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Synthesis of the human respiratory syncytial virus nucleoprotein in yeast *Saccharomyces cerevisiae*

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The development of a simple, efficient and cost-effective system for generation of respiratory syncytial virus (RSV) nucleoprotein (NP) might help to upgrade reagents for virus serology, and facilitate an investigation of viral replication and RNA encapsidation mechanisms. To this aim, the gene encoding human RSV NP of antigenic subgroup A was cloned into the yeast *Saccharomyces cerevisiae* expression vector pFGG3 under control of *GAL*7 promoter. For the recombinant nucleoprotein the yeast expression system proved to be efficient. After purification by CsCl gradient centrifugation, the recombinant RSV–A N protein from yeast yielded 0.9–1.2 mg/1g of wet yeast biomass. A similar electrophoretic mobility of rN and native N proteins was exhibited by SDS-PAGE analysis. Recombinant nucleoproteins reacted with immune sera in a Western blot assay. Electron microscopy of purified recombinant nucleoproteins demonstrated an assembly of herring-bone structures typical of viruses of the family *Paramyxoviridea*. The yeast-expressed RSV-A N protein appeared to be stable during purification and storage. A change of amino acid Y337 to C precluded formation of nucleocapsid-like particles.

Key words: human respiratory syncytial virus, nucleoprotein, yeast, nucleocapsid-like particles

INTRODUCTION

Respiratory syncytial virus (RSV) is an enveloped, single-stranded negative-sense RNA virus from the genus *Pneumovirus* of the family *Paramyxoviridea,* and is a major cause of a serious respiratory illness in infants [1]. RSV encodes 11 proteins, namely, three transmembrane surface proteins (G, F, and SH); the virion matrix protein M; the nucleocapsid and polymerase proteins N, P, M2-1, and L; the putative transcription-replication regulatory factor M2-2; and two nonstructural proteins, NS1 and NS2 [2]. The RSV genome is completely encapsidated by virus-encoded nucleocapsid (N) protein, rendering the genome nuclease-resistant. The morphology of N-bound RNA (hereafter referred to as the nucleocapsid) is considered to be one of the defining features of the *Paramyxoviridae* and is described as having a herring-bone appearance when imaged under electron microscope. RSV nucleocapsid is significantly smaller (diameter, 13 to 14 nm) than those described for other paramyxoviruses (18 nm). Electron microscopic studies indicate that RSV nucleocapsids are less rigid and narrower than those of measles virus and Simian virus 5 [3].

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Respiratory syncytial virus (RSV) is the most important viral etiologic agent of a serious pediatric respiratory tract disease worldwide. RSV is also receiving increasing recognition as an important cause of respiratory tract disease in the elderly, in immunocompromised patients such as bone marrow transplant recipients, and in the general population, and remains one of the pathogens deemed most important for diagnostics and vaccine development [2]. RSV exists as a single serotype but has two antigenic subgroups, A and B. The RSV N protein is a target for CTLs in rodents and humans, the protective effects associated with a (vaccine-induced) immune response to N appear to be CTL-mediated but short-lived [4]. The nucleocapsid protein of the bovine respiratory syncytial virus in the baculovirus expression system was evaluated as a source of antigen in an enzyme-linked immunosorbent assay for the detection of respiratory syncytial virus antibodies [5]. Here we have presented a high-level expression system for generation of human respiratory syncytial virus N protein in yeast which thereinafter is going to be used for generation of specific monoclonal antibodies and in the development of virus-specific enzyme-linked immunosorbent assay for serum analysis.

After the sequencing of several hRSV-A N genes, which were amplified using cDNA isolated from patients infected with human respiratory syncytial virus, we had

found that some of the sequenced N genes contain a single amino acid substitution at the position 377 (Y→C). We expressed the substitution containing hRSV-A N gene in yeast in order to find out whether it is a naturally existing polymorphism or the amplification mistake which may affect recombinant N proteins.

MATERIALS AND METHODS

Generation of yeast expression plasmids

All DNA manipulations were performed according to the standard procedures [6]. Enzymes and kits for DNA manipulations were purchased from Fermentas (Vilnius, Lithuania).

The hRSV–A N gene was amplified from cDNA isolated from patients infected with a hRSV A2 strain (the major antigenic subgroup A) (GenBank Acc. No. M11486). cDNA was kindly provided by Dr. M. Coiras. Primers used in the amplification of hRSV–A N gene included a SpeI site for subcloning into the yeast vector, a single ATG codon in the forward primer, and a stop TAA codon in the reverse primer. The primer sequences containing the SpeI sites (in bold) and the start and stop codons (underlined) are forward (5'→3'): GCA**ACTAGT**A CAATGGCTCTTAGCAAAGTCAAGTTG, and reverse (5'→3'): TTT**ACTAGT**TTAAAGCTCTACATCATTATCT.

The amplified hRSV-A N gene was digested with a restriction endonuclease SpeI, gel-purified and cloned into the SpeI site of the yeast expression vector pFGG3 under control of *GAL7* promoter [7]. The cloned N gene was sequenced using specific primers flanking the pFGG3 SpeI cloning site. The following primers were used:

Forward (5'→3') ATTATGCAGAGCATCAACATG.

Reverse (5'→3') GTCTTTGTAGATAATGAATCTG.

Resulting plasmids pFGG3-hRSV-A-N and pFGG3-hRSV-A-N* (Y337→C) were used for transformation of yeast *S. cerevisiae* strain AH22 (*leu*2 *his*4 *ura*3). Yeast cells were grown in YEPD medium (yeast extract 1%, peptone 2% and glucose 2%) supplemented with 5 mM formaldehyde or in induction medium containing YEPG (yeast extract 1%, peptone 2% and galactose 2.5%).

Expression and purification of nucleoproteins

The procedure used for expression and purification of recombinant N proteins was similar to that described for recombinant mumps nucleoprotein [8]. Briefly, *S. cerevisiae* cells harbouring pFGG3-hRSV-A-N and pFGG3-hRSV-A-N*(Y337→C) were inoculated separately into YEPD medium supplemented with 5 mM formaldehyde, grown overnight and reinoculated into YEPG induction medium and cultured for 24 h at 28 °C. Cells were harvested by centrifugation and their wet weight was recorded. Cells were suspended in disruption buffer DB (0.05 M phosphate buffered saline, PBS, pH 7.2, 10 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, PMSF) and the amount of glass beads (Sigma, U.K.) equal to two weights of wet cells was added and cyclically disrupted twelve times by homogenization in a blender at 3000 rpm and 4 °C for 1 min. After centrifugation at 1500 × *g* and 4 °C, rN proteins were purified from the supernatant by successive ultracentrifugation through a 30% sucrose cushion followed by two CsCl gradients ranging from 1.23 to 1.38 g/mL. CsCl fractions were analyzed on 12% PAGE gels under reducing conditions in SDS-Tris-glycine buffer [6]. Fractions containing recombinant N proteins were pooled and dialyzed against PBS for immunological analysis. The buoyant density of fractions was determined with a refractometer.

SDS PAGE and Western blotting

Protein samples were separated on 12% SDS-PAGE gels and transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, USA) by semi-dry blotting. The membrane was blocked in TBS-T (20mM Tris-HCl, 8g/l NaCl, pH7.6, 0.1% Tween-20) and incubated with RSV specific polyclonal antibodies (Chemicon, Temecula, USA) diluted 1:1000 in TBS-T and thereafter with anti-goat IgG-HRP conjugate (Sigma-Aldrich, Steinheim, Germany) diluted 1:1000 in TBS-T. The membrane was stained with 4-chloro-naphthol supplemented with H_2O_2 (Sigma, Steinheim, Germany).

Proteins on SDS-PAGE gels were visualized by staining with Coomassie brilliant blue dye.

Electron microscopy

After purification by CsCl centrifugation, suspensions of recombinant N proteins were placed on 400-mesh carbon-coated palladium grids. Samples were stained with 2% aqueous uranyl acetate solution and examined with the JEM-100S electron microscope.

RESULTS AND DISCUSSION

Expression of the recombinant nucleoprotein was analyzed in SDS-PAGE. SDS-PAGE analysis of crude lysates of *S. cerevisiae* harbouring pFGG3-hRSV-A-N, after induction with galactose revealed the presence of an additional ~43–44 kDa protein band (Fig. 1 A: lane 2) consistent with the molecular weight of N protein of human respiratory syncytial virus reported in literature [9]. No additional band of a corresponding molecular size was observed in crude lysates of *S. cerevisiae*, harbouring the integrated vector pFGG3 (Fig. 1 A: lane 1).

After centrifugation through 30% sucrose cushion recombinant N proteins were found in pellets (data not shown), which is consistent with their large size and multimeric organization. Further, ultracentrifugation of the resolubilised pellets in CsCl gradient and analysis revealed rNs in fractions with a buoyant density of 1.28–1.29 g ml^{-1}. An interval of the buoyant density ranging from 1.28 g ml^{-1} to 1.31 g ml^{-1} is characteristic of most virus-like particles or NLPs, with or without RNR [10]. Fractions of this buoyant density contained highly purified N proteins with only minor contaminants present as observed in SDS-PAGE. The SDS-PAGE analysis of purified N proteins after centrifugation in CsCl revealed a band of ~43–44 kDa (Fig. 1 A: lane 3), which was also observed in crude lysates (Fig. 1 A: lane 2). The identity

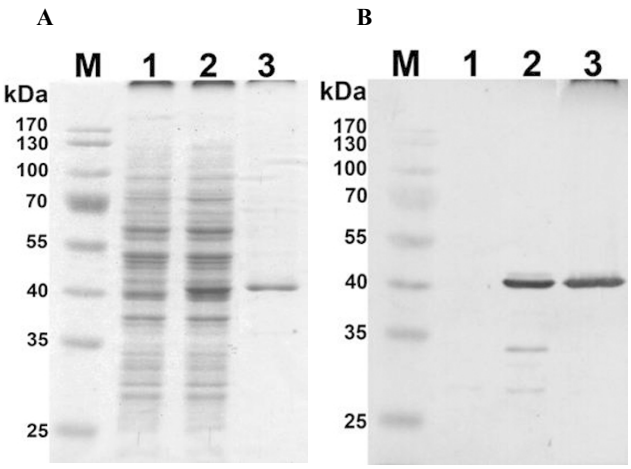

Fig. 1. SDS-PAGE analysis of yeast lysates and samples of yeast expressed hRSV-A N protein after ultracentrifugation in CsCl. A – Comassie blue stained gel. B – Western blot using hyperimmune goat sera generated by immunizing goats with purified virions (Chemicon, Temecula, USA). Preparations loaded onto gels were as follows: lane 1 – *S. cerevisiae* [pFGG3] lysate; lane 2 – *S. cerevisiae* [pFGG3-hRSV-A-N] lysate; lane 3 – CsCl purified hRSV-A N protein; M — protein molecular mass marker (Fermentas UAB, Vilnius, Lithuania) 170, 130, 100, 70, 55, 40, 35, 25 kDa

of recombinant hRSV-A Ns was confirmed by immunoblot with hyperimmune goat sera generated by immunizing goats with purified virions (Fig. 1 B).

In our study, yeast expressed rN proteins form typical herring-bone and ring-like structures that were evident after electron microscopy analysis (Fig. 2). Similar nucleocapsid-like structures form yeast expressed N proteins of various viruses of family *Paramyxoviridae* [11]. Nucleocapsids isolated after the expression of the recombinant hRSV-A nucleoprotein appeared to be loosely ordered and highly flexible helices. Weakly organized helical nucleocapsid is a characteristic feature of the genus *Pneumovirinea* [3]. It is interesting to note that analysis of electron micrographs of the purified yeast expressed hRSV-A N proteins, containing single amino acid substitution at the position 377 (Y→C), showed no detectible particle-like structures (Fig. 3). Current data suggest that the domain responsible for N-N interactions is located in the N-terminal portion of the N protein (~100 N-terminal amino acids) [12]. Cystein residue at the position 377 (C-terminal end of N protein) may form unusual disulfide bond interfering with normal protein folding.

In several preparative procedures, the yield of recombinant hRSV-A N was found to be ~0.9–1.2 mg/1g of wet yeast biomass. The yield obtained resembles that observed previously for N proteins of different other viruses [7].

The hRSV-A N protein was stable in CsCl at 4 ˚C. Treatment with 10mM EDTA, 10mM EGTA or 10mM DTT did not cause dissociation of NLPs indicating that the assembled structure did not require divalent ions or disulphide bonds for stabilization of the structure. The

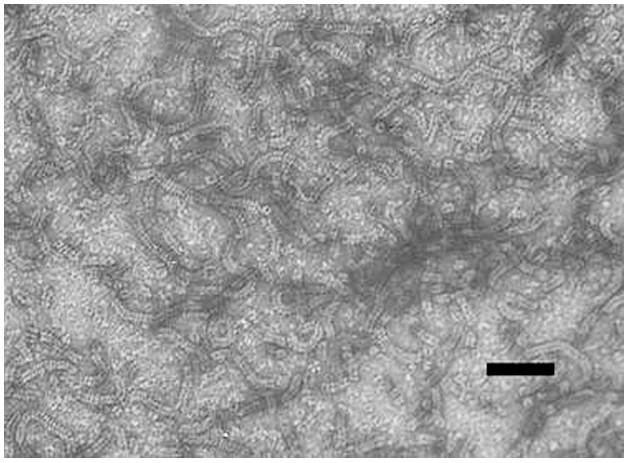

Fig. 2. Electron micrograph of negatively stained hRSV-A N protein isolated from *S. cerevisiae* (black bar corresponds to 100nm)

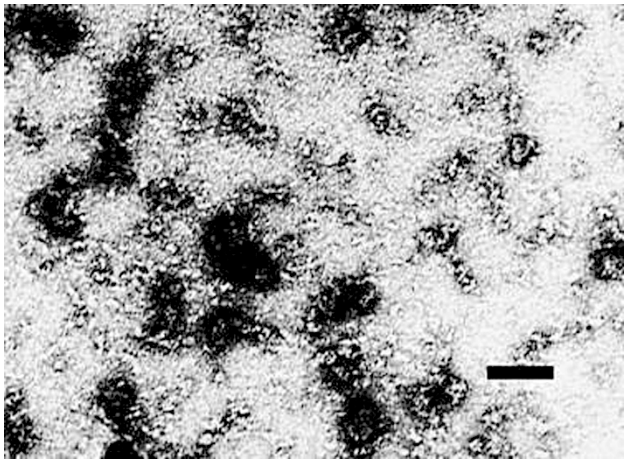

Fig. 3. Electron micrograph of negatively stained hRSV-A N*(Y337→C) protein isolated from *S. cerevisiae* (black bar corresponds to 100nm)

particles were stable after dialysis and long-term storage in 50% glycerol at –20 ˚C.

CONCLUSIONS

In our study was shown that the hRSV-A N protein might be efficiently synthesized in yeast expression system. Yeast expressed hRSV nucleoproteins proved to be very similar to native N proteins and might help to upgrade reagents for virus serology, facilitate investigation of viral replication and RNA encapsidation mechanisms. We also find out that the change of amino acid Y337 to C in the recombinant hRSV-A N protein precluded formation of nucleocapsid-like particles.

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ŽMONIŲ KVĖPAVIMO SINCITINIO VIRUSO NUKLEOKAPSIDĖS BALTYMO SINTEZĖ MIELĖSE *SACCHAROMYCES CEREVISIAE*

Santrauka

Siekiant sukurti žmonių kvėpavimo viruso (hRSV) nukleokapsidės (N) baltymo sintezės sistemą buvo sukonstruotos plazminės, skirtos šio baltymo ekspresijai mielėse *S.cerevisiae*. A antigeniniam pogrupiui priklausančio hRSV viruso N baltymą koduojantis genas buvo įklonuotas į mielių ekspresijos vektorių pFGG3 po galaktoze indukuojamo promotoriaus sekos. Nustatyta, kad mielių ekspresijos sistema puikiai tinka hRSV N baltymo sintezei. Gauta 0,9–1,2 mg/1 g šlapios baltymų biomasės išgryninto rekombinantinio hRSV-A N baltymo išeiga. NDS-PAGE analizė rodo, kad rekombinantinio ir autentiško hRSV N baltymo elektroforetinis mobilumas yra labai panašus. Su rekombinantiniu hRSV nukleokapsidės baltymu atlikta imunologinė analizė. Išgrynintų baltymų elektroninė mikroskopija rodo, kad jie formuoja nukleokapsidines daleles (NKD), kurios yra labai panašios į *Paramyxoviridea* šeimos virusų N baltymų formuojamas daleles. Iš rekombinantinių N baltymų suformuotos NKD yra patvarios ir nesuyra gryninimo metu. Nustatyta, kad mielėse susintetintas hRSV-A N baltymo variantas su 337 pozicijoje pakeista amino rūgštimi (iš Y į C) neformuoja nukleokapsidinių dalelių.