARS N-protein produced in M15 cells and purified was refolded by gradual dialysis and the engineered $(His)_6$-tag was removed using Qiagen DAPase as described in Materials and methods. Ten microliters of the protein mixtures were loaded onto 12% SDS–PAGE and stained using Coomassie blue. Lane M, molecular weight markers; lane 1, SARS N-protein in 8 M urea; lane 2, 0.5 M urea; lane 3, 0 M urea; lane 4, 0 M urea; lane 5, 10 min following DAPase addition; lane 6, 20 min following DAPase addition; lane 7, 30 min following DAPase addition. (B) SARS N-protein was produced and refolded without DAPase. Samples were separated on 12% SDS–PAGE and stained with Sypro Ruby Red lane 1, SARS N-protein in 2 M urea; lane 2, 1 M urea; and lane 3, 0.5 M urea.