Production of the M2 protein of influenza A virus in insect cells is enhanced in the presence of amantadine

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Recombinant baculoviruses that express the M2 protein from the genes of either the amantadine-sensitive, influenza A/Ann Arbor/6/60 virus or a laboratory-derived, amantadine-resistant mutant of this virus were constructed. Addition of amantadine or rimantadine at 2 μg/ml to cultures of Sf9 cells infected with the recombinant baculoviruses increased the yield of the M2 protein from the amantadine-sensitive virus approximately 10-fold, but did not increase the yield of the M2 protein from the amantadine-resistant virus. Flow cytometry demonstrated that the increased production of M2 in the presence of amantadine resulted in increased cell surface expression of the M2 protein. Pulse–chase experiments indicated that whereas the rate of synthesis of the M2 protein increased in the presence of amantadine, the M2 protein was stable in both the presence and absence of amantadine. Addition of amantadine to Sf9 cells as late as 72 h after infection with the recombinant virus increased the production of M2 protein. These data suggest that the M2 protein exerts some biological activity in Sf9 cells.

The RNA segment 7 of influenza A virus codes for the membrane protein (M1) and an integral membrane protein, M2, which is expressed abundantly at the surface of infected cells and is present in small quantities on the surface of mature virions (Lamb et al., 1985; Zebedee et al., 1985). The 97 amino acid-long M2 protein contains an 18 amino acid hydrophobic membrane-spanning domain (amino acids 25 to 43) and exists as a disulphide-linked homotetramer (Sugrue & Hay, 1991; Holsinger & Lamb, 1991) in infected cells. M2 protein (Pinto et al., 1992), as well as a peptide derived from the membrane-spanning domain of M2 (Duff & Ashley, 1992), function as proton channels. Late in infection, this activity serves to regulate the pH of the trans-Golgi vesicles in cells infected with avian influenza viruses and prevents the low pH-induced conformational change of the haemagglutinin molecule from occurring during its maturation (Hay et al., 1986; Hay, 1989; Sugrue et al., 1990; Steinhauer et al., 1991; Ciampor et al., 1992a, b; Grambas & Hay, 1992). In addition, studies with human influenza virus strains suggest that the M2 protein functions early in the infection process by regulating the pH within the virion to facilitate uncoating (Skehel et al., 1978; Bukrinskaya et al., 1982; Hay et al., 1986).

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The transmembrane portion of M2 has been identified as the target of the antiviral drug amantadine. Single amino acid changes in this domain can result in viruses that are resistant to this drug at either early or late times after infection (Belshe et al., 1988; Belshe & Hay, 1989). The ion channel activity of M2 and the subsequent ability of M2 to regulate vesicular pH are inhibited by amantadine (Ciampor et al., 1992a; Steinhauer et al., 1991; Pinto et al., 1992; Duff & Ashley, 1992; Grambas & Hay, 1992).

We have previously reported the construction of a recombinant baculovirus (Bac-M2) that expresses the M2 protein of an amantadine-sensitive strain of influenza A/Ann Arbor/6/60 virus (Black et al., 1993). This recombinant M2 protein was used to detect antibody to M2 in serum samples from naturally infected individuals. In this report, we describe the increased expression of the M2 protein by this recombinant baculovirus in the presence of amantadine.

In addition to recombinant Bac-M2 we constructed a recombinant baculovirus expressing the M2 gene from an amantadine-resistant strain of influenza A/Ann Arbor/6/60 (H2N2) virus. A/Ann Arbor/6/60 virus was grown and plaqued three times in MDCK cells in the presence of 1 μg/ml amantadine. The final stock of resistant virus demonstrated no reduction in plaquing efficiency in the presence of the drug. Comparative
Fig. 1. Increased yield of baculovirus-expressed M2 protein in the presence of amantadine. SF9 cells were infected with the recombinant baculoviruses (lanes 1, 2: Bac-M2; lanes 3, 4: Bac-M2R; lanes 5, 6: Bac-ANP; lane 7: SF9 cell control) in the presence (lanes 1, 3, 5, 7) or absence (lanes 2, 4, 6) of amantadine (2 μg/ml). Cell lysates were prepared at 96 h post-infection and analysed by Western blotting as described by Black et al. (1993) using monoclonal antibodies to M2 (14C-2 kindly provided by Dr R. Lamb) (lanes 1, 2, 3, 4) or to ANP (lanes 5, 6, 7). Lanes 1 to 4 each contained 36 μg of protein, lanes 5 and 6, 90 μg and lane 7, 50 μg.

sequence analysis of the M2 coding regions of the amantadine-resistant and amantadine-sensitive viruses identified a single coding change, leucine to phenylalanine, at residue number 26 in the transmembrane domain of the resistant virus. An amino acid change at this position had not been reported to be associated with resistance. However, during the 1989 to 1990 influenza season, from an influenza outbreak in a nursing home where amantadine was used resistant viruses were isolated that also had the leucine to phenylalanine change at amino acid 26 (Roumillat et al., 1990).

The M2 gene from the amantadine-resistant strain of A/Ann Arbor/6/60 virus was amplified by PCR and cloned into the baculovirus transfer vector pAcYM1-B1 as described previously (Black et al., 1993). A recombinant baculovirus expressing the M2 protein from the amantadine-resistant virus, Bac-M2R, was generated using standard techniques (Summers & Smith, 1986). Expression of M2 protein by Bac-M2R was confirmed by indirect immunofluorescence (not shown) on both acetone- and paraformaldehyde-fixed SF9 cells using a monoclonal antibody, 14C-2, that recognizes the amino-terminal portion of the M2 protein (Zebedee et al., 1985).

During our initial characterization of the recombinant baculoviruses, it was observed by using phase-contrast microscopy that the c.p.e. in SF9 cells infected with Bac-M2 advanced more rapidly than the c.p.e. in SF9 cells infected with either Bac-M2R or a recombinant baculovirus expressing the influenza type A virus nucleoprotein (Bac-ANP) (Rota et al., 1990) at equivalent m.o.i. However, this enhanced c.p.e. was not observed in cells infected with Bac-M2 when amantadine (2 μg/ml) was added to the culture medium at the time of infection. No differences in c.p.e. were observed in cells infected with Bac-M2R or Bac-ANP in the presence or absence of the drug (data not shown).

Western blot analysis of lysates prepared from cells infected with Bac-M2 indicated that more M2 protein was produced in the presence of 2 μg/ml of amantadine (Fig. 1). In contrast, neither the amount of M2 produced by Bac-M2R nor the amount of nucleoprotein produced by Bac-ANP were affected by amantadine (Fig. 1). Similar results were obtained when 2 μg/ml of rimantadine was substituted for amantadine. Quantitative slot blot analysis (Fig. 2) revealed that the M2...
protein could be detected easily at 48 h post-infection in lysates of Sf9 cells infected with Bac-M2 when amantadine was added to the medium. At 3 and 4 days post-infection, M2 protein was barely detectable in lysates from cells infected in the absence of amantadine, and 10 to 15 times more M2 was detectable in lysates of cells infected in the presence of the drug (Fig. 2).

Flow cytometric analysis of Sf9 cells infected with Bac-M2 confirmed a previous observation made by immunofluorescence (Black et al., 1993) that M2 protein was expressed at the surface of the infected Sf9 cell. In the presence of amantadine, the infected cells showed increased fluorescence intensity indicating that eight to 10 times more M2 was present on the surface of the cells infected with Bac-M2 (Fig. 3). The surface expression of M2 by Bac-M2R was not affected by the addition of amantadine (not shown). Therefore, addition of amantadine to cells infected with Bac-M2 resulted in the increased accumulation of M2 protein as well as increased transport of M2 to the cell surface.

Pulse-chase experiments confirmed that the rate of accumulation of M2 in Sf9 cells infected with Bac-M2 was greater in the presence of amantadine (Fig. 4). These experiments also confirmed that the M2 protein is stable in Sf9 cells in the presence and absence of amantadine and that the inability to detect M2 in the absence of the drug was not due to increased turnover of the protein. By comparison, the rate of accumulation of M2 in Sf9 cells

Fig. 3. Flow cytometric analysis of Sf9 cells infected with Bac-M2 at 72 h post-infection in the presence (b, peak 2) or absence (b, peak 1) of 2 μg/ml amantadine. Cells were harvested, washed with PBS (pH 6.4) and incubated with 14C-2 and fluorescein isothiocyanate-labelled goat anti-mouse IgG. The washed cells were fixed in 1% formalin before being analysed using a Becton-Dickinson Facscan flow cytometer. Scans of uninfected Sf9 cells incubated in the presence (peak 1) or absence (peak 2) of amantadine (a).

Fig. 4. Pulse-chase analysis of the expression of the M2 protein by Bac-M2 in the presence and absence of amantadine. Sf9 cells were infected with Bac-M2 and incubated in either the absence (a) or presence (b) of amantadine. At 48 h post-infection, cells were labelled with [35S]cysteine for 45 min and chased as indicated. Cell lysates were analysed by immunoprecipitation with monoclonal antibody 14C-2 and then by SDS-PAGE.
infection. At 120 h post-infection, cells were harvested and lysates were analyzed by Western blotting using monoclonal antibody 14C-2 as described by Black et al. (1993). Lane 1 shows lysate of cells infected with Bac-M2 in the absence of amantadine; all lanes contain approximately 90 µg cell lysate.

infected with Bac-M2R was not affected by the presence of amantadine in the culture medium (data not shown).

Since we were unable to detect differences in cell viability after infection with Bac-M2 and Bac-M2R in the presence or absence of amantadine (data not shown), the effect of adding amantadine at various times after infection was investigated. Addition of amantadine to Sf9 cells as late as 72 h post-infection with Bac-M2 was able to restore the increased production of the M2 protein (Fig. 5). These data suggest that the expression of the M2 protein in the absence of amantadine does not result in cell death but possibly inhibits further synthesis of M2.

Recent findings indicate that M2 tetramers function as a proton channel which can regulate the pH in vesicles of the trans-Golgi network during the maturation of the influenza virus haemagglutinin and that amantadine interacts directly and reversibly with M2 to inhibit this pH regulation (Sugrue et al., 1990; Sugrue & Hay, 1991; Steinhauer et al., 1991; Ciampor et al., 1992a; Duff & Ashley, 1992; Pinto et al., 1992). Our finding that amantadine increased the amount of M2 protein produced by the recombinant baculovirus expressing the M2 gene from the amantadine-sensitive virus but not the amount of M2 from the amantadine-resistant virus is consistent with previous findings and suggests that the M2 protein expressed has biological activity in insect cells and possibly mimics its activity in cells infected with influenza A virus. The most probable explanation for our results is that M2 protein which is being synthesized under the control of the strong polyhedrin promoter becomes inserted into vesicular membranes of the Sf9 cells and, acting as an ion channel, disrupts the normal ionic balance within the cell. If this effect is inhibited or delayed by amantadine it is suggested that more M2 can accumulate in cells and subsequently be transported to the cell surface before the cells are eventually destroyed by baculovirus replication. The inhibition of Sf9 cell functions by M2 in the absence of amantadine is also manifested by visible changes in cell morphology and by decreased ability to support the replication of the baclovirus.

These observations allow the alternative explanation that M2 protein is metabolized in Sf9 cells into a toxic product, and that this mechanism is inhibited by amantadine. However, this explanation seems less likely taking into account the lack of any known direct effect of amantadine on cellular metabolic pathways. Also, since the influenza virus nucleoprotein was produced normally in the presence or absence of the drug, it is unlikely that amantadine had any toxic effect on Sf9 cells at the concentration used.

The ability to express the M2 protein using cloned DNA has provided the opportunity to study its properties without the involvement of other influenza virus proteins. Observations reported here suggest that at least some of the biological activity of the M2 protein is retained in insect cells. This system should facilitate future studies to investigate the interaction of amantadine and other antiviral drugs with the M2 protein as well as to explore pathways of signal transduction and the role that the M2 protein plays in cells infected with influenza A virus.

The authors are grateful to Robert Lamb for providing the M2-specific monoclonal antibody and Petra Neubauer for technical assistance. This work was presented, in part, at the Eighth International Congress of Virology in Berlin, 1990. A. P. K. gratefully acknowledges the receipt of a Senior Scientist Award from the Alexander von Humboldt Foundation. This work was partially supported by the Deutsche Forschungsgemeinschaft (SFB 286) and Fonds der Chemischen Industrie.

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Short communication 1677


(Received 20 October 1992; Accepted 22 March 1993)