Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 366 (2008) 308–313
BBRC
www.elsevier.com/locate/ybbrc

The RSV F and G glycoproteins interact to form a complex on the surface of infected cells

Kit-Wei Low [a], Timothy Tan [a], Ken Ng [a], Boon-Huan Tan [b], Richard J. Sugrue [a,*]

[a] School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore
[b] Detection and Diagnostics Laboratory, Defence Medical and Environmental Research Institute, DSO National Laboratories, 27 Medical Drive, #13-00, Singapore 117510, Singapore

Received 7 November 2007
Available online 26 November 2007

Abstract

In this study, the interaction between the respiratory syncytial virus (RSV) fusion (F) protein, attachment (G) protein, and small hydrophobic (SH) proteins was examined. Immunoprecipitation analysis suggested that the F and G proteins exist as a protein complex on the surface of RSV-infected cells, and this conclusion was supported by ultracentrifugation analysis that demonstrated co-migration of surface-expressed F and G proteins. Although our analysis provided evidence for an interaction between the G and SH proteins, no evidence was obtained for a single protein complex involving all three of the virus proteins. These data suggest the existence of multiple virus glycoprotein complexes within the RSV envelope. Although the stimulus that drives RSV-mediated membrane fusion is unknown, the association between the G and F proteins suggest an indirect role for the G protein in this process.

Keywords: Respiratory syncytial virus; Fusion protein; Attachment protein; Protein cross-linking; Biotinylation; Surface labelling; Virus glycoproteins

Respiratory syncytial virus (RSV) is the most important respiratory virus causing lower respiratory tract infection in the young children and neonates, being responsible for up to 64 million reported cases and 160,000 deaths each year [1]. The mature virus remains largely cell-associated, forming filamentous structures on the surface of infected cells that are referred to as virus filaments [2]. The virus genome is surrounded by a lipid-envelope that is derived from the host cell, within which three virus-encoded proteins are embedded.

The G protein is thought to mediate the attachment of the virus to the cell during virus entry [3], while the role played by the SH protein during virus replication remains to be established. The F protein plays a pivotal role during virus infection by mediating fusion of the virus and host cell membranes. It is initially synthesised as an inactive precursor, F_0 (75 kDa), which undergoes proteolytic cleavage by furin in the *trans*-Golgi compartment [4–6]. This generates the mature and active form of the protein which consists of two disulphide-linked subunits, called F_1 (55 kDa) and F_2 (20 kDa) [7].

In several well characterized paramyxoviruses, membrane fusion is initiated by a protein complex that involves the respective virus fusion and attachment proteins. In these viruses, the binding of the attachment protein to the cell surface receptor induces a conformational change in the F protein, which in turn mediates membrane fusion (reviewed in [8,9]). In the case of RSV, no convincing evidence for an association between its fusion and attachment proteins has been demonstrated, and the trigger that initiates membrane fusion is still not understood. As a consequence, biochemical mechanisms have been proposed to explain how the F protein is able to mediate membrane fusion without the aid of other virus proteins (e.g. [10]). In this report, we provide the first direct evidence that the RSV F and G proteins are able to interact to form a protein complex on the surface of virus particles. This

* Corresponding author.
E-mail address: rjsugrue@ntu.edu.sg (R.J. Sugrue).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.11.042

implies that the RSV fusion process may be similar to that reported for other paramyxoviruses, and suggests the G protein may play an ancillary role in the virus-mediated membrane fusion process.

Materials and methods

Cells and viruses. The RSV A2 strain was used throughout this study. The HEp-2 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) and antibiotics.

Antibodies. The F protein monoclonal antibody (MAb169) and anti-SH have been described previously [11]. MAb19 was a gift from Geraldine Taylor (IAH, Compton, UK) and anti-G was purchased from Chemicon.

Fluorescence microscopy. This was performed as described previously [4]. Briefly, RSV-infected cells grown on glass coverslips were fixed with 4% paraformaldehyde and then washed extensively with PBS. The cells were permeabilised using 0.1% saponin and the cells incubated with MAb19, followed an anti-mouse mouse antibody conjugated to FITC. The stained cells were viewed in a Zeiss LSM510 Axiovert confocal microscope.

Protein biotinylation. Cell monolayers were washed with PBS8 (PBS adjusted to pH 8.0), after which they were incubated with 0.5 mg/ml sulpho-NHS-LC-LC-biotin in PBS8 for 30 min. Prior to detergent extraction the monolayers were extensively washed with PBS8 (supplemented with 2 mM lysine).

In situ cross-linking. This was performed using Dithiobis[succinimidylpropionate] (DSP) (Pierce Perbio Ltd.) as described previously [12]. Briefly, the biotinylated cell monolayers were incubated in PBS8, and DSP added to give the required concentration. Prior to detergent extraction the monolayers were extensively washed with PBS8 (supplemented with 2 mM lysine).

Immunoprecipitation. This was performed as described previously [4]. Briefly, cell lysates were prepared at 4 °C using either RIPA buffer (1% NP-40, 0·1% SDS, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 2 mM lysine, 20 mM Tris–HCl, pH 7.5) or NP40 buffer (1% NP40, 150 mM NaCl, 1 mM EDTA, 2 mM lysine, 2 mM PMSF, 20 mM Tris–HCl, pH 7.5) and clarified by centrifugation (13,000*g*, 10 min 4 °C). Aliquots of the lysates were incubated with the relevant primary antibody, and the immune complexes were isolated with protein A–Sepharose. The immune complexes were then resuspended in boiling mix and analysed further.

Western blotting. The proteins were separated by SDS–PAGE, transferred by Western blotting on to PVDF membranes, and the membranes incubated in blocking buffer (1% Marvel™, 0.05% Tween in PBS). The membranes were then either incubated with the relevant primary antibody and a suitable secondary antibody conjugated to horse radish peroxidases (HRP), or alternatively, incubated with streptavidin conjugated to HRP (Amersham) for the detection of biotinylated protein species. Protein bands were visualised using the ECL protein detection system (Amersham). Apparent molecular masses were estimated using Kaleidoscope (Biorad) prestained protein markers in the molecular weight range, 14–200 kDa.

Density gradient centrifugation. All steps were performed at 4 °C. A RSV-infected cell monolayer was treated with NP40 buffer, and the resulting lysate clarified by centrifugation (13,000*g*, 10 min). The clarified lysate was layered onto a 5–30% sucrose gradient prepared in PBS + 0.2% Triton X-100. The gradient was centrifuged for 20 h at 210,000*g* in a P40ST rotor in a CP90WY preparative ultracentrifuge (Hitachi), after which the gradient was fractionated, and the individual fractions analysed further.

Preparation and analysis of [^{3}H]glucosamine-labelled virus. Sub-confluent T75 flasks containing HEp-2 cells were infected with RSV at a multiplicity of infection equal to 0.1, and incubated at 33 °C. At 6 h post-infection the cells were washed with PBS, and the incubation continued in DMEM minus glucose (Gibco) containing 100 μCi/ml [^{3}H]glucosamine hydrochloride (Amersham) for an additional 44 h. All subsequent steps were performed at 4 °C. The tissue culture medium and cells were harvested by gentle agitation using glass beads, after which the medium was clarified by low speed centrifugation (2000*g*, 10 min). The clarified medium was harvested, and again centrifuged at 2000*g* for 10 min. Equal volumes (12 ml) of the clarified media were applied to 40% sucrose cushion (prepared in Hanks buffered saline solution) and centrifuged at 140,000*g* for 10 h in a P28S rotor. The resulting pellet containing the virus particles was resuspended in NP40 buffer using a sonicating water bath at 4 °C. The radiolabelled immunoprecipitated virus proteins were separated by SDS–PAGE, and detected by autoradiography.

Results and discussion

Virus filaments form on the surface of RSV-infected cells, and confocal microscopy demonstrated that the F protein expressed on the surface of infected cells was concentrated within these structures (Fig. 1A). A similar staining pattern was also observed following G protein-labelling of virus-infected cells [2]. Infected cell monolayers were surface labelled using sulpho-NHS-LC-LC-biotin, and either non-treated or treated with 0.05 mM DSP. The optimal DSP concentration was determined by prior titration of the DSP concentration on infected cell monolayers (data not shown). The cell monolayers were detergent-extracted using RIPA buffer, and the F protein isolated by immunoprecipitation using MAb19 (recognises the F_1 subunit). The F protein was separated by reducing SDS–PAGE, transferred by Western blotting onto PVDF membranes, and the biotinylated proteins bands detected using streptavidin–HRP (Fig. 1B). In the absence of DSP, a labelled band corresponding in size to the F_1 subunit was detected, together with small amounts of a 90 kDa labelled protein (Fig. 1B, lane −). Following DSP treatment, the level of the 90 kDa protein appeared to increase (Fig. 1B, lane +), suggesting that the 90 kDa protein is associated with the F protein, and that this protein complex is stabilised by DSP during the detergent extraction procedure. In addition, the total protein in the non-cross-linked and DSP-treated lysates were analysed by immunoblotting using MAb19 (Fig. 1C). This revealed a prominent protein species corresponding in size to the F_1 subunit. The 90 kDa protein species was not detected, suggesting that the 90 kDa biotinylated protein did not arise due to cross-reactivity of MAb19 with another virus or cell protein, nor did it represent a previously undescribed form of the F protein.

The size of this co-precipitating 90 kDa protein was consistent with that of the G protein [13], and this possibility was investigated. Biotinylated mock and virus-infected cells were treated with NP40 buffer at 4 °C, and the F and G proteins isolated by immunoprecipitation using either MAb19 (Fig. 1D, i) or anti-G (Fig. 1D, ii). This detergent buffer does not contain SDS, and it therefore represents a milder extraction condition than the more commonly used RIPA buffer. Immunoprecipitation using MAb19 revealed a protein species corresponding in size to the F_1 subunit, together with the 90 kDa protein. Similarly, immunoprecipitation of the lysate using anti-G revealed a protein species corresponding in size to the G protein, together with a corresponding 55 kDa protein, the expected size for the F1 subunit. To confirm the identity of the co-precipitating