The influenza A virus M2 protein forms a proton-selective transmembrane ion channel, which is activated at acidic pH (Pinto et al., 1992; Chizhmakov et al., 1996; Mould et al., 2000) and is the specific target of the anti-influenza drugs amantadine and rimantadine. The M2 channel plays a role in the uncoating of influenza virions in endosomes (Martin & Helenius, 1991; Wharton et al., 1994). In addition, during infection by the highly pathogenic influenza A virus subtypes H7 and H5, M2 is required to act as a proton-leak channel to elevate pH within the trans-Golgi network; this is important to prevent premature acid activation of newly synthesized haemagglutinin (HA), which is cleaved intracellularly, and consequent inactivation of progeny virus (Sugrue et al., 1990; Steinhauer et al., 1991; Ciampor et al., 1992; Grambas & Hay, 1992). The M2 proteins of different viruses vary in their ability to alter trans-Golgi pH. In particular, differences in the activities of two closely related strains of avian H7 viruses, Rostock and Weybridge, correlate with differences in the fusion pH of their HAs (Grambas et al., 1992). The greater activity of the Rostock M2 in reducing acidity in the trans-Golgi complements the lower acid stability of Rostock HA, with a fusion pH of 5.9 compared with 5.3 for Weybridge HA (Grambas & Hay, 1992).

Furthermore, electrophysiological studies have shown that the M2 channels of the two viruses differ in two important respects, representing mechanistic changes in the channel (Chizhmakov et al., 2003): (i) the Rostock M2 possesses a sevenfold greater proton conductance, corresponding to its greater pH-modulating activity; and (ii) the two channels differ in activation characteristics. More specifically, they differ in the direction of rectification induced by high pH (>7). Whereas the Weybridge M2, like that of human viruses, deactivates in response to external (but not internal) high pH, the Rostock M2 deactivates in response to internal (but not external) high pH. The latter difference in activation characteristics was shown to be determined by three amino acid differences, V27I, F38L and D44N, within the transmembrane domain, which distinguish the Weybridge and Rostock proteins, respectively. Substitution of all three residues was required to transform the Weybridge phenotype into that of the Rostock M2 and, conversely, single substitutions in Rostock M2 were sufficient to effect the opposite phenotypic change. However, these mutagenesis experiments did not resolve which of the amino acid differences affected the ion flux through the channel. To answer that question, we have used a semi-quantitative HA–M2 co-expression assay to assess the effects of single and double mutations at these three positions, aa 27, 38 and 44, on the pH-modulating activity of M2 and to determine whether the differences in ion flux and activation are genetically linked.

DNA copies of coding sequences for the HA of influenza virus strain A/chicken/Germany/34 (H7N1, Rostock strain) and the M2 proteins of the Rostock strain, A/chicken/Germany/27 (H7N7, Weybridge strain) and A/PR/8/34 (H1N1) were inserted into plasmid pVOTE.1 (kindly provided by B. Moss, National Institutes of Health, Bethesda, MD, USA) to generate pVOTE.1-HA and
pVOTE.1-M2, respectively. Plasmids encoding the mutant M2 proteins I27V, L38F, I27V+L38F, N44D and D44N (Fig. 1) were prepared by using four-primer PCR. Sequences of oligonucleotide primers and details of cloning are available upon request. CV-1 cells were infected for 1 h with recombinant vaccinia virus vTF7.3 (10 p.f.u. per cell) (Fuerst et al., 1986), transfected for 4 h with plasmids mixed with Lipofectin (Life Technologies) and incubated for a further 16 h in minimal essential medium containing 20 % fetal calf serum and 40 μg arabinose ml⁻¹, with or without 5 μM amantadine.

Proteins in 24-well plates were analysed by 12.5 % SDS-PAGE and Western blot analysis was done as described by Grambas et al. (1992), using anti-M2 serum, protein A–horseradish peroxidase (HRP) conjugate (Bio-Rad) and labelling with TMB stabilized substrate for HRP (Promega). The amount of protein expressed depended on the amount of pVOTE.1-X plasmid that was used for transfection (data not shown) and was roughly equivalent for similar amounts (0-5 μg) of plasmid DNA, as shown in Fig. 2. The variation observed, less than twofold, was within the variation seen between different experiments.

ELISA to detect HA co-expressed with M2 was performed on CV-1 cells in 96-well plates (duplicate wells) following transfection with 0.25 μg pVOTE.1-HA together with increasing amounts of plasmid DNA encoding wt or mutant M2 proteins, essentially as described by Grambas & Hay (1992). Three mAbs were used: HC58, specific for the native form of HA; H9, specific for the low-pH form; and HC2, which recognizes both forms of HA. The effectiveness of M2 in elevating trans-Golgi pH and protecting HA against low pH-induced changes depended on the ratio of HA and M2 proteins expressed and was indicated by corresponding increases in the proportion of native HA and decreases in the proportion of low-pH HA (shown for wt Rostock M2 in Fig. 3a). The maximum percentage change in A450 obtained with HC58 and H9 antibodies relative to HC2 was recorded at the optimum HA/M2 ratio; further increases in expression of M2 reduced expression of total HA. The optimum HA/M2 ratio was similar for all M2 proteins. Mean values from six experiments were used to compare the pH-modulating activities of the different M2 proteins (Fig. 3b).

Co-expression of HA with the wt Rostock M2 (R-M2) resulted in an increase of approximately 24 % in native HA and a corresponding decrease of approximately 23 % in the low-pH form of HA, compared with HA expressed in the absence of M2 protein (Fig. 3b). The changes conferred by the wt Weybridge M2 (W-M2) were approximately half those found with R-M2 (approx. ±13 %) and were comparable to those obtained with M2 of the human influenza virus A/PR/8/34 (PR8-M2; approx. ±16 %) (Fig. 3b). The activities of R-M2 and W-M2 were inhibited specifically by amantadine (5 μM), whereas PR8-M2 was resistant, consistent with previously published data.

The mutant Rostock proteins containing single amino acid substitutions of I27V or L38F possessed a pH-modulating activity that was indistinguishable from that of the wt protein (Fig. 3b), as did the double mutant I27V+L38F (data not shown). Substitution of aspartic acid for arginine 44 (N44D), however, caused a significant decrease in activity (approximately to the levels found for W-M2), indicating that the change in this residue alone could account for the difference in the pH-modulating activities of the two wt channel proteins. Furthermore, substitution of asparagine for aspartic acid 44 in the M2 PR8-M2 (D44N) caused an increase in activity (resistant to amantadine) to a level comparable to that of the wt Rostock protein, confirming the more general influence of this particular substitution at residue 44 on M2 activity.

Fig. 1. Amino acid sequences of the M2 proteins of Rostock strain A/chicken/Germany/34 (R-M2), Weybridge strain A/chicken/Germany/27 (W-M2), A/PR/8/34 and transmembrane mutants of R-M2 and PR8-M2. The transmembrane domain is shown in bold.

Fig. 2. Expression of wt and mutant M2 proteins. CV-1 cells in 24-well plates, transfected with 0.5 μg pVOTE.1-X, were lysed and analysed by 12.5 % SDS-PAGE and Western blotting with anti-M2 antibody. Lane 1, wt Rostock M2 (R-M2); lane 2, R-M2 I27V; lane 3, R-M2 L38F; lane 4, R-M2 N44D; lane 5, wt Weybridge M2; lane 6, A/PR/8/34 (PR8-M2); lane 7, PR8-M2 D44N; lane 8, non-transfected cells.
Thus, whereas any one of the single amino acid substitutions – I27V, L38F or N44D – caused a switch in the activation characteristics of Rostock M2 to those of the Weybridge channel (Chizhmakov et al., 2003), only the change in residue 44 between asparagine and aspartic acid was necessary and was sufficient to account for the difference between the pH-modulating activities (proton flux) of the two channel proteins. It was apparent, therefore, that there was no strict genetic correlation between the two phenotypic properties and that they represented separable functional characteristics.

Previous studies of mutant viruses have shown that a number of single amino acid substitutions, at residues 26, 30, 31 and 34, as well as 27, can cause increases or decreases in pH-modulating activity, as assayed by HA–M2 co-expression, e.g. I27T and I27S substitutions caused increases in activity of Rostock M2, whereas I27N, like I27V in the present study, caused no detectable difference (Grambas et al., 1992). Furthermore, it was also observed, by passage in cell culture, that mutations that altered the fusion pH of the HA or the pH-modulating activity of M2 could influence selection of changes in the corresponding properties of M2 and HA, respectively. Thus, for example, a Weybridge mutant with an increased HA fusion pH selected a mutation in M2, G34E, that caused a compensatory increase in pH-modulating activity.

As for the significance of the differences in phenotype, there was a clear correlation between the greater pH-modulating activity and proton flux of the M2 channel and the higher HA fusion pH of the Rostock virus. However, there was no clear indication as to the advantage conferred by the ‘unusual’ activation characteristics of its M2 channel protein, especially with respect to modulation of trans-Golgi pH. As acquisition of the latter property by the Rostock M2 required two unusual amino acid substitutions in residues 27 and 44 (among known M2 sequences), whereas the former alteration was effected by the change in residue 44 (N44D) alone, it appeared likely that the change in activation characteristics was selected for in addition to, and not coincidentally with, the increase in M2 channel activity. We do not know, however, how the Rostock virus with these peculiar properties (in HA and M2) emerged – whether in vivo or as a result of extensive passage in vitro – or how the sequence in which the (complementary) characteristics of HA and M2 were acquired. That the change in activation characteristics is apparently not associated with the change in modulation of trans-Golgi pH points to its greater significance for the activity of M2 in virus entry. It may be that removal of the ‘strict’ regulation by pH outside the virion (in the case of Weybridge M2) is necessary to facilitate uncoating of the Rostock virion at a higher endosomal pH, consistent with the higher pH at which fusion between the virus and endosomal membranes is promoted by the Rostock HA. As the change in activation affects the direction of the pH-induced effect (from outside, the N terminus of M2, to inside, the C terminus) and not the intrinsic nature of the pH-induced change in the voltage dependence of channel conductance, this appears to provide a mechanism for ‘switching off’ the normal activation property. Furthermore, maintenance of the intrinsic conductance characteristics of the channel emphasizes their importance. However, the mechanistic significance of reduced outward H+ flux at high external pH in, for example, promoting H+ transfer into the virion or preserving the

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**Fig. 3.** pH-modulating activity of wt and mutant M2 proteins. Change in HA (%) recognized by HC58 (native form of HA) or H9 (low-pH form of HA), respectively, was estimated from the ratios of $A_{450}$ as follows: HC58 (%) = $\frac{[HC58/HC2 - 2m\times100]}{[HC58/HC2 - 2m\times100]}$ or H9 (%) = $\frac{[H9/HC2 - 2m\times100]}{[H9/HC2 - 2m\times100]}$, respectively. (a) Dependence of changes in HA on amount of wt R-M2 (ng). □, HC58; ■, H9; △, HC58 + amantadine; ●, H9 + amantadine. (b) Maximum changes (%) obtained with HC58 and H9 antibodies relative to HC2. Results are shown for wt Rostock M2 (R), mutant Rostock proteins (I27V, L38F, N44D), wt Weybridge M2 (W), A/PR/8/34 (PR8) and mutant A/PR/8/34 (PR8-D44N). Filled bars, HC58; open bars, H9; dark hatched bars, HC58 + amantadine; pale hatched bars, H9 + amantadine. Results are shown as means ± SD of six experiments.
integrity of the RNA genome of virus exposed to an alkaline environment, has yet to be elucidated.

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