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Influenza virus M2 protein modifies membrane permeability in *E. coli* cells

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Abstract

The M2 protein of influenza virus is an integral membrane protein with ion channel activity. This protein has been expressed in *E. coli* cells in an inducible manner. Expression of the M2 protein causes rapid lysis of BL21(DE3) pLysS *E. coli* cells upon induction with IPTG. M2 protein increases membrane permeability to a number of hydrophylic molecules, such as ONPG, uridine or impermeant translation inhibitors. The behaviour of M2 in bacteria resembles that of other viral proteins, such as poliovirus 3A and Semliki Forest virus 6K.

Key words: Influenza virus; Ion channel; Influenza M2 protein; Membrane permeability

1. Introduction

Influenza A virus is a negative-sense, single-stranded RNA virus with a genome fragmented into eight different pieces that encode at least 10 proteins (13). M2 protein is synthesized from a spliced mRNA derived from genome RNA segment 7 [17]. Once synthesized, M2 becomes membrane associated and is present in abundance on the surface of infected cells, although only a few molecules are incorporated into virions [11,31]. M2 is an integral membrane protein that contains an extracellular domain consisting of approximately 24 N-terminal amino acids, a 19 residue trans-membrane domain and a cytoplasmic domain of 54 C-terminal amino acids [32]. The native form of the M2 protein is a homotetramer consisting of either a pair of disulfide-linked dimers or four disulfide-linked monomers [10,27]. The protein is acylated, as occurs with other integral membrane proteins and is phosphorylated [10,26,28]. Despite the presence of a potential glycosylation site, M2 is not glycosylated.

M2 protein localizes to the Golgi apparatus of influenza virus-infected cells and is transported to the cell surface by a pathway similar to that of other integral membrane proteins [32]. Some clues on the role of M2 in the influenza virus replication cycle come from studies on the mechanism of action of the antiviral compound amantadine [23] since M2 protein is the target of inhibition by amantadine hydrochloride [9]. Characterization of amantadine-resistant mutants of influenza virus has indicated that the resistance trait is located within the membrane-spanning domain of M2 [9]. However, the precise mechanism of the antiviral activity of amantadine remains unclear. It seems that amantadine blocks influenza virus at two different steps of its growth cycle. Early during virus entry, the drug acts by inhibiting endosomal acidification and at a later stage of virus infection, by preventing virus release [12,21,23]. It has been claimed that amantadine induces a premature conformational change in HA that occurs in the trans-Golgi complex during the transport of HA to cell surface [25]. This premature conformational change in HA is though to be detrimental to the release of virus particles [21].

Membrane permeability is enhanced by influenza virus infection, leading to an imbalance of ions in the cytoplasm [4,18]. Expression of M2 in *Xenopus* oocytes increases membrane permeability to monovalent ions [20,29], suggesting that M2 is endowed with ion channel activity. Since M2 is able to oligomerize [10,27], it seems plausible that the oligomers located at the cell surface form hydrophylic pores, as with phage lytic proteins [30]. The present work shows that influenza M2 protein is able to increase membrane permeability when it is inducibly expressed in *E. coli* cells. These results resemble those reported with other viroporins, such as poliovirus 3A or the Semliki Forest virus 6K protein [6,14,22].

2. Materials and methods

2.1. Construction of M2 expression plasmid

Construction of the vectors containing the M2 sequence was carried out by standard cloning procedures [19]. The M2 cDNA was kindly provided by Dr. R. Lamb (Howard Hughes Medical Institute, Illinois) and was cloned at the *Bam*HI site of pGEM3. Two oligonucleotides were synthesized to amplify the M2 gene by polymerase chain reaction to create two unique *Nde*I and *Bam*HI restriction sites, while also adding an additional translation stop codon. After amplification, the products were digested with the appropriate enzymes (*Bam*HI and

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*Nde*I) and ligated to the pET3 vector containing a T7 promoter, signals for efficient translation in *E. coli* and a transcription termination sequence from bacteriophage T7 [15,24]. After transformation of DH5 cells and restriction enzyme analysis of plasmid pET-M2, the region of pET3 encoding M2 was sequenced by the dideoxy method. Plasmid pET-M2 was then used to transform BL21(DE3) *E. coli* cells or BL21(DE3) pLysS *E. coli* cells. Plasmid pET11-M2 was generated by insertion of the M2 fragment obtained by *Nde*I/*Bam*HI digestion of pET3-M2.

2.2. Growth and induction of recombinant bacteria

Single clones of BL21(DE3) cells or BL21(DE3) pLysS cells containing the indicated plasmid, were grown overnight at 37°C in LB medium in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol in the case of pLysS cells. The cells were then diluted 100-fold in M9 medium supplemented with 0.2% glucose and antibiotics. When the culture reached 0.5–0.6 A_{660} the cells were induced by addition of 1 mM IPTG. Rifampicin (Sigma) was used, when indicated, at 150 μg/ml to inhibit transcription by *E. coli* RNA polymerase.

2.3. Labelling and analysis of bacterial proteins

To label the proteins synthesized by recombinant bacteria, 1 ml aliquots of bacteria cultures were collected and incubated with 2 μCi/ml [^{35}S]methionine (1.45 Ci/mmol, Amersham) for 10 min at 37°C. The labelled bacteria were centrifuged for 1 min at 12,000 rpm in an Eppendorf microfuge and dissolved in lysis buffer (1% SDS, 17% glycerol, 100 mM dithiothreitol, 0.37 M Tris-HCl (pH 6.8) and 0.024% Bromophenol blue). After SDS-PAGE, fluorography was carried out with 1 M salicylic acid for 1 h, and after drying, the gels were exposed to XAR films (Kodak) at −70°C.

2.4. Uridine release from preloaded E. coli *cultures.*

Cells were grown as described before and, 60 min before induction (when A_{660} reaches approx. 0.2–0.3), were loaded with 4 μCi/ml [^{3}H]uridine (27.3 Ci/mmol, Amersham) for 1 h. After loading, the cells were pelleted and washed twice with uridine-free pre-warmed growth medium. Cells were then resuspended in the initial volume of growth medium and incubated at 37°C for 15 min. The cells were induced to express the target protein by addition of 1 mM IPTG. At the times indicated 0.2 ml aliquots of culture were centrifuged. Scintillation liquid (formula 989, Du Pont–New England Nuclear) was added to the supernatant to estimate radioactivity.

2.5. β-galactosidase assays

1 ml of bacteria culture was taken at different times after induction and the cells were centrifuged for 1 min at 12,000 rpm in an Eppendorf microfuge. To measure extracellular β-galactosidase, 0.2 ml of 12 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added to the supernatant. Reactions were carried out for 10 min at 30°C and stopped by addition of 0.4 ml 1 M sodium carbonate. A_{420} was estimated to monitor the formation of the cleaved product. The pellet was used to measure the entry of ONPG into bacterial cells after resuspension in 1 ml of growth medium and mixing with 0.2 ml of 12 mM ONPG. Incubation was performed as indicated above, then cells were centrifuged to avoid interference due to the scattering of light by intact cells, and A_{420} was measured. To assay the total amount of β-galactosidase present in cells, 1 ml of growth culture was taken and 1 ml toluene was added to the medium. Addition of ONPG and further steps were performed as indicated above.

2.6. Entry of hygromycin B

Bacteria were grown as described above. At the indicated times after induction with IPTG, 1 ml of culture was taken and 1 mM hygromycin B and 2 μCi/ml [^{35}S]methionine were added to the medium. Cells were incubated for 10 min at 37°C, bacteria were centrifuged and the proteins analyzed by SDS-PAGE as indicated.

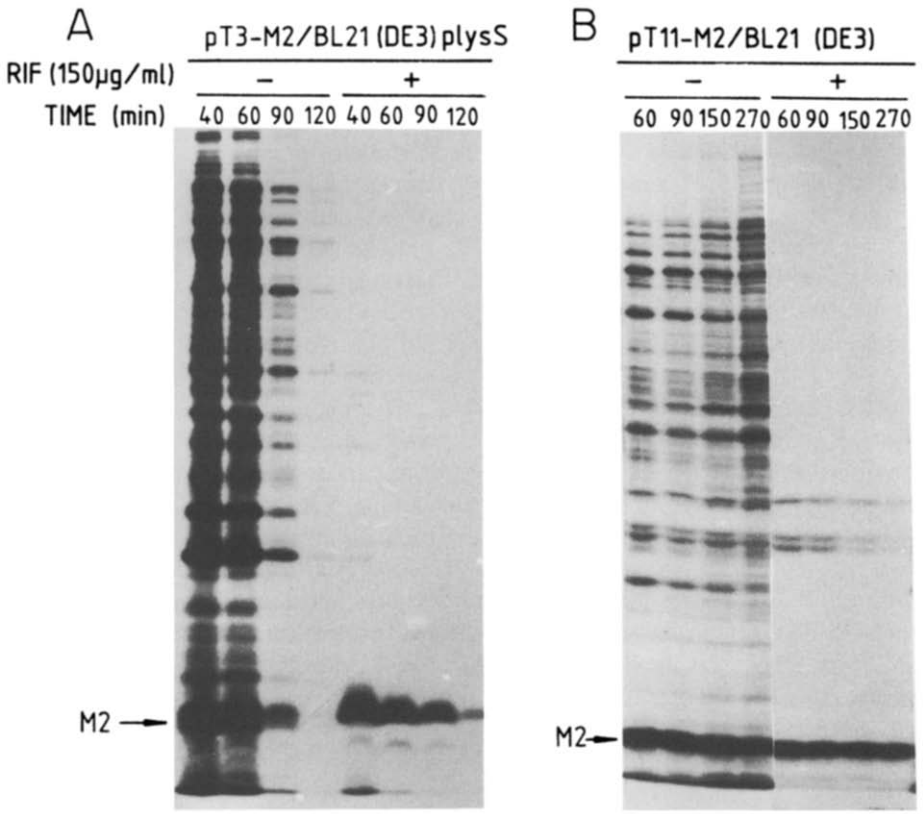

Fig. 1. Induction of influenza virus M2 protein in *E. coli* cells. BL21 (DE3) pLysS cells containing pET3-M2 plasmid or BL21(DE3) cells containing pET11-M2 plasmid were grown and induced with 1 mM IPTG as described in section 2. At the indicated times post-induction proteins were labelled with [^{35}S]methionine and then analyzed by SDS-PAGE. Where indicated, 150 μg/ml rifampicin were added to the cultures 30 min after addition of IPTG. The position of M2 protein is indicated.

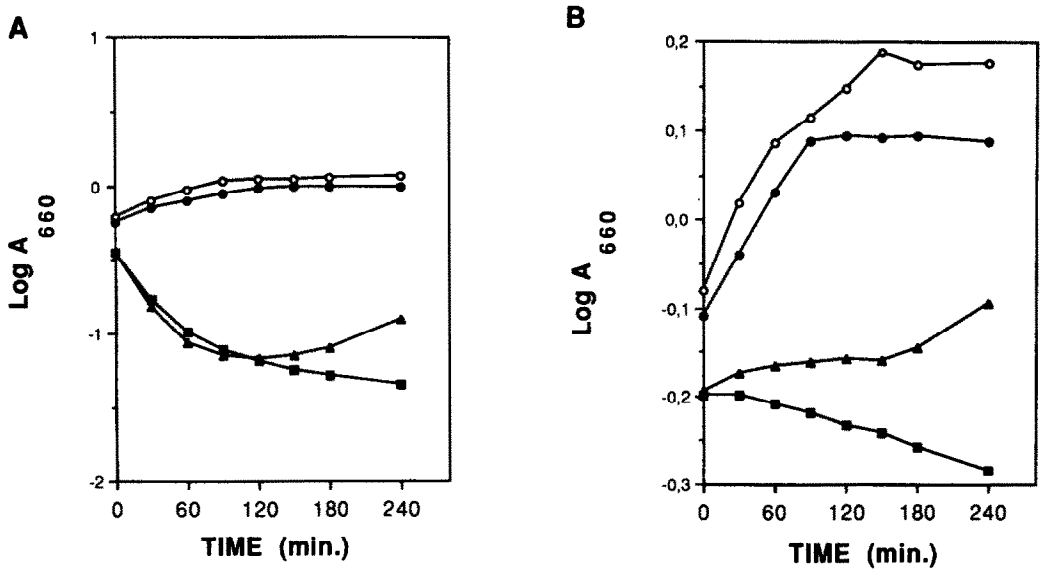

Fig. 2. Growth curve of BL21(DE3) pLysS cells and BL21(DE3) cells expressing M2 protein. *E. coli* cells were grown as indicated in section 2. At zero time, cells were induced with 1 mM IPTG and cell density ($A_{660\ nm}$) was measured at the indicated times. Panel A: pET3 (○), pET3-M2 (●), pET3-M2 plus IPTG (▲), pET3-M2 plus IPTG plus rifampicin (■) in BL21(DE3) pLysS cells. Panel B: pET11 (○), pET11-M2 (●); pET11-M2 plus IPTG (▲); pET11-M2 plus IPTG plus rifampicin (■) in BL21(DE3) cells.

3. Results

3.1. Cloning and expression of influenza virus M2 protein in E. coli cells

The M2 influenza virus protein was cloned in pET plasmids. The inducible expression of the protein was achieved using two different systems [24]. Either the protein was cloned under the control of gene ϕ 10 promoter of T7 bacteriophage (pET3 plasmid) generating the pET3-M2 plasmid or under the control of a *lac*/T7 hybrid promoter containing a *lac* operator before the M2 gene (pET11 plasmid) generating the pET11-M2 plasmid. These constructs were transformed into BL21(DE3) *E. coli* cells which contain the integrated T7 RNA polymerase gene under the *lac UV5* promoter. BL21(DE3) pLysS *E. coli* cells were also used; these cells are lysogens which express the T7 phage lysozyme, a natural inhibitor of the T7 RNA polymerase, at low levels [24]. We have used this system successfully to express toxic proteins from animal viruses [14–16,22].

Addition of IPTG to BL21(DE3) pLysS cells containing pET3-M2 plasmid induces the expression of a polypeptide migrating on SDS-PAGE with the mobility expected for influenza M2 protein. This is the only protein that is synthesized in the presence of rifampicin which blocks transcription by *E. coli* RNA polymerase but not by T7 polymerase (Fig. 1A). Expression of the influenza M2 protein in the presence of lysozyme is very toxic since synthesis of M2 protein could not be detected after 90–120 min post-induction. However, synthesis of M2 from pET11-M2 plasmid in BL21(DE3) cells devoid of phage T7 lysozyme, takes place to higher levels and synthesis of M2 was detected after longer post-induction times (Fig. 1B).

3.2. Expression of M2 protein induces cell lysis

To measure cell lysis upon M2 expression, the A_{660} of cultures was determined at various times after induction of cells bearing plasmid pET3-M2 or pET11-M2 (Fig. 2). A drastic fall in cell density at 60 min after IPTG induction is observed in the pET3-M2 plasmid/BL21(DE3) pLysS cell system in the presence or absence of rifampicin (Fig. 2A). This lytic effect could be a consequence of membrane permeability alterations allowing the leakage of T7 lysozyme from the cytoplasm to the periplasmic space, where its lytic activity would be exerted.

The expression of M2 protein in BL21(DE3) cells which do not contain lysozyme was slightly toxic after longer post-induction times (Fig. 2B). Lysis in this case, was observed upon induction with IPTG plus rifampicin, but not with IPTG alone, suggesting that inhibition of *E. coli* gene expression helps the lytic capacity of influenza M2 protein. This result indicated that high levels of M2 expression for a long period of time is toxic for bacteria, as occurred with the synthesis of poliovirus 3A protein, or SFV 6K protein [6,14,15,22].

3.3. Modification of E. coli membrane permeability by the expression of the M2 protein

To analyze permeability changes in response to influenza virus M2 protein, different assays were performed. We first tested the leakage of radioactivity from [^{3}H]uridine-preloaded cells. The release of [^{3}H]uridine was moni-

tored after induction of M2 synthesis (Fig. 3A). Substantial amounts of radioactivity are released to the medium and are detected soon after induction of M2 synthesis. As a control, bacteria bearing pET3 plasmid, induced in parallel do not show that behaviour.

Modifications in membrane permeability by a number of agents is usually observed in both directions (influx and efflux) [5]. Thus, not only the release of ions or small metabolites from the cell interior to the medium takes place, but also compounds normally readily excluded enter into cells [5]. Hence, the entry of ONPG into the cytoplasm was measured during the synthesis of M2. Induction of M2 expression caused an increased ONPG entry into bacteria as compared with control cells carrying the parental plasmid and treated with IPTG (Fig. 3B). No difference in the total amount of β-galactosidase activity between the two clones was found (Fig. 3C). Moreover, induction of M2 expression gives rise to increased β-galactosidase activity in the extracellular medium after 60 min post-induction (Fig. 3B), probably as a consequence of cell lysis.

Some translation inhibitors do not pass the membrane barrier of intact cells, but these compounds readily cross the cellular membrane after modification by membrane active agents or viral infection [2,3,5]. Hygromycin B has been one of the most non-permeant inhibitors used, in such a way that inhibition of protein synthesis by this antibiotic constitutes a very sensitive test to assay modifications of membrane permeability in intact cells [7,8]. The previous experiments were performed in the pET3-M2/BL21(DE3) pLysS system. Because the BL21(DE3) pLysS cells lysed rapidly upon M2 expression, we used both systems: pET3-M2/BL21(DE3)pLysS and pET11-M2/BL21(DE3) to assay the entry of hygromycin B. Fig. 4 shows that hygromycin B does not inhibit protein synthesis in control cells containing pET3 or pET11 vectors, whereas protein synthesis was powerfully blocked in the recombinant clones that express M2. Thus, the expression of M2 protein gives rise to a strong and rapid modification in membrane permeability, even at early times (30 min) post-induction, when cell lysis is not detected even in pLysS cells. Therefore, these results lead us to the conclusion that M2 permeabilizes *E. coli* cells to a number of compounds non-specifically.

4. Discussion

During the process of animal virus infection, an increase in membrane permeability takes place [5,6]. Late in infection, the lipidic barrier constituted by the plasma membrane is destroyed and cell lysis ensues. Although the phenomenology of these changes is fairly well known, the viral proteins involved in these modifications remain poorly characterized [5,6].

Recent findings suggest a role for poliovirus 3A protein and togavirus 6K protein in the modification of membrane permeability of infected cells [6,14,22]. Both proteins share similarities in their hydrophobic profiles and both contain a hydrophobic region at the carboxy terminus that can form an amphipathic helix [6,6,32]. A similar structure is also found in influenza M2 protein [6]. It has been speculated that late during influenza virus infection, M2 protein would regulate the pH within the Golgi apparatus to facilitate transport of the native acid-sensitive HA to the plasma membrane of infected cells, a process necessary for the release of virus particles [25].

Influenza virus induces membrane leakiness in the infected cells [4,18], but the relationship between the expression of any influenza protein, including M2, and

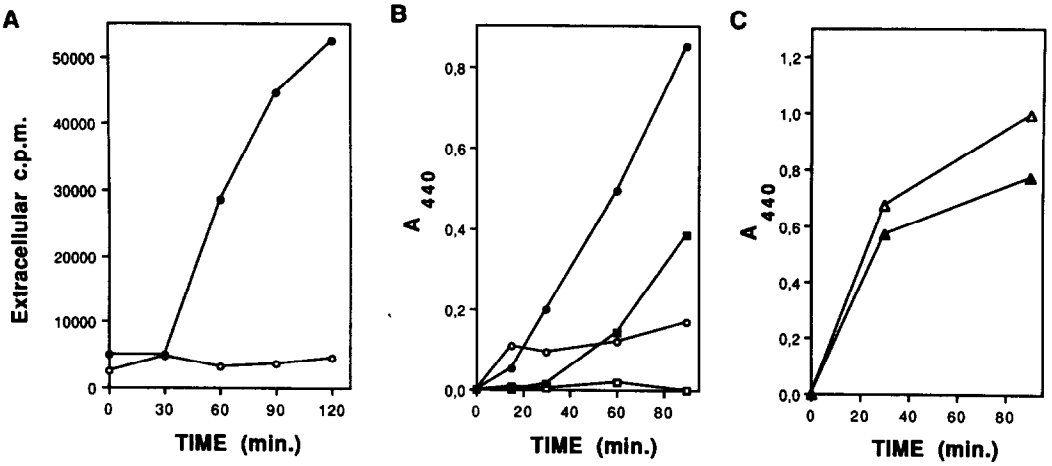

Fig. 3. Permeability changes in BL21(DE3) pLysS cells after induction of M2 expression. Panel A: BL21(DE3) pLysS cells containing pET3 (○) or pET3-M2 plasmids (●) were preloaded for 1 h with 4 μCi/ml [^{3}H]uridine and at zero time were induced with 1 mM IPTG. At the times post-induction indicated the level of radioactivity in the culture medium was measured. Panel B: BL21(DE3) pLysS cells containing pET3 (open symbols) or pET3-M2 (closed symbols) plasmids were induced with 1 mM IPTG at zero time. At the indicated times the entry of ONPG (●/○) or β-galactosidase activity recovered from the extracellular medium (■/□) was analyzed. Measurements are given as $A_{440\ nm}$. Panel C: total amount of β-galactosidase activity in cells containing pET3(△) or pET3-M2 (▲).

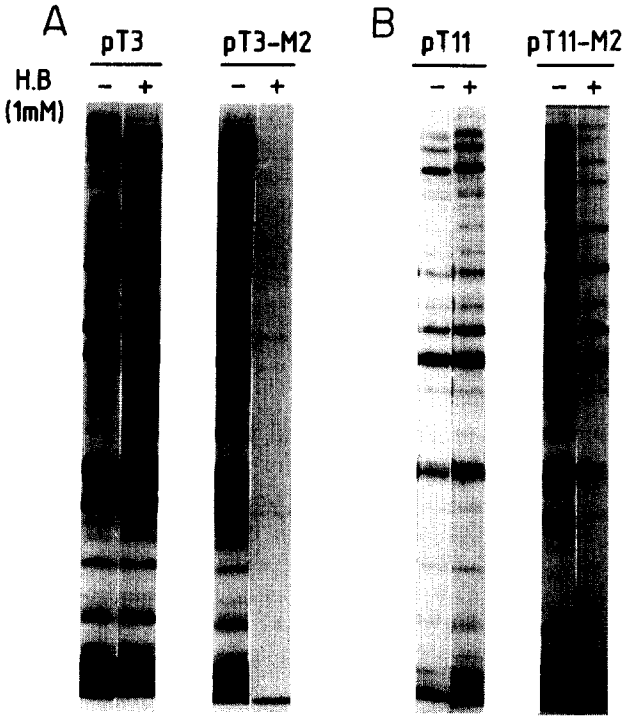

Fig. 4. Effect of hygromycin B on protein synthesis in cells expressing the influenza virus M2 protein. Cells were grown and induced as indicated in section 2. Half an hour after IPTG induction, proteins were labelled by addition of [^{35}S]methionine for 10 min at 37°C. 1 mM hygromycin B was present during the labelling period. Cells were then centrifuged and the proteins analyzed by SDS-PAGE. Panel A: pET3 or pET3M2 plasmids/BL21(DE3) pLysS cells. Panel B: pET11GT pET11-M2 plasmid/BL21(DE3) cells.

membrane modifications in mammalian cells has not been analyzed yet. The lytic potential of M2 protein in *E. coli* and its behaviour as an ion-channel, when expressed in *Xenopus* oocytes [20] makes M2 a good candidate for the protein involved in modifying membrane permeability in influenza virus-infected cells. However, further analyses on the mode of action of M2 in mammalian cells is still necessary to reach this conclusion. Our present results together with previous findings on poliovirus 3A and togavirus 6K proteins lend support to the concept that animal viruses encode specific proteins, the viroporins, involved in modifying membrane permeability and causing cell lysis by forming pores in the plasma membrane [1,3]. A relationship between the role of M2 in inducing permeability changes and the budding or release of virus particles can be envisaged. Finally, the efficient expression of M2 in the bacterial system described in this work should facilitate future research on the mode of action of M2 and its interaction with the antiviral compound amantadine.

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