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An Influenza A Vaccine Based on Tetrameric Ectodomain of Matrix Protein 2*[S]

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Matrix protein 2 (M2) of influenza A is a tetrameric type III membrane protein that functions as a proton-selective channel. The extracellular domain (M2e) has remained nearly invariable since the first human influenza strain was isolated in 1933. By linking a modified form of the leucine zipper of the yeast transcription factor *GCN4* to M2e, we obtained a recombinant tetrameric protein, M2e-tGCN4. This protein mimics the quaternary structure of the ectodomain of the natural M2 protein. M2e-tGCN4 was purified, biochemically characterized, and used to immunize BALB/c mice. High M2e-specific serum IgG antibody titers were obtained following either intraperitoneal or intranasal administration. Immunized mice were protected fully against a potentially lethal influenza A virus challenge. Antibodies raised by M2e-tGCN4 immunization specifically bound to the surface of influenza-infected cells and to an M2-expressing cell line. Using a M2e peptide competition enzyme-linked immunosorbent assay with M2-expressing cells as target, we obtained evidence that M2e-tGCN4 induces antibodies that are specific for the native tetrameric M2 ectodomain. Therefore, fusion of an oligomerization domain to the extracellular part of a transmembrane protein allows it to mimic the natural quaternary structure and can promote the induction of oligomer-specific antibodies.

Influenza has one of the highest infection rates of all human viruses and can kill healthy persons of all ages (1). It is estimated that influenza infection during seasonal epidemics kills 1 in 1000 infected individuals, whereas an unpredictable pandemic is likely to kill millions. In addition, increased hospitalization and absenteeism from school and work are direct consequences of the flu. At present, the best way to protect against influenza is to vaccinate against the ever-mutating strains (2). However, antigenic drift and occasional shift of the two major membrane glycoproteins, hemagglutinin and neuraminidase, make vaccine production cumbersome and necessitate yearly revision of the vaccine seed strains by the World Health Organization.

Influenza A also encodes a third integral membrane protein, M2,[2] a homotetramer, the subunit of which has a small external domain (M2e) of 23 amino acid residues (3). Natural M2 protein is present in a few copies in the virus particle but in abundance on virus-infected cells (4). In contrast to hemagglutinin and neuraminidase, M2e is almost nonimmunogenic (5), and its sequence is highly conserved. Capitalizing on these properties, we developed a universal influenza A vaccine by linking the M2e peptide to a virus-like particle based on the hepatitis B virus core (HBc) (6). In this context, M2e is highly immunogenic, and the M2e-HBc vaccine induces antibodies that protect mice against influenza-induced death and morbidity.

Oligomeric proteins found in vaccines derived by inactivating or attenuating a pathogen often function as their major antigenic determinant. Conformational epitopes embedded in the quaternary structures may critically contribute to immunogenicity, but the oligomeric status of the antigenic structures may change during vaccine preparation, leading to aggregation or disassembly into monomers. For example, when producing influenza split vaccines, the hemagglutinin and neuraminidase oligomeric antigens may lose their oligomeric structure during the virus disruption step, or they may form aggregates. Specific protein oligomerization is critical for the function of many proteins. For example, influenza virus hemagglutinin is a homotrimer (7), and neuraminidase is a homotetramer composed of two disulfide-linked dimers (8, 9, 10, 11). Remarkably, the enzymatic activity of neuraminidase is associated only with the tetrameric form (12). Furthermore, tetrameric neuraminidase molecules are considerably more immunogenic than the monomers and dimers. The quaternary structure of an oligomeric protein is often determined by a subdomain with strong oligomerization properties. In many instances, an oligomerization subdomain can be substituted by a heterologous motif with similar conformation-inducing properties. For example, the p53 tetramerization domain can be replaced with a tetrameric coiled-coil motif, in this case an engineered leucine zipper that

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: M2, matrix protein 2; M2e, extracellular domain of M2; BM2e, influenza B M2 protein ectodomain; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BS3, bis(sulfosuccinimidyl) suberate; HBc, hepatitis B virus core; DSP, dithiobis(succinimidyl propionate); MALDI, matrix-assisted laser desorption ionization; MDCK, Madin-Darby canine kidney; HEK, human embryonic kidney; Bistris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

assembles as a four-stranded coiled-coil, with regain of full function (13).

Influenza M2e is the entry site of the proton channel. To obtain a tetrameric structure with the conformation of the native M2 ectodomain, we fused M2e to a sequence variant of the leucine zipper domain from the yeast transcription factor *GCN4*, forming all parallel four-chain coiled-coils (15). The fusion protein, M2e-tGCN4, was evaluated as a vaccine antigen both biochemically and biologically. M2e-tGCN4 is a potent vaccine that induces in mice complete protective immunity against a lethal challenge with influenza virus. Finally, by using a competitive ELISA, we provide evidence that this vaccine elicits antibodies that bind specifically to conformational epitopes on the ectodomain of natural M2 protein.

EXPERIMENTAL PROCEDURES

Expression and Purification of M2e-tGCN4 and BM2e-tGCN4—Two overlapping synthetic oligonucleotides (Invitrogen) were used to generate the coding sequence for a modified version of the leucine zipper domain *GCN4*. This variant (tGCN4) forms four-stranded parallel coiled-coils (15). We used standard PCR protocols to fuse M2e or the influenza B M2 ectodomain (BM2e) at the N terminus of tGCN4 and cloned the fusions in the bacterial expression vector pLT32H under control of the left promoter of phage lambda (16).[3] The sequences of the primers used are available upon request. The resulting expression constructs, pLT32hM2etGCN4 and pLT32hBM2etGCN4, were sequence verified and used to transform *Escherichia coli* BL21 RIL (Invitrogen) containing the transcription regulatory plasmid pICA2.[3] The deduced amino acid sequences of M2e- and BM2e-tGCN4 are shown in supplemental Fig. S1. M2e- and BM2e-tGCN4 proteins were expressed and purified in the same way. A 30-ml preculture grown at 28 °C in Luria broth was used to inoculate 1 liter of fresh medium. At an A_{600} of 0.6–0.8, the cells were treated with 1 mM isopropyl 1-thio-β-D-galactopyranoside, incubated for another 4 h, and then collected by centrifugation (6000 × g, 20 min, 4 °C). The bacterial pellet was resuspended in 20 mM Bistris propane buffer, pH 6.8, and sonicated. Bacterial debris was pelleted by centrifugation (20,000 × g, 1 h, 4 °C). The supernatant was applied to a DEAE column pre-equilibrated with 20 mM Bistris propane, pH 6.8 (buffer A). M2e- and BM2e-tGCN4 were eluted by a two-step gradient going from 0–40% buffer B (20 mM Bistris propane, pH 6.8, 1 M NaCl) and 40–100% buffer B. Fractions containing M2e- or BM2e-tGCN4 were pooled, adjusted to 25% ammonium sulfate saturation, and applied to a phenyl-Sepharose column pre-equilibrated with 25% ammonium sulfate, 50 mM Tris, pH 8 (buffer C). Bound proteins were eluted with a two-step gradient. The two-step elution was performed with 0–40 and 40–100% 50 mM Tris buffer, pH 8 (buffer D). The fractions containing M2e- or BM2e-tGCN4 were concentrated by ultrafiltration (10-kDa cut-off) and loaded on a Superdex 75 column. Gel filtration was performed in phosphate-buffered saline (PBS), and the fractions containing M2e- or BM2e-tGCN4 were pooled and stored at −70 °C.

[3] N. Mertens, unpublished data.

Estimation of Molecular Weight by Nondenaturing PAGE—The molecular weights of the M2e- and BM2e-tGCN4 proteins were estimated using a modification of the methods of Bryan (17) and Davis (18). Briefly, the chimeric protein and molecular weight standards were resolved by gel electrophoresis using various polyacrylamide concentrations. Electrophoretic mobility (R_F) was determined in each gel relative to the tracking dye, and 100 log ($R_F \times 100$) values were plotted against the percent gel concentration for the reference proteins as well as M2e- and BM2e-tGCN4. The slope for each protein was determined and the logarithm of the negative slope was plotted against the logarithm of the molecular weight of each standard protein. This produces a linear plot from which the molecular weight of an unknown protein can be deduced.

Cross-linking of M2e-tGCN4—Cross-linking was carried out in PBS, pH 8.0. Freshly made 65 mM bis(sulfosuccinimidyl) suberate (BS3; Pierce) was added to 20 μg of purified M2e-tGCN4 to final concentrations of 10 to 0.1 mM in a final volume of 24 μl. The reactions were incubated for 1 h at room temperature, quenched by adding Tris buffer, pH 8.0, to a final concentration of 50 mM, and incubated for 15 min. The cross-linker dithiobis(succinimidyl propionate) (DSP; Pierce) was used similarly, but a 25 mM stock solution in Me_2SO was used. After cross-linking, an equal volume of reducing (200 mM dithiothreitol, 20% glycerol, 50 mM Tris buffer, pH 6.8) or nonreducing (20% glycerol, 50 mM Tris buffer, pH 6.8) loading buffer was added. The samples were boiled and then resolved by SDS-PAGE with a prestained broad range molecular weight marker as reference (Bio-Rad).

Mass Spectrometric Analysis of M2e-tGCN4—We used mass spectrometry to analyze M2e-tGCN4 alone and cross-linked by either BS3 or DSP. A 1-μl sample containing 0.3 μg of protein was mixed with 1 μl of saturated α-cyano-4-hydroxycinnamic acid that had been diluted 5-fold, and 0.5 μl of this mixture was dried on the MALDI target of an Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany). Before measurement, the target was briefly rinsed with 10 mM ammonium citrate. MALDI mass spectra were generated by accumulation of 200 laser shots on the best target spots.

Generation of M2-expressing HEK Cells and HEK-M2 ELISA—M2 cDNA was cloned in the pENTR3C vector (Invitrogen) under the control of the cytomegalovirus promoter and recombined into pDWPI using the Gateway® system. Recombinant lentivirus was produced by cotransfection of HEK293T cells with the pDWPI-M2 expression plasmid and the packaging plasmids pMGD and pδR (19). The resulting recombinant lentivirus was used to transduce HEK293 cells. Green fluorescent protein-positive cells were sorted by fluorescence-activated cell sorting, which generated HEK-M2 cells. These were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, penicillin, streptomycin, and 10 μM amantadine. For ELISA, HEK-M2 cells were seeded at 15,000 cells/well in a 96-well plate. After 24 h, the medium was removed, and the cells were fixed with 100 μl of 0.05% glutaraldehyde in PBS for 20 min at room temperature. Fixed cells were washed with PBS and blocked with 1% bovine serum albumin in PBS. The plates were washed and incubated for 90 min with the indicated immune serum samples or with M2e-spe-

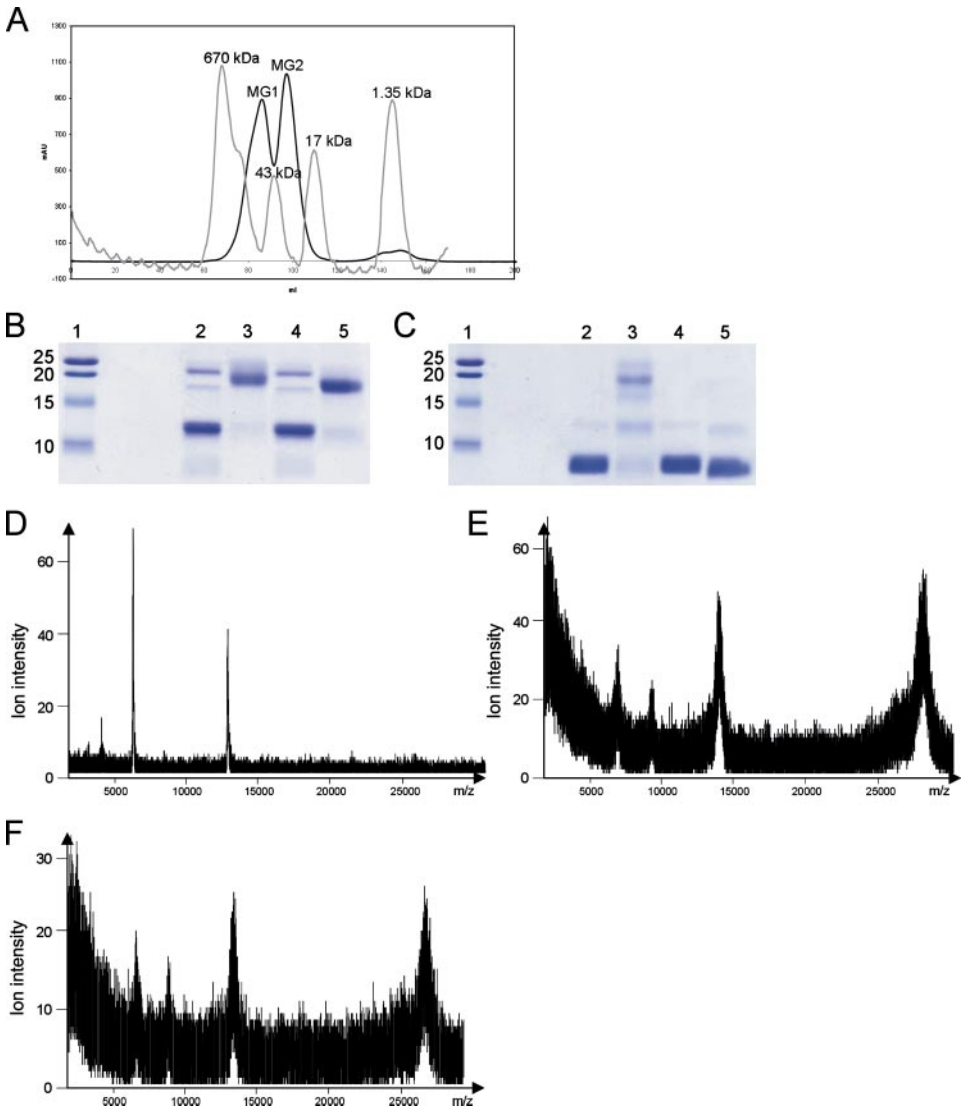

FIGURE 1. **M2e-tGCN4 analysis.** *A*, final purification step: gel filtration on a precalibrated Superdex 75 column. Molecular masses of calibration proteins are indicated. *Peak MG1* corresponds to M2e-tGCN4 that is associated with bacterial contaminants. *Peak MG2* contains pure M2e-tGCN4 and was used throughout the study. *B* and *C*, nonreducing and reducing SDS-PAGE, respectively, of purified protein samples cross-linked by BS3 or DS. *Lane 1*, molecular mass markers; *lane 2*, M2e-tGCN4; *lane 3*, M2e-tGCN4 + 10 mM BS3; *lane 4*, M2e-tGCN4 + Me_2SO; *lane 5*, M2e-tGCN4 + 1 mM DSP. *D–F*, MALDI mass spectra of noncross-linked M2e-tGCN4 (*D*), DSP-cross-linked M2e-tGCN4 (*E*), and BS3-cross-linked M2e-tGCN4 (*F*).

cific monoclonal ^{14}C2 (Affinity BioReagents) diluted in PBS + 1% bovine serum albumin. Washing of the plates was followed by incubation with a peroxidase-labeled goat anti-mouse IgG; detection was with tetramethylbenzidine substrate. The reaction was stopped by adding 50 μl of 1 M H_2SO_4. For competition assays, immune serum was preincubated for 1 h with serial dilutions of the M2e peptide or M2e- or BM2e-tGCN4 before transfer to the HEK-M2 plates.

Immunization and Challenge of Mice—Specific pathogen-free female BALB/c mice were obtained from Harlan and housed in a temperature-controlled environment with 12-h light/dark cycles and given free access to food and water. At the age of 8 weeks they were injected intraperitoneally with 10 μg of M2e- or BM2e-tGCN4 with trehalose dicorynomycolate + monophosphoryl lipid A (Sigma) or Alhydrogel (Brenntag Biosector A/S) adjuvant or intranasally with the mucosal adjuvant CTA1-DD and boosted twice at 3-week intervals, as described previously (6, 20, 21).

Two weeks after each immunization, blood samples were collected from the ventral tail vein. The final bleeding was performed by cardiac puncture. Blood was left to clot at 37 °C for 30 min, and serum was collected by taking the supernatant from two consecutive centrifugations. The titers of IgG subtypes produced against the M2e peptide, M2e-tGCN4, and BM2e-tGCN4 were determined by ELISA (20). Mice were challenged under light isoflurane anesthesia with 4 LD_{50} mouse-adapted X47 for the follow-up study. X47 is a H3N2 recombinant influenza A virus strain (A/Victoria/3/75 (H3N2) × A/Puerto Rico/8/34 (H1N1)). X47 was adapted to mice by several lung passages as described (6). All animal experiments were authorized by the Institutional Ethics Committee on Experimental Animals and conducted under conditions specified by law (European Directive and Belgian Royal Decree of November 14, 1993).

RESULTS

Purification and Characterization of M2e-tGCN4—We constructed a bacterial expression vector to produce M2e fused at its C terminus to the *GCN4*-derived tetramerizing leucine zipper (supplemental Fig. S1*A*). As a control we used BM2e, the biological counterpart of influenza A M2e (14). Recombinant M2e-tGCN4 was purified to ~98% (supplemental Fig. S1*B*, *lane 5*). BM2e-tGCN4 was purified according to the same protocol (supplemental Fig. S2*A*). Under reducing SDS-PAGE conditions, M2e-tGCN4 migrated as a predominant band with apparent molecular mass of 7 kDa (Fig. 1*C*, *lane 2*). However, under nonreducing conditions, M2e-tGCN4 appeared as a band of 26 kDa corresponding to a tetramer and a minor band of ~13 kDa corresponding to a dimer (Fig. 1*B*, *lane 2*). Because M2 is a tetrameric protein with subunits linked by disulfide bridges in the M2e domain, we speculated that these intermolecular bonds were also present in M2e-tGCN4. In the size exclusion step on a Superdex 75 column, fractions containing M2e-tGCN4 appeared as two peaks with approximate molecular masses of 47 kDa (*peak MG1*) and 35 kDa (*peak MG2*) (Fig. 1*A*). The former peak corresponded to M2e-tGCN4 associated with bacterial protein contaminants and was dis-

carded (data not shown). The calculated molecular mass of purified M2e-tGCN4 is 25.8 kDa, but it was estimated by nondenaturing PAGE at 33 kDa (supplemental Fig. S2). These experimentally derived values suggest that M2e-tGCN4 and BM2e-tGCN4 have tetrameric structures.

To prove that M2e-tGCN4 is indeed a tetramer, chemical cross-linking experiments were performed with the homobifunctional cross-linkers BS3 and DSP. Following cross-linking, nonreducing SDS-PAGE analysis revealed a major band with an apparent molecular mass of 18 kDa (Fig. 1*B*, *lanes 3* and *5*). When decreasing amounts of cross-linker were used, a band of 26 kDa reappeared (supplemental Fig. S3).

Mass spectrometric analysis of M2e-tGCN4 allowed unambiguous determination of the oligomerization state. The MALDI spectrum of nontreated M2e-tGCN4 revealed two components with estimated masses of 6320 ± 13 and 12,664 ± 25 Da. These values correspond with the monomeric and dimeric forms of M2e-GCN4 lacking the initiator methionine (Fig. 1*D*). When samples cross-linked by BS3 or DSP were analyzed, we noticed broader peaks centered at molecular masses 26,533 ± 53 and 27,068 ± 54 Da, respectively, together with residual monomer and dimer. Peak broadening is most likely due to different numbers of cross-linker molecules attached to the M2e-tGCN4 protein. The second, less intense peaks had masses of ~13,800 (BS3) and 14,000 (DSP) Da and presumably correspond to double-charged cross-linked tetramers or dimers (Fig. 1, *E* and *F*). Taken together, we conclude that M2e-tGCN4 is a soluble, tetrameric protein consisting of disulfide-linked dimers, and a fraction is even further stabilized by disulfide cross-links between the dimers.

M2e-tGCN4 Immunization Protects Mice from Challenge with Influenza A Virus—We next determined whether immunization with M2e-tGCN4 could induce a protective immune response. BALB/c mice were immunized three times with M2e-tGCN4 together with alum, Ribi, or CTA1-DD adjuvants as described previously for the M2e-HBc vaccine (6, 20, 21). BM2e-tGCN4 was used as a control. M2e-specific serum IgG1 and IgG2a antibodies were induced in all M2e-tGCN4-treated groups, as quantified by the M2e peptide ELISA (Fig. 2, *A* and *B*). A strong antibody response against the *GCN4* moiety was also apparent from ELISA using BM2e-tGCN4-coated plates. Importantly, M2e-tGCN4-immunized mice were protected against a potentially lethal challenge with 4 LD_{50} of mouse adapted X47 virus (Fig. 2*C*).

M2e-tGCN4-specific Antibodies Recognize Native Tetrameric M2—Because M2e is presented as a tetrameric complex by M2e-tGCN4, we investigated whether antibodies raised by immunization could bind to M2e tetramer-specific conformational epitopes. Immunofluorescence staining of X47-infected MDCK cells with M2e-tGCN4 immune serum demonstrated specific staining of M2 present on the cell surface (Fig. 2*D*, *upper right panel*). BM2e-tGCN4 control immune serum did not bind to infected MDCK cells (Fig. 2*D*). We used a lentiviral vector system to generate HEK-M2 cells that constitutively express M2 (supplemental Fig. S4*A*) and can be kept in culture for a long time, provided amantadine, an M2 proton channel inhibitor, is present in the medium. As in the case of infected cells, M2e-tGCN4, but not BM2e-tGCN4, immune serum specifically bound nonpermeabilized HEK-M2 cells (supplemental Fig. S4, *B* and *C*).

To provide direct evidence for the presence of M2e tetramer-specific antibodies, we used an HEK-M2 cell-based ELISA to analyze binding of anti-M2e-tGCN4 serum in the absence or presence of different concentrations of competing M2e peptide or M2e-tGCN4 using both competitors in the same M2e molar range. Binding of mouse immune serum was almost completely inhibited by M2e-tGCN4, whereas free M2e peptide only partially inhibited the binding to HEK-M2 cells. In contrast, both inhibitors completely blocked the binding of the monoclonal antibody ^{14}C2 to HEK-M2 cells (Fig. 2, *F* and *G*). As expected, BM2e-tGCN4 did not interfere with the binding of ^{14}C2 or anti-M2e-tGCN4 immune serum (Fig. 2*E*). This indicates that serum IgG antibodies induced by immunization with M2e-tGCN4 recognize epitopes in the ectodomain of native M2 that are absent in the linear M2e peptide.

DISCUSSION

The purpose of our study was to obtain a well defined antigenic structure suitable for influenza vaccination and closely mimicking the native conformation of the tetrameric external domain of the influenza A M2 protein. In the native configuration, interactions in the intramembrane domain, and possibly even in the intracellular domain of M2 protein, contribute to the tetrameric assembly. To obtain a fully characterized molecular structure, we replaced the transmembrane segment by a defined, tetrameric scaffold. The resultant M2e-tGCN4 was expressed efficiently in bacteria as a soluble protein and was purified and characterized in detail.

The status of the native form as a tetramer was proven by gel electrophoresis, gel filtration, and cross-linking experiments in combination with mass spectrometry. The difference between the theoretically calculated molecular mass and the value estimated by gel filtration or native gel analysis can be explained by the fact that the *GCN4*-derived tetramerizing leucine zipper adopts a cylindrical shape (15). Reduction with dithiothreitol resulted in dissociation of the dimers into monomers, indicating that the monomers are linked by disulfide bonds involving at least one of the two cysteines of M2e. A fraction of the tetramers does not dissociate by ionic detergent treatment alone but does so under reducing conditions, indicating some disulfide cross-links between dimers as well. Hence, the structure of the M2e domain of M2e-tGCN4 is very similar to the structure of native M2 protein (23, 25).

Mass spectrometry provided unambiguous molecular mass values. M2e-tGCN4 revealed two peaks in the MALDI mass spectrometry, one corresponding to the theoretical size of the monomer (6320 kDa) and the other corresponding to the size of a dimer (12,664 Da), both lacking the N-terminal methionine. Most likely the tetrameric structure was destroyed by the energy of the ionizing laser. Mass spectrometric analysis of M2e-tGCN4 cross-linked by BS3 or DSP revealed a major peak corresponding to a tetramer. The observed molecular mass was slightly larger than the calculated value, undoubtedly because of bound cross-linker.

M2e-tGCN4 was designed not only to increase the valency of the target antigen but also to mimic the natural conformation of

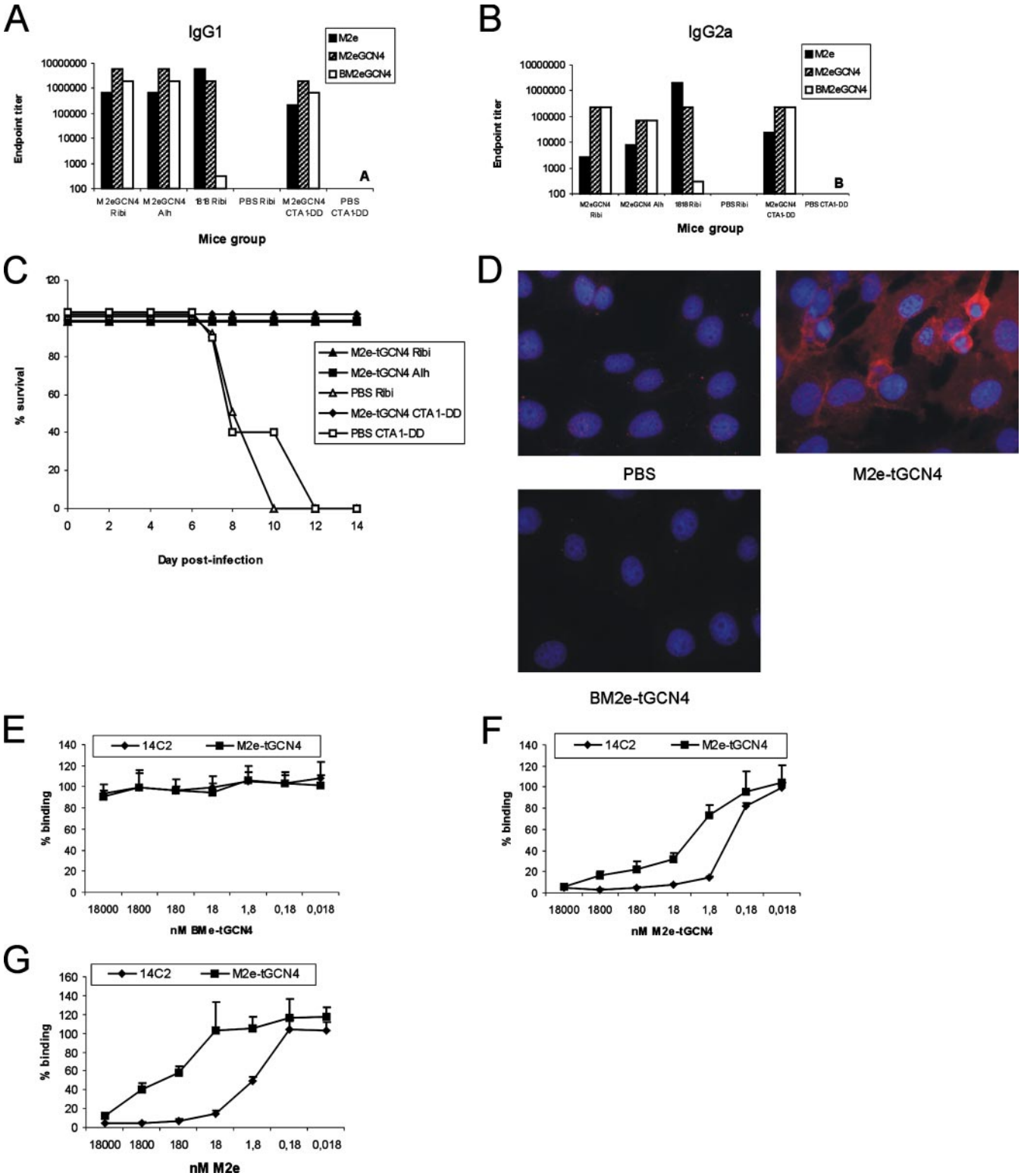

FIGURE 2. *A–C*, immunization with M2e-tGCN4. Five groups of 11 BALB/c mice were immunized with 10 μg of M2e-tGCN4 vaccine or PBS in the presence of Ribi adjuvant (intraperitoneal), Alhydrogel (*Alh*; intraperitoneal), or CTA1-DD (intranasal) as indicated. Three weeks after the boost, mice were challenged with 4 LD_{50} mouse-adapted X47 virus. *A* and *B*, IgG1 and IgG2a titers, respectively, of mice after the second boost. ELISA plates were coated with the M2e peptide, M2e-tGCN4, or BM2e-tGCN4 as indicated. *C*, mortality after challenge. *D*, M2e-tGCN4 immune serum specifically binds to native M2. Indirect immunofluorescent detection of the M2 ectodomain on the surface of infected MDCK cells is shown. MDCK cells were infected with influenza A strain X47, and 24 h later they were fixed in 4% formaldehyde for 10 min. Cells were stained with serum from PBS-treated mice (*upper left panel*), M2e-tGCN4 immune serum (*upper right panel*), or BM2e-tGCN4 immune serum (*lower panel)* followed by staining with goat anti-mouse IgG labeled with Alexa Fluor 594. *E–G*, competition of antibody binding to HEK-M2 in the presence of increasing concentrations of BM2e-tGCN4 (*E*), M2e-tGCN4 (*F*), or the M2e peptide (*G*). All plates contained HEK-M2 cell monolayers as target and were treated with either 100 ng/ml monoclonal antibody ^{14}C2 or 1:5000 dilution of M2e-tGCN4 immune serum. These dilutions of ^{14}C2 and M2e-tGCN4 immune serum have the same reactivity against HEK-M2 cells in ELISA. Decreasing concentrations of competitor were used to assess inhibition of binding expressed as percentage relative to binding in the absence of competitor. The results were obtained from experiments performed in quadruplicate.

the tetrameric M2 protein ectodomain. Combining M2e-tGCN4 with Ribi or Alhydrogel as adjuvant led to high IgG1 titers (Fig. 2) comparable with those obtained with the M2e-HBc vaccine described previously (6, 20). The titers of M2e-specific serum IgG2a were highest in the group immunized intranasally with M2e-tGCN4 plus CTA1-DD, in agreement with our previous results using the same mucosal adjuvant (21). Unlike for IgG1, the IgG2a titers were lower compared with the M2e-HBc vaccine. The induced immunity fully protected mice from a lethal challenge. The antibodies in the immune serum bound specifically to influenza A virus-infected MDCK cells and to M2-expressing HEK cells.

The M2e-specific monoclonal antibody ^{14}C2 binds to the M2e peptide and also inhibits viral replication in passively immunized mice (22). However, we anticipated that the tetrameric M2e domain presented to the immune system by M2e-tGCN4 would mimic the native M2 structure and would elicit not only antibodies binding to linear epitopes but also antibodies recognizing conformational epitopes. Using a competition ELISA with M2-expressing cells as target, we demonstrated that a substantial portion (*i.e.* the fraction noncompetable by the M2e peptide) of antibodies in the immune serum specifically recognize conformational epitopes on the ectodomain of native M2. Feng *et al.* (5) used a similar cell-based ELISA to study the specificity of M2e-directed antibodies present in immune serum. Interestingly, the same group had immunized mice with a multiple antigenic peptide vaccine presenting M2e as a monomer (24) and showed that anti-M2e serum IgG could bind specifically to M2-expressing cells and that binding was completely abolished in the presence of free M2e peptide. By contrast, antibody binding using sera from mice immunized by repeated infections was far less prone to competition by the M2e peptide in this assay with >60% of binding remaining even at the highest concentration of competing M2e peptide. However, serum antibody levels specific for M2e remain low in humans following infection or vaccination with licensed flu vaccines (5, 26, 27). Our results suggest that an M2e-tGCN4-based vaccine approach induces an immune response that resembles in quality the anti-M2e response following exposure to natural M2, but vaccination with M2e-tGCN4 induces much higher titers. Moreover, the antibody subtype response can be optimized by improvements in vaccine formulation and delivery protocol. In addition to the advantage of the strong conservation of M2e, a subfraction of antibodies specific to the M2e tetramer might also be able to block the proton channel function of M2 and in this way further contribute to protection against influenza A. Experiments are ongoing to test this possibility.

In summary, the tetrameric M2e-tGCN4 vaccine induces M2e-specific IgG antibodies that recognize the natural M2 ectodomain on infected or transfected cells and protects mice against a lethal influenza challenge. Additional experiments using an optimized M2e-tGCN4 construct, vaccine formulation, and delivery protocol are needed to evaluate the merit of this vaccine relative to that of the M2e-HBc vaccine documented previously (6, 20, 21). The correct assembly of oligomeric extracellular domains of membrane proteins by virtue of a heterologous oligomerizing domain attached to their C or N terminus leads to a surrogate structure that mimics the oligomeric ectodomain of a natural oligomeric protein. Such a convenient, simplified structure, when used as a vaccine, improves the quality of the humoral adaptive immune response by presenting conformational epitopes determined by the quaternary conformation of the complex.

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