

# Temperature dependent characteristics of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells

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**ABSTRACT:** A recombinant infectious hematopoietic necrosis virus (IHNV) glycoprotein (G protein) was produced in insect cells using a baculovirus vector (*Autographa californica* nuclear polyhedrosis virus). Characteristics of this protein were evaluated in relation to native viral G protein. A full-length (1.6 kb) cDNA copy of the glycoprotein gene of IHNV was inserted into the baculovirus vector under control of the polyhedrin promoter. High levels of G protein (approximately 0.5 µg/1 × 10<sup>5</sup> cells) were produced in *Spodoptera frugiperda* (Sf9) cells following recombinant baculovirus infection. Analysis of cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot revealed a recombinant IHNV G of slightly higher mobility on the gel than the viral G protein. Differences in mobility were abrogated by endoglycosidase treatment. When the recombinant G protein was produced in insect cells at 20°C (RecG<sub>low</sub>), immunostaining and cell fusion activity demonstrated surface localization of the protein. In contrast, when recombinant protein was produced at 27°C (RecG<sub>high</sub>), G protein was sequestered within the cell, suggesting that at the 2 different temperatures processing differences may exist. Eleven monoclonal antibodies (MAbs) were tested by immunoblotting for reactivity to the recombinant G protein. All 11 MAbs reacted to the reduced proteins. Four MAbs recognized both RecG<sub>high</sub> and RecG<sub>low</sub> under non-reducing conditions; however, 1 neutralizing MAb (92A) recognized RecG<sub>low</sub> but failed to react to RecG<sub>high</sub> under non-reducing conditions. This suggests that differences exist between RecG<sub>low</sub> and RecG<sub>high</sub> which may have implications in the development of a properly folded recombinant G protein with the ability to elicit protective immunity in fish.

**KEY WORDS:** IHNV · G protein · Baculovirus · Recombinant · Conformational alterations · Monoclonal antibodies

## INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus causing severe disease in trout and salmon populations, results in large economic impacts to public and private aquaculture. Epizootics occur primarily in juvenile sockeye salmon (kokanee) *Oncorhynchus nerka*, chinook salmon *O. tshawytscha* and rainbow trout (steelhead) *O. mykiss* (Wolf 1988). Once considered enzootic to countries bordering the North

Pacific Rim, the virus has spread and is now found in continental Europe (Hattenberger-Baudouy et al. 1989). No chemotherapeutic treatments exist, and commercially licensed vaccines aimed at mass immunization of juvenile fish are not available. Only through avoidance is the disease controlled (Winton 1991).

Vaccine development against IHNV has included formalin-killed whole virus preparations (Nishimura et al. 1985), attenuated virus (Leong et al. 1988), recombinant subunit vaccines (Gilmore et al. 1988), and more recently, DNA vaccines (Anderson et al. 1996). Killed and live virus vaccines provide protection, but regulatory concerns, cost of production, and storage problems have inhibited commercial licensing. This situation has prompted development of recombinant

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subunit vaccines expressing epitopes of the glycoprotein of IHNV in *Escherichia coli*. These have been reported to provide protection against viral challenge in the laboratory (Gilmore et al. 1988, Xu et al. 1991). The glycoprotein (G) of IHNV is the only viral protein required to elicit neutralizing antibodies in salmonids (Engelking & Leong 1989). Likewise, the surface glycoproteins of rabies and vesicular stomatitis virus (VSV) are responsible for eliciting neutralizing antibodies and protective immunity in mammals (Kelley et al. 1972, Cox et al. 1977, Wiktor et al. 1984). The glycoprotein of viral hemorrhagic septicemia virus (VHSV), another fish rhabdovirus that is prevalent in European trout farms and has an economic impact similar to that of IHNV, is also responsible for inducing protective immunity (de Kinkelin et al. 1984).

Unlike bacterial expression, insect cells provide post-translational modifications such as glycosylation. Recombinant proteins produced in cultured insect cells often retain biological activity and are often functionally similar to their authentic counterparts (Luckow & Summers 1988, Maeda 1989, Luckow 1991, King & Possee 1992). For these reasons, it was thought that a full-length glycosylated G protein produced in insect cells may provide a more complete IHNV vaccine. Koener & Leong (1990) successfully expressed the glycoprotein gene of IHNV in insect cells using a baculovirus vector. The resulting protein migrated similarly to the viral G protein and reacted with anti-IHNV glycoprotein antiserum in a Western blot; however, no further characterization or testing of this recombinant protein has been reported. Research on other rhabdovirus G proteins suggests that glycosylation may be critical for neutralizing antibody formation (Machamer & Rose 1988, Prehaud et al. 1989). Development of a baculovirus vector for expression of a full-length IHNV G gene may provide a glycosylated protein structurally similar to the viral G protein. The objectives of this study were to express the IHNV G gene as a full-length recombinant protein in insect cells using a baculovirus vector and to characterize the product in relation to the wild-type viral glycoprotein.

## MATERIALS AND METHODS

**Virus and cells.** Recombinant baculoviruses were propagated and assayed in confluent monolayers or suspensions of *Spodoptera frugiperda* (Sf9) cells grown in serum-free media Sf-900 II (Gibco, BRL). Sf9 cells, originally obtained from the American Type Culture Collection (ATCC #CRL-1711), were supplied by Gibco BRL and maintained at 20 or 27°C. Culture and infection of cells with recombinant baculovirus stocks followed recommendations by Gibco BRL. Recombinant baculovirus stocks were obtained by centrifugation of

infected Sf9 suspension cultures (100 × g) for 5 min, harvesting supernatant and storing at 4°C in the dark for later use. Purified IHNV (Round Butte I isolate; previously described in Hsu et al. 1986, Ristow & Arnzen de Avila 1991) was used for electrophoretic analysis.

**Baculovirus vector construction.** Recombinant baculoviruses were constructed using the Bac-to-Bac™ Expression System (Gibco BRL). Briefly, a cDNA copy of the full-length glycoprotein gene of IHNV was excised from the pCMV4-G plasmid (kindly provided by Dr Jo-Ann Leong, Oregon State University) by digestion with the restriction endonuclease *Pst*I. The digested products were separated in low melting agarose and the 1.6 kb glycoprotein gene was excised. The gene was cloned into the multiple cloning site of the pFastbac-HT donor plasmid (Gibco BRL) just downstream of the polyhedrin promoter. A control gene (Gus) encoding β-glucuronidase (provided by Gibco BRL), was cloned and expressed in parallel with the G gene. Donor plasmids containing each of the target cDNA inserts (Gus and IHNV G) were transformed into competent *Escherichia coli* DH10Bac cells (Gibco BRL) harboring a baculovirus shuttle vector (bacmid), a recombinant virus that replicates as a large plasmid and is infective to lepidopteran insect cells. Transformation occurred by site-specific transposon-mediated insertion of the target gene from the donor plasmid into a mini-*att*Tn7 site on the bacmid (Luckow et al. 1993). Four bacterial colonies containing the recombinant bacmid were chosen for transfection into Sf9 cells. Bacmid DNA from colonies 4, 6, 7, and 8 was isolated and transfected into Sf9 cells using Cellfectin (Gibco BRL) reagent following the manufacturer's instructions. Transfection media was removed after incubation for 5 h at 27°C and replaced with fresh media. Supernatants were harvested 72 h post-transfection. Virus titers were determined by plaque assay (Gibco BRL), and suspension cultures of Sf9 cells were infected at a multiplicity of infection (MOI) of 0.01 to 0.1 for amplification of virus stock. The resulting recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) containing the gene encoding the G protein or the Gus (β-glucuronidase) control were designated rAcG497, rAcG697, rAcG797, rAcG897, and rAcGus. Production of target proteins was evaluated following infection of suspension cultures of Sf9 cells at an MOI of 5 to 10.

**Recombinant glycoprotein production.** Separate cultures of Sf9 cells, grown in suspension at 27°C, were initially mock infected or infected with 1 of the 4 recombinant baculoviruses. Cells were harvested by centrifugation at 8 d post-infection (dpi) and analyzed for protein production by electrophoresis and immunoblotting (described in detail below). Based on initial protein production, rAcG897 was chosen and used for all subsequent expression experiments.

Recombinant G protein produced in Sf9 cells cultured at either 20 or 27°C was designated RecG<sub>low</sub> or RecG<sub>high</sub>, respectively. Infection with rAcGus and expression of the positive control gene was accomplished at 27°C. Protein production for RecG<sub>low</sub> or RecG<sub>high</sub> was evaluated by removal of 5 ml of media plus cells daily from Day 2 to Day 14 following infection of suspension cultures of Sf9 cells with rAcG897. Harvest of cells for electrophoretic analysis was accomplished by centrifugation for 5 min at 100 × *g* of baculovirus infected or mock infected cell suspensions. Following centrifugation, cell pellets were resuspended in phosphate buffered saline (PBS) and stored at -20°C.

**Electrophoresis and immunoblotting.** Protein production was demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to previously described methods (Laemmli 1970). Recombinant proteins were evaluated by staining gels for total protein with coomassie brilliant blue or by immunoblotting. Following coomassie blue staining, protein concentrations were estimated by comparison of G specific bands to a band produced by 1 µg of a bovine serum albumin (BSA) standard. Spot densitometry analysis was accomplished with an AlphaImager-2000 image analyzer (Alpha Innotech Corporation) and computer software (Alpha Imager-2000 3.23).

Samples were prepared for electrophoresis under both reducing and non-reducing conditions. Those samples to be analyzed under reducing conditions were boiled for 5 min in dissociation buffer (Laemmli 1970) containing 2% SDS, 3% glycerol, and 2% 2-mercaptoethanol (2-ME), while samples prepared under non-reducing conditions were treated identically except 2-ME was omitted from the dissociation buffer. Samples were loaded into wells, and SDS-PAGE was performed using pre-cast polyacrylamide gels (Bio-Rad) containing a 4% stacking gel and either 10 or 20% separating gel and subjected to electrophoresis at 150 V for 1 h. Electrophoresed proteins were transferred to nitrocellulose membranes (Bio-Rad) at 100 V for 1 h along with rainbow pre-stained protein molecular weight markers (Amersham Life Science). Blots were blocked by incubation in 5% non-fat milk for 1 h. They were then incubated for 1 h or overnight with polyclonal rabbit anti-IHNV glycoprotein antiserum or supernatant from mouse hybridoma cultures containing monoclonal antibodies (MAbs) specific for the G protein of IHNV. Eleven different G-specific MAbs were used in this study (Ristow & Arnzen de Avila 1991). MAbs were tested individually and as a pool. The MAb pool was prepared by combining equal volumes of hybridoma culture supernatants from MAbs 92A(IgG<sub>2b</sub>), 131A(IgM), 127B(IgM), 135L(IgG<sub>2b</sub>), 136J(IgG<sub>2b</sub>), 111A(IgG<sub>1</sub>), 15A(IgM), 15B(IgM), 2F(IgM), 10B(IgM), and 151K(IgG<sub>3</sub>). Blots were incubated for 1 h

with either affinity purified goat anti-rabbit or rabbit anti-mouse (IgG + IgA + IgM) antibodies (Zymed Laboratories, Inc) conjugated to horseradish peroxidase (HRP) and diluted 1:2000. Between all incubations, blots were washed for 10 min in PBS containing 0.05% tween (PBST). Protein bands were visualized by exposure to chemiluminescence reagents (Dupont NEN Research Products) for 1 min. Finally, film was exposed to the blots and developed by autoradiography.

**Deglycosylation.** Purified IHNV or Sf9 cells producing recombinant G proteins (RecG<sub>low</sub> or RecG<sub>high</sub>) were deglycosylated by endoglycosidase treatment. Samples were reduced by boiling for 5 min in 1% SDS and 0.5% 2-ME. Preparations were diluted 2-fold in 20 mM-EDTA and 0.6% Triton X-100 in PBS to yield a cell concentration of approximately 1 × 10<sup>5</sup> cells ml<sup>-1</sup>. Samples were incubated with or without 1 U of an endoglycosidase F/N-glycosidase F preparation (Boehringer Mannheim) overnight at 37°C and analyzed by immunoblotting.

**Immunostaining and β-glucuronidase (Gus) staining of Sf9 cells.** An alkaline phosphatase immunostaining procedure, slightly modified from previous methods (Cain et al. 1996), was utilized for visualization of recombinant G proteins using light and fluorescence microscopy. Monolayers of Sf9 cells were grown to confluency in 96-well flat bottom tissue culture plates (Falcon), and infected with rAcG897, rAcGus, or mock infected with serum free media. Cells were maintained at either 20 or 27°C and fixed at 10 dpi. Sf9 cells were rinsed briefly with PBS and fixed in either 10% neutral buffered formalin or methanol for 15 min. Fixation with a formalin based fixative leaves the cell impermeable to antibodies and allows visualization of surface antigens (Biberfeld et al. 1974). Following fixation, cells were again rinsed in PBS and placed at 4°C until staining could be performed. Staining for recombinant antigen was accomplished by blocking cells for 1 h in 5% non-fat milk and then incubating overnight with an MAb (136J) specific to the G glycoprotein of IHNV (Ristow & Arnzen de Avila 1991). Cells were washed with several changes of PBST, and a 1:400 dilution of biotinylated goat anti-mouse IgG (Dako Corporation) secondary antibody was applied for 1 h at room temperature (RT). After washing with PBST and a final rinse with PBS, ABC-APase (Vector Laboratories) was applied for 30 min at RT; then the Vector Red substrate (Vector Laboratories) was applied, and color developed in the dark for 5 to 10 min. The reaction was stopped by rinsing for 5 min in distilled water. Cells were counterstained in Mayer's hematoxylin for 2 min followed by a tap water rinse for 5 min. Positive cells appeared red against a blue background and were visualized using standard light microscopy. The stain is also highly fluorescent and was distinguished with a fluorescence microscope using a rhodamine filter.

Staining of rAcGus-infected cells was performed using a  $\beta$ -glucuronidase specific staining procedure following the manufacturer's instructions (Gibco BRL, Bac-to-Bac™ Baculovirus Expression System). Briefly, cells were washed with PBS containing calcium and magnesium (Gibco BRL) and fixed for 5 min at room temperature with a solution of PBS containing 2% formaldehyde and 0.05% glutaraldehyde. Cells were washed twice and a substrate solution (1 mg ml<sup>-1</sup> of X-glucuronide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl<sub>2</sub> in PBS) was added and incubated for 2 to 18 h at 37°C depending on color development. Cells were rinsed in PBS, and  $\beta$ -glucuronidase positive cells (stained blue) were identified and counted. Plates were fixed in 10% neutral buffered formalin for 10 min, rinsed with PBS and stored in PBS at 4°C.

**Cell-associated fusion activity.** The ability of recombinant G proteins to induce fusion of Sf9 cells was evaluated. Monolayers of Sf9 cells grown in 24-well tissue culture plates (Corning) were mock infected, infected with rAcG897 at an approximate MOI of 10, or infected with rAcGus at an approximate MOI of 1. Virus inocula were allowed to adsorb to cells for 1 h, at which time media was aspirated from wells. Fresh media at pH 6.4 (normal), 6.2, 6.0, 5.8 and 5.6, was added to designated wells, and cultures were maintained at 20 or 27°C for 10 d. Syncytia formation (measured as the percentage of cells fusing/well) was monitored daily with phase-contrast microscopy.

## RESULTS

### Glycoprotein production

Production of the recombinant form of the IHNV G protein was demonstrated in Sf9 cells infected with

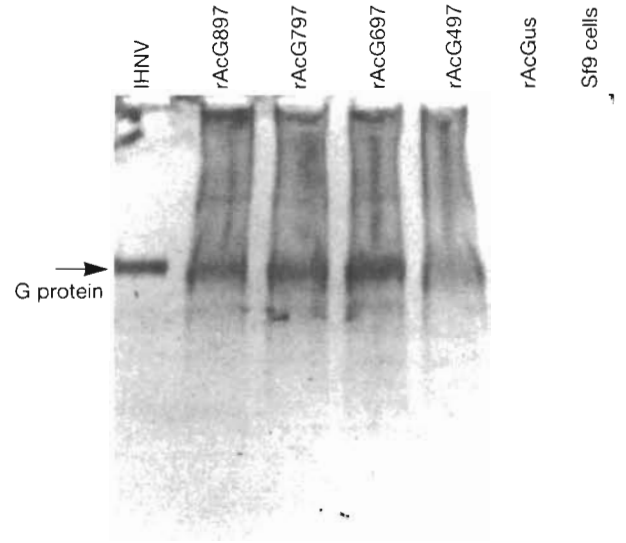


Fig. 1. Demonstration of recombinant IHNV G protein production in Sf9 cells infected with baculoviruses rAcG897, rAcG797, rAcG697, rAcG497. Proteins were transferred to a nitrocellulose membrane and detected with polyclonal rabbit anti-IHNV glycoprotein Ab

each of the 4 initial recombinant baculoviruses containing a 1.6 kb cDNA insert. Immunoblotting with polyclonal rabbit anti-IHNV glycoprotein antiserum showed that the recombinant G protein subjected to SDS-PAGE migrated slightly lower in molecular weight (MW) than the viral G protein of IHNV (Fig. 1). No reaction was observed with Sf9 cells or cells infected with a baculovirus containing the  $\beta$ -glucuronidase gene (rAcGus). Coomassie blue staining of proteins from Sf9 cells infected with rAcG897 and harvested at 4, 6, 8, and 10 dpi following SDS-PAGE revealed bands of overproduced protein slightly lower

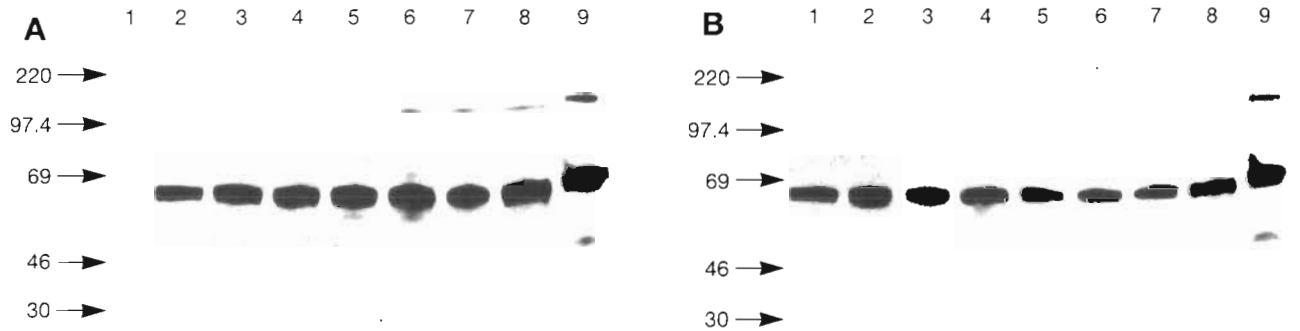


Fig. 2. Time of expression of RecG<sub>low</sub> and RecG<sub>high</sub> analysed by immunoblotting with a pool of 11 IHNV G-specific MABs. (A) Lanes 1 to 8, approximately  $1 \times 10^4$  Sf9 cells producing RecG<sub>low</sub> at 2 to 9 dpi, respectively; lane 9, 2  $\mu$ g purified IHNV. (B) Lanes 1 to 8, approximately  $1 \times 10^4$  Sf9 cells producing RecG<sub>high</sub> at 2 to 9 dpi, respectively; lane 9, 2  $\mu$ g purified IHNV

in MW than the IHNV glycoprotein (data not shown). Average density of bands corresponding to the G protein in the 4, 6, 8, and 10 dpi samples was estimated by spot densitometry analysis and compared to 1  $\mu$ g of a BSA standard. Approximately 0.5  $\mu$ g of recombinant G protein/ $1 \times 10^5$  infected Sf9 cells was produced.

Immunoblotting with a pool of 11 different G specific MAbs (Ristow & Arnzen de Avila 1991) showed delayed production of RecG<sub>low</sub> when compared to RecG<sub>high</sub>. Production of RecG<sub>low</sub> was minimal prior to 72 h post-infection, while RecG<sub>high</sub> was detected at 48 h post-infection (Fig. 2). All 11 MAbs reacted in a Western blot to IHNV G, RecG<sub>high</sub>, and RecG<sub>low</sub> following disulfide bond reduction in the presence of 2-ME. Only 111A, 2F, 151K, and 136J reacted in the Western blot with the non-reduced form of both RecG<sub>high</sub> and RecG<sub>low</sub>, while all MAbs recognized the non-reduced IHNV G protein (Table 1). Of the 4 MAbs recognizing both RecG<sub>high</sub> and RecG<sub>low</sub> under non-reducing conditions, reactions were primarily to glycoprotein aggregations at the top of the gel (not shown). An additional MAb (92A) showed little reaction to protein aggregations but recognized distinct bands corresponding to reduced and non-reduced wild-type IHNV G protein and RecG<sub>low</sub> (Fig. 3). In all cases, samples were loaded in an identical manner. In lane 2 of the figure, 3 bands are recognized and most likely represent small amounts of pro-

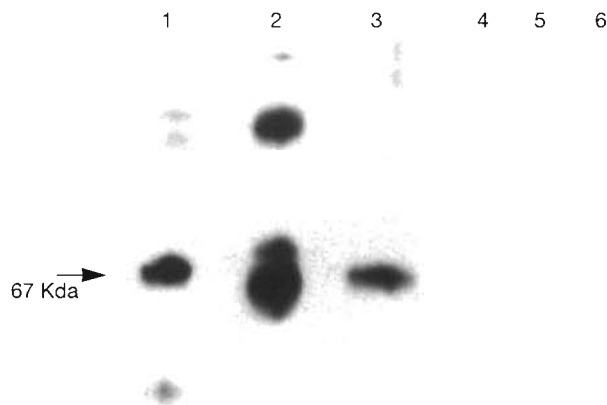


Fig. 3. Recognition of non-reduced RecG<sub>low</sub> by MAb 92A. Lane 1, reduced IHNV; lane 2, non-reduced IHNV; lane 3, non-reduced RecG<sub>low</sub>; lane 4, non-reduced RecG<sub>high</sub>; lane 5, Sf9 control cells; lane 6, Gus control cells

Table 1. Properties of 11 monoclonal antibodies (MAbs) against the IHNV glycoprotein, and their specificity to recombinant G protein, produced at 20°C (RecG<sub>low</sub>) or 27°C RecG<sub>high</sub>). +: positive, +/-: weak positive, -: negative. R: reduced; N: non-reduced

MAb	RecG <sub>low</sub> <sup>a</sup>		RecG <sub>high</sub> <sup>a</sup>		IHNV		IHNV neutralization <sup>b</sup>	IHNV immunofluorescence
	R	N	R	N	R	N		
92A	+	+	+	-	+	+	+	+
131A	+	-	+	-	+	+	-	+
127B	+	-	+	-	+	+	+	+
135L	+	-	+	-	+	+	+/-	+
136J	+	+	+	+	+	+	-	+
111A	+	+	+	+	+	+	-	+
15A	+	-	+	-	+	+	-	+
15B	+	-	+	-	+	+	-	+
2F	+	+	+	+	+	+	-	+
10B	+	-	+	-	+	+	-	+
151K	+	+	+	+	+	+	-	-

<sup>a</sup>Recognition of immunoblotted (RecG<sub>low</sub> or RecG<sub>high</sub>) separated by SDS-PAGE under R or N conditions and transferred to a nitrocellulose membrane  
<sup>b</sup>As measured against Round Butte I isolates of IHNV collected from steelhead (anadromous form of rainbow trout) at the Round Butte hatchery, Oregon, USA (Ristow & Arnzen de Avila 1991)

tein present as dimers and trimers in the non-reduced sample. 92A was the only neutralizing MAb that reacted to the recombinant G protein under non-reducing conditions, but recognition was specific to RecG<sub>low</sub> and not RecG<sub>high</sub>.

Deglycosylation by overnight incubation with endoglycosidase F revealed a band for each recombinant G protein that reacted to MAbs in an immunoblot and corresponded to the deglycosylated IHNV G protein (Fig. 4). Whereas the glycoprotein of purified IHNV was completely deglycosylated, the G protein from recombinant Sf9 cells appeared only partially deglycosylated after 18 h of digestion. This was most likely due to the presence of high concentrations of other competing cellular glycoproteins (a possible alternate substrate for the endoglycosidase).

#### Surface localization of the recombinant glycoprotein on Sf9 cells

Immunostaining of formalin-fixed Sf9 cells using MAb 136J demonstrated localization of the G protein on the surface of cells grown at 20°C (Fig. 5B). Only rare surface staining of individual cells was observed when cells were grown at 27°C (Fig. 5C), and no reaction to 136J was detected in uninfected Sf9 controls or rAcGus-infected cells grown at either temperature. Cells fixed in methanol allowed some permeability and entry of reagents through the plasma membrane. This resulted in similar staining of surface G protein at 20°C (Fig. 5E), and cytoplasmic staining of recombinant G was evident in cells grown at 27°C (Fig. 5F).

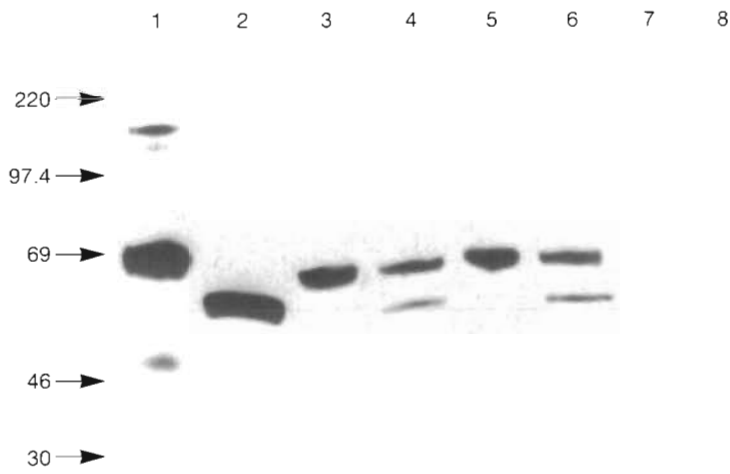
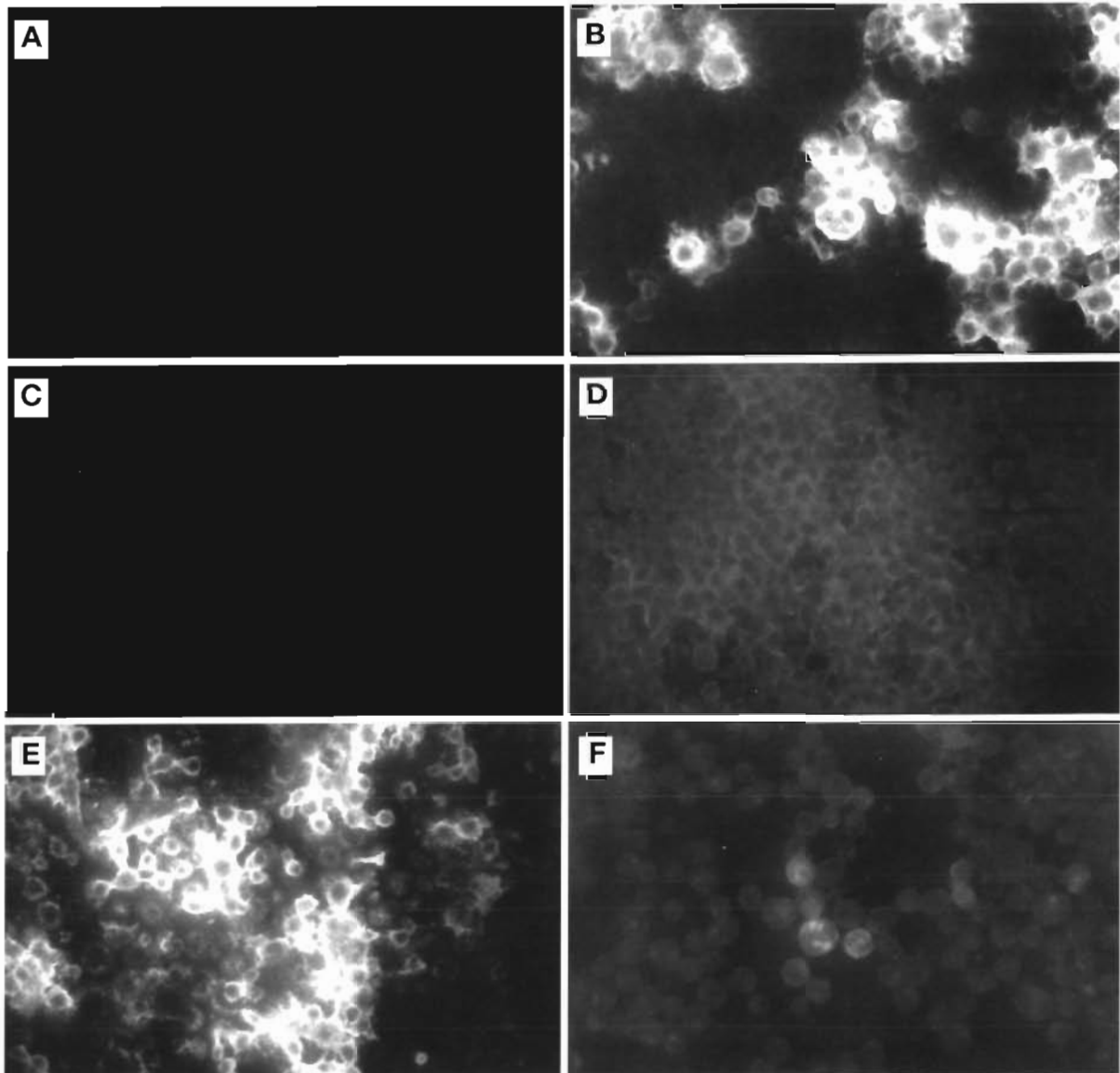


Fig. 4. Deglycosylation of RecG<sub>high</sub>, RecG<sub>low</sub>, and IHNV. Lane 1, IHNV; lane 2, deglycosylated IHNV; lane 3, RecG<sub>high</sub>; lane 4, deglycosylated RecG<sub>high</sub>; lane 5, RecG<sub>low</sub>; lane 6, deglycosylated RecG<sub>low</sub>; lane 7, Sf9 controls; lane 8, rAcGus-infected Sf9 cells. All samples were incubated overnight (18 h) with or without endoglycosidase F/N-glycosidase F at 37°C



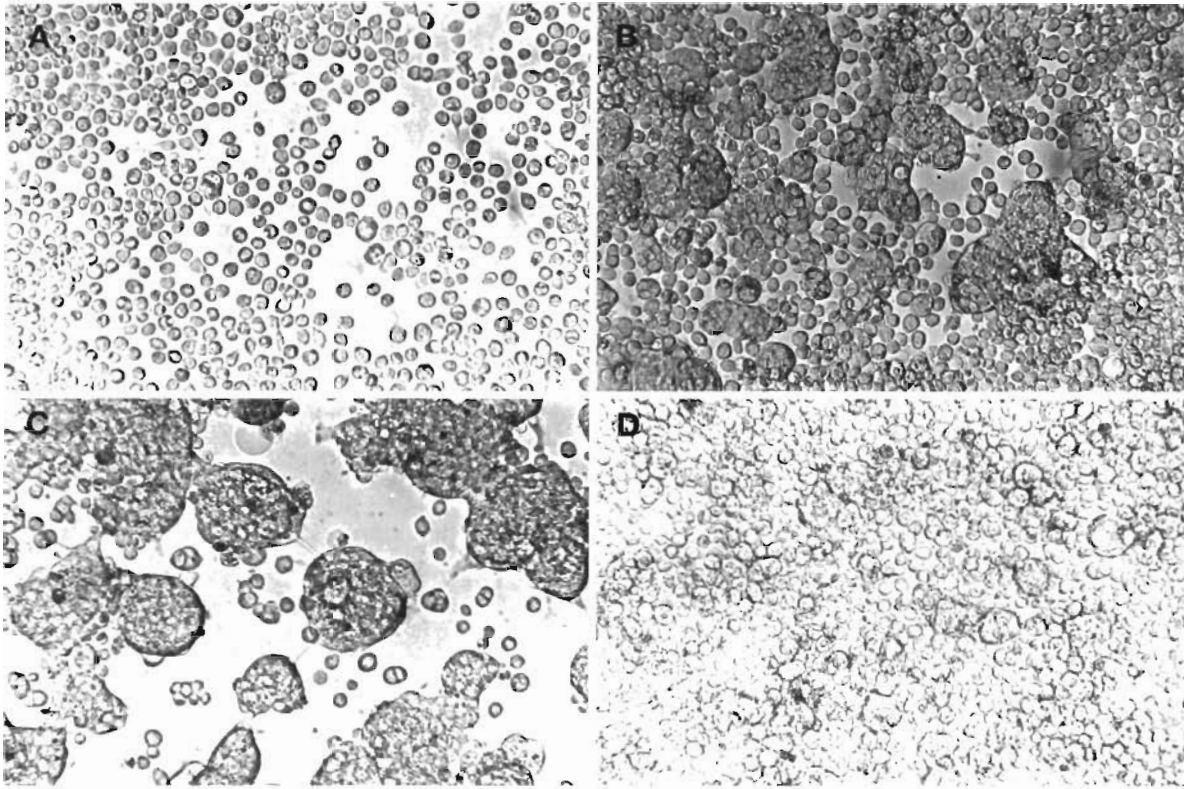


Fig. 6. Cellular fusion activity of RecG<sub>low</sub> infected cells at different pHs. (A) Sf9 cells producing RecG<sub>low</sub> (6 dpi) at pH 6.4 (normal); (B) Sf9 cells producing RecG<sub>low</sub> (6 dpi) at pH 5.8; (C) Sf9 cells producing RecG<sub>low</sub> (10 dpi) at pH 5.6; and (D) Sf9 cells producing RecG<sub>high</sub> (10 dpi) at pH 5.6

Fusion of Sf9 cells producing the recombinant IHNV glycoprotein at 20°C was initially observed by 5 dpi at pHs ≤ 6.2. Cell fusion was monitored out to 10 dpi, and increased steadily in cells producing RecG<sub>low</sub>, but minimal fusion was observed in parallel cultures of RecG<sub>high</sub> cells (Fig. 6). By 9 dpi, between 25 and 100% of RecG<sub>low</sub> cells exhibited fusion at all pH ranges (6.4 to 5.6). Fusion of cells expressing RecG<sub>high</sub> was <25% at all pH values tested in the study and was highest at 10 dpi and pH 5.6 (Fig. 6D). No fusions were observed at either temperature with uninfected Sf9 cells, while cells infected with rAcGus and expressing the glycoprotein (β-glucuronidase) only exhibited fusions at 27°C.

## DISCUSSION

Koener & Leong (1990) originally expressed the IHNV G gene using baculovirus vectors and found that

MWs of the recombinant protein corresponded to the viral G protein. In earlier work, the deduced MW of the unglycosylated G protein was 56 795 daltons and 5 potential glycosylation sites were identified (Koener et al. 1987). In the present study a recombinant IHNV G protein was produced in insect cells using a commercially available baculovirus expression system (Bac-to-Bac™ Gibco BRL), and was found to be only partially glycosylated. The baculovirus derived G protein migrated faster during SDS-PAGE than the viral IHNV glycoprotein, and MW differences were due to glycosylation differences. It was therefore concluded that insect cells process the IHNV glycoprotein differently than fish cells. Similar studies of another fish rhabdovirus (VHSV) have also shown decreased levels of glycosylation when recombinant G protein or wild-type virus were produced in insect cells (Lecocq-Xhonneux et al. 1994, Lorenzen & Olesen 1995). It is known that insect cells lack certain glycosylation features

Fig. 5. Immunofluorescence microscopy using MAb 136J and showing surface localization of recombinant G protein in Sf9 cells. (A) Formalin-fixed Sf9 cells; (B) formalin-fixed Sf9 cells producing RecG<sub>low</sub>; (C) formalin-fixed Sf9 cells producing RecG<sub>high</sub>; (D) methanol-fixed Sf9 cells; (E) methanol-fixed Sf9 cells producing RecG<sub>low</sub>; and (F) methanol-fixed Sf9 cells producing RecG<sub>high</sub>

such as sialyl transferase activities (Prehaud et al. 1989), and it was found that oligosaccharide side chains of the hemagglutinin of influenza virus are smaller when produced in insect cells than when virions are produced in vertebrate hosts (Kuroda et al. 1990). Nevertheless, insect cells are generally able to produce functionally immunogenic proteins (Luckow 1991).

Effects of temperature have been reported to influence cellular processing of rhabdoviral G proteins (Gibson et al. 1981, Kotwal et al. 1986). Temperature effects in relation to the recombinant IHNV G protein were investigated in this study. Production of IHNV G protein in insect cells was found to be delayed slightly when Sf9 cells were grown at 20°C (RecG<sub>low</sub>) compared to 27°C (RecG<sub>high</sub>). This delayed production may be attributed to a decrease in metabolic processing at lower temperatures. Other temperature influences have been demonstrated by Lorenzen (1997) in relation to a recombinant VHSV G protein produced in insect cells. The protein appeared to fold correctly and was recognized by a conformational dependent MAb only when produced at lower temperatures. Based on Lorenzen's findings, similar temperature influences were evaluated for the glycoprotein of IHNV. Interestingly, dramatic differences were observed in the ability of insect cells to efficiently transport/localize RecG<sub>high</sub> and RecG<sub>low</sub> to the cell surface (Fig. 5). Cell surface expression, characteristic of rhabdoviral G proteins, was demonstrated by immunostaining of formalin-fixed Sf9 cells and was only evident in cells producing RecG<sub>low</sub>. Formalin or methanol fixation allowed the detection of either cell surface or intracellular antigen, respectively. Little or no G protein was present on the surface of cells producing RecG<sub>high</sub>, but intracytoplasmic staining was observed in methanol fixed cells producing RecG<sub>high</sub>. The recombinant protein is therefore confined within cells grown at high temperatures, but expression at low temperature results in transport to the surface of cells. The temperature-associated differences observed here indicate that low temperature production of this baculovirus product may lead to a more native rhabdoviral G protein. Lecocq-Xhonneux et al. (1994) reported that only a fraction of recombinant VHSV G protein was correctly processed and exported to the surface of insect cells. They also reported that the recombinant G protein was detected within cytoplasmic inclusions by immunofluorescence.

Surface localization and other rhabdoviral G protein characteristics were further demonstrated by examination of cellular fusion activity in recombinant baculovirus-infected Sf9 cells (Fig. 6). Cells producing RecG<sub>low</sub> exhibited some level of cell fusion by 6 dpi at pH values between 5.6 and 6.2 with cells at the lower pH range showing the highest level of fusion. Fusion of

cells expressing RecG<sub>high</sub> was minimal (<25%) and only observed at pH 5.6. The fusion activity observed with RecG<sub>low</sub> is consistent with low pH fusion activity of other rhabdovirus G proteins (Tuchiya et al. 1992), and the limited fusion observed with RecG<sub>high</sub> supports the lack of surface expression at 27°C. These characteristics are considered to be an important part of the natural viral infection process. It is known that G proteins form trimers that are activated at low pH and expose hydrophobic regions at the surface which allows fusion to occur (Gaudin et al. 1993). During infection, once endocytosis occurs, fusion of the viral and host membrane occurs only after the pH is lowered to approximately 5 in VSV (Schlegel et al. 1982, Rigaut et al. 1991), and rabies (Superti et al. 1984). At this pH it has been shown that isolated VSV G protein undergoes conformational changes that allow the protein to simultaneously interact with the viral and host cell membranes (Brown et al. 1988). Although rhabdoviruses infecting mammals may not be directly comparable to fish rhabdoviruses, it appears that similar types of changes must occur for RecG<sub>low</sub> to be present at the cell surface and produce similar fusion characteristics. This fusion activity has also been observed in Sf9 cells producing the recombinant G protein of rabies (Tuchiya et al. 1992) and VHSV (Lecocq-Xhonneux et al. 1994). Altered post-translational processing associated with high temperature or glycosylation differences is known to result in misfolding of G proteins (Gallione & Rose 1985). Since both RecG<sub>high</sub> and RecG<sub>low</sub> produced an underglycosylated product, it is interesting that only RecG<sub>low</sub> appeared to be transported to the cell surface in a correct manner.

Further analysis of the recombinant G protein by immunoblotting with 11 different MAbs showed that linear epitopes were recognized for both RecG<sub>high</sub> and RecG<sub>low</sub> with no apparent differences. Interestingly, when non-reduced samples were evaluated, an epitope recognized by MAb 92A was present only for RecG<sub>low</sub> and not RecG<sub>high</sub>. 92A is an IHNV neutralizing MAb (Ristow & Arzen de Avila 1991), and recognition of only RecG<sub>low</sub> under non-reduced conditions suggests this epitope may be hidden at non-permissive temperatures. Huang et al. (1994) used similar Western blot analyses to identify MAbs specific for conformation-dependent epitopes on the G protein of IHNV. The site identified by 92A may map to an immunogenically important region that is conformationally correct only when processed at lower temperatures in insect cells. All 11 MAbs tested recognized viral IHNV G protein under both reducing and non-reducing conditions at the concentrations used, which indicates that these epitopes are linear in nature and are not normally hidden due to protein folding. The fact that the recombinant G proteins reacted differently to the



MAbs is of concern and may represent unavoidable differences in protein folding associated with insect cell production in general.

The demonstration of surface localization of the recombinant IHNV G protein is considered important because this is an important factor in the infection of the cell by rhabdoviruses (Coll 1995). The data presented here provides evidence that insect cells produce a more native IHNV glycoprotein when cultured at temperatures closer to the physiological optimum of the virus. Although vaccine trials have shown that RecG<sub>low</sub> lacks immunogenicity associated with attenuated strains of IHNV (Cain et al. 1998), the present results provide a further understanding of the complex nature of the IHNV glycoprotein. Increased knowledge of the structural and functional characteristics of the G protein may assist in the future development of an efficacious IHNV vaccine.

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