Rapid Communication

Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface

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Abstract

The influenza B virus BM2 protein contains 109 amino acid residues and it is translated from a bicistronic mRNA in an open reading frame that is +2 nucleotides with respect to the matrix (M1) protein. The amino acid sequence of BM2 contains a hydrophobic region (residues 7–25) that could act as a transmembrane (TM) anchor. Analysis of properties of the BM2 protein, including detergent solubility, insolubility in alkali pH 11, flotation in membrane fractions, and epitope-tagging immunocytochemistry, indicates BM2 protein is the fourth integral membrane protein encoded by influenza B virus in addition to hemagglutinin (HA), neuraminidase (NA), and the NB glycoprotein. Biochemical analysis indicates that the BM2 protein adopts an $N_{out}C_{in}$ orientation in membranes and fluorescence microscopy indicates BM2 is expressed at the cell surface. As the BM2 protein possesses only a single hydrophobic domain and lacks a cleavable signal sequence, it is another example of a Type III integral membrane protein, in addition to M_2, NB, and CM2 proteins of influenza A, B, and C viruses, respectively. Chemical cross-linking studies indicate that the BM2 protein is oligomeric, most likely a tetramer. Comparison of the amino acid sequence of the TM domain of the BM2 protein with the sequence of the TM domain of the proton-selective ion channel M_2 protein of influenza A virus is intriguing as M_2 protein residues critical for ion selectivity/activation and channel gating (H^{37} and W^{41}, respectively) are found at the same relative position and spacing in the BM2 protein (H^{19} and W^{23}).
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Introduction

The *Orthomyxoviridae* family of viruses includes the genera Influenzavirus A and Influenzavirus B. Human influenza A virus was first isolated in 1933 (Smith et al., 1933), and in 1940 Francis (1940) detected a new type of influenza virus that had no antigenic relationship to influenza A virus which was called type B (Horsfall et al., 1940). Influenza B virus, unlike influenza A virus, does not undergo periodic major antigenic shifts in its major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). In part this may be due to the inability of influenza B

virus to undergo genetic reassortment with influenza A virus (Compans and Choppin, 1975) coupled with the absence of an animal reservoir for influenza B virus (Kilbourne, 1975). Morphologically, influenza A and B viruses are very similar, both exhibiting pleomorphic enveloped particles with surface glycoprotein spike projections.

At the biochemical level, influenza A and B viruses both have negative-stranded RNA genomes consisting of eight RNA segments. For influenza B virus the genome chain length totals 14,639 nucleotides, whereas for influenza A virus (A/PR/8/34) the genome totals 13,588 nucleotides. The difference in genome size is largely due to the fact that the 5′ and 3′ untranslated regions of influenza B virus are longer than those of influenza A virus. For both influenza A and B viruses RNA segments 1–3 encode the three polymerase proteins, PB1, PB2, and PA, RNA segment 4 encodes HA; RNA segment 5 encodes the nucleocapsid pro-

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tein (NP); RNA segment 6 encodes NA; RNA segment 7 encodes the matrix protein (M_1) and RNA segment 8 encodes two proteins NS1 and NEP that are translated from unspliced and spliced mRNAs using overlapping reading frames (reviewed in Lamb and Krug, 2001).

Despite these genome RNA segment coding similarities for proteins with similar functional properties, the genomes of influenza A and B viruses also differ in coding for additional proteins that distinguish the influenza A and B virus types. Most strains of influenza A virus (but not all) encode on RNA segment 2, using an overlapping reading frame, an 87 residue protein called PB1-F2, and the available evidence suggests PB1-F2 is involved in virus-induced apoptosis (Chen et al., 2001). For all influenza A viruses RNA segment 7, in addition to encoding the M_1 protein, also encodes the M_2 integral membrane protein (Lamb and Choppin, 1981; Lamb et al., 1985). The M_1 and M_2 proteins are translated from unspliced and spliced mRNAs, respectively, using overlapping reading frames (Lamb et al., 1981). The influenza B virus genome encodes two extra proteins. Influenza B virus RNA segment 6, in addition to coding for the B/NA protein, also encodes via a bicistronic mRNA and by using an overlapping reading frame the 100 residue NB glycoprotein (Shaw et al., 1983). Initiation of translation of NB occurs using an AUG codon positioned four nucleotides before the AUG codon used to initiate the NA protein (Williams and Lamb, 1989). Influenza B virus RNA segment 7 in addition to encoding the M1 protein encodes the BM2 protein (Horvath et al., 1990).

The influenza B virus BM2 protein is translated from an open reading frame that is +2 nucleotides with respect to the reading frame of the M1 protein and it is conserved in all isolates of influenza B virus. The BM2 protein contains 109 residues and in influenza B virus-infected cells the BM2 protein has an apparent M_r of 12–15 kDa (Horvath et al., 1990). A mutational analysis indicates that the BM2 protein initiation codon overlaps with the termination codon of the M1 protein in a translational stop-start pentanucleotide UAAUG and that expression of the BM2 protein requires termination of M1 synthesis adjacent to the 5′ end of the BM2 coding region; thus, termination of translation and the reinitiation event are tightly coupled (Horvath et al., 1990). Further characterization of the BM2 protein by Odagiri and co-workers indicates that it is posttranslationally modified by phosphorylation, that BM2 is incorporated into purified virions, and that the BM2 protein is localized to the cytoplasm (Odagiri et al., 1999).

When we examined the amino acid sequence of the BM2 protein using several protein prediction algorithms, we observed that BM2 residues 7–25 (ILSICSFILSALHF-MAWTI) formed a hydrophobic domain that could act as a transmembrane (TM) segment. We report here the results of cell biology experiments that indicated the BM2 protein has properties expected of an integral membrane protein and that BM2 is expressed at the surface of virus-infected cells with an $N_{out}C_{in}$ orientation.
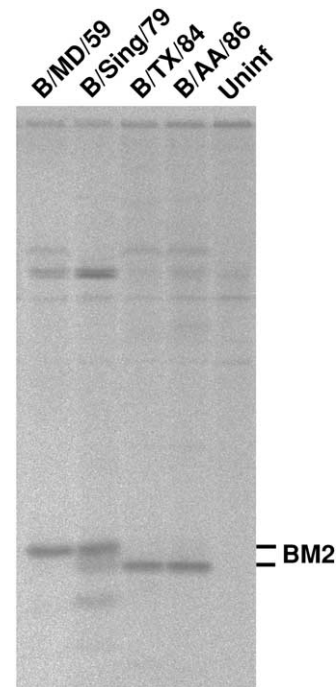

Fig. 1. Synthesis of BM2 protein in influenza B virus-infected cells. MDCK cells were infected with various strains of influenza B virus at a multiplicity of infection of 5 PFU per cell. At 7 h postinfection cells were metabolically labeled with Pro-mix L[^{35}S] in vitro cell-labeling mix (150 μCi/ml) in DMEM Met$^-$Cys$^-$ for 2 h. Cells were lysed in RIPA buffer; lysates were immunoprecipitated with anti-BM2 sera and polypeptides were analyzed by SDS–PAGE on a 17.5% acrylamide gel containing 4 M urea. The strains of influenza B virus used are indicated. Uninf = uninfected cell lysate.

Results

Influenza B virus BM2 protein has properties of an integral membrane protein

The expression in MDCK cells of the influenza B virus BM2 protein by various influenza B virus strains was confirmed by immunoprecipitating [^{35}S]methionine/cysteine-labeled cell lysates with rabbit antisera (anti-BM2) generated to a β-galactosidase–BM2 open reading frame fusion protein (Horvath et al., 1990). As shown in Fig. 1, BM2 ($M_r \sim 15$ kDa) was immunoprecipitated from virus-infected cells. BM2 protein exhibited strain-specific electrophoretic mobility differences which probably reflect amino acid sequence differences among influenza B virus BM2 proteins (approximately 85% conservation of sequence from B/Lee/40 (Briedis et al., 1982) to B/Shiga/98 (Lindstrom et al., 1999)). The multiple bands observed for B/Sing/79 BM2 may represent breakdown products of BM2. Although the anti-BM2 sera precipitated nonspecifically some other proteins, the sera was judged suitable for further analysis of the BM2 protein. Influenza B/MD/59 virus was selected as a virus strain for investigation of properties of the BM2 protein.

To test whether the BM2 protein had properties expected

of an integral membrane protein, a postnuclear supernatant from [^{35}S]methionine/cysteine metabolically labeled virus-infected cells was treated with detergent and high salt concentrations and was separated by centrifugation (150,000 g for 30 min) into a pellet and supernatant and aliquots were immunoprecipitated with HA (a known integral membrane protein) antisera or BM2 antisera. As shown in Fig. 2A, the solubility properties of BM2 paralleled those of HA in that neither protein was soluble after 0.5 M NaCl treatment; both HA and BM2 were soluble after 2% Triton X-100 treatment but the greatest extent of solubility was obtained after a combined treatment of 0.5 M NaCl and 2% Triton X-100. Another test to show that a protein is an integral membrane protein as compared to a peripherally associated membrane protein is to examine the solubility of the protein after pH 11.5 "alkali" treatment (Steck and Yu, 1973). As shown in Fig. 2B, after alkali treatment of a microsomal fraction derived from [^{35}S]methionine/cysteine metabolically labeled virus-infected MDCK cells, the BM2 protein was found predominantly in the pellet fraction, indicative that BM2 is an integral membrane protein. To show further that BM2 had properties expected of an integral membrane protein, a [^{35}S]methionine/cysteine metabolically labeled postnuclear supernatant derived from virus-infected MDCK cells was subjected to flotation centrifugation on Optiprep gradients (Lindwasser and Resh, 2001). As shown in Fig. 2C, regardless of the precise subcellular origin of each fraction, the bulk of HA and virtually all of BM2 were found in fractions of a density of 1.058 to 1.163 as expected for proteins associated with membrane fractions.

Determination using biochemistry that BM2 adopts an $N_{out}C_{in}$ orientation in membranes

The determination of the orientation of an integral membrane protein is facilitated by obtaining site-specific antisera to the N-terminal and C-terminal sides of the TM domain(s) (Lamb et al., 1985). However, for the BM2 protein the precise boundaries of the single BM2 TM domain are not known. Furthermore, the determination of the BM2 protein orientation is confounded by the fact that the number of N-terminal residues preceding the TM domain is small, likely ranging from four to seven residues. Thus, we resorted to surrogate markers to examine the orientation of BM2 in membranes and to infer the expression of BM2 at the cell surface. To provide an N-terminal domain marker we substituted the first four residues of BM2 (MLEP) with 25 residues (M2g) (MSNLTEVETPIRNEWGCRC-NDSSNP) derived from the ectodomain of a modified influenza A virus M_2 protein and we designated this protein M2gBM2 (Fig. 3A). The M2g domain provides a site for addition of N-linked carbohydrate (NLT), which we have shown previously is used in another protein, gM$_2$ (Sakaguchi et al., 1996), and the M2g domain provides the epitope for the 14C2 M_2-specific monoclonal antibody (MAb) (M2Tag). To provide a C-terminal marker the epitope
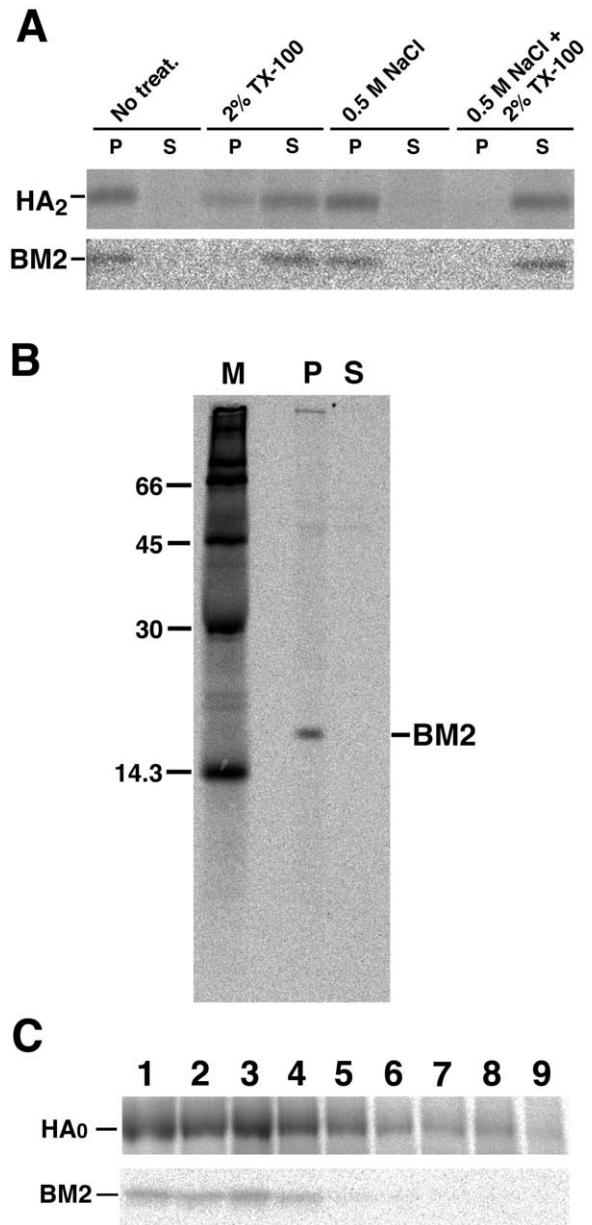

Fig. 2. BM2 protein has properties of an integral membrane protein. (A) Influenza B virus-infected MDCK cells were metabolically labeled as described in the legend to Fig. 1. A postnuclear supernatant was subjected to no treatment, 2% Triton X-100 treatment, 0.5 M NaCl treatment, or 0.5 M NaCl plus 2% Triton X-100 treatment. Pellet and supernatant fractions were made as described under Materials and Methods and proteins were immunoprecipitated with anti-HA sera or anti-BM2 sera. Polypeptides were analyzed by SDS–PAGE on a 17.5% acrylamide gel containing 4 M urea. P = pellet; S = supernatant. (B) The BM2 protein is resistant to extraction from microsomal membranes with alkali pH 11.5. Microsomes from metabolically labeled influenza B virus-infected cells were prepared and a pH 11.5 extraction performed. P = pellet; S = supernatant; M = marker polypeptides. (C) The BM2 protein is associated with the membrane fraction of cells. A cell homogenate from influenza B virus-infected MDCK cells (as above) was subjected to flotation centrifugation on Optiprep gradients. Nine fractions were collected and immunoprecipitated with anti-HA and anti-BM2 sera.
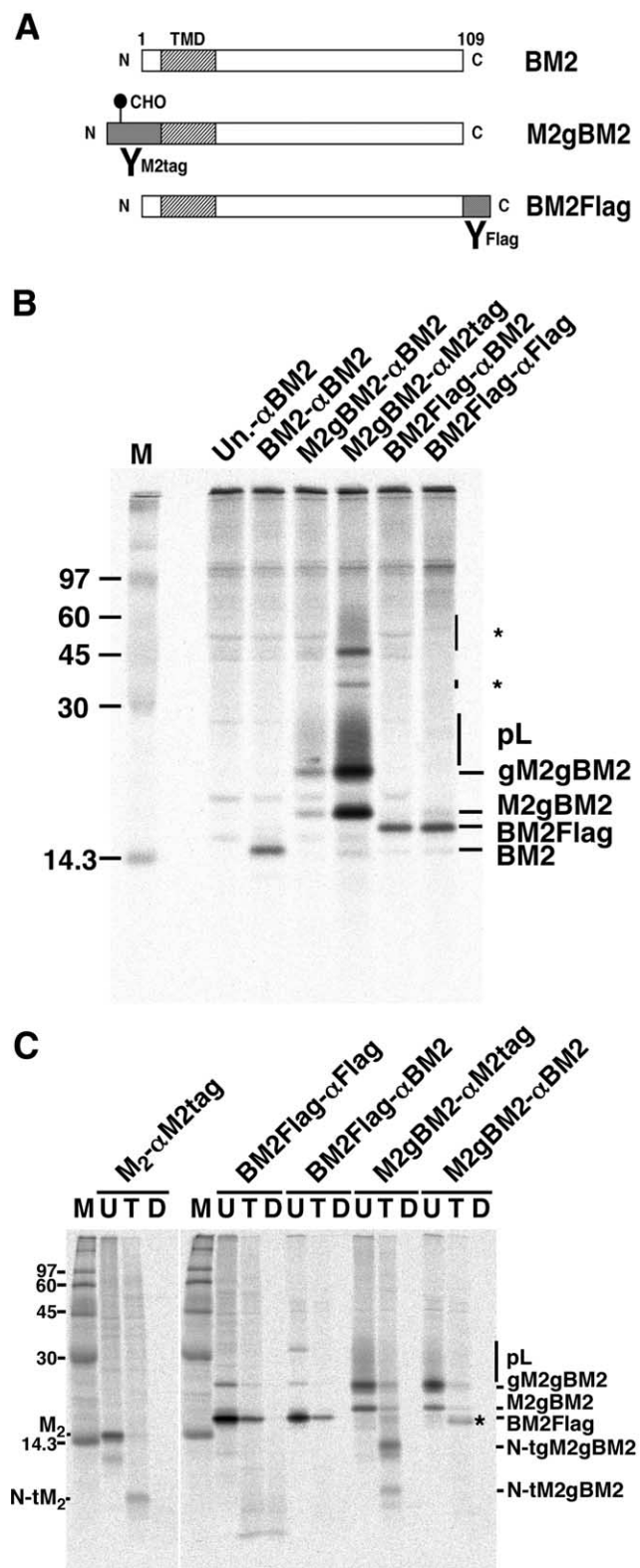
Fig. 3. Transient expression of BM2, M2gBM2, and BM2-Flag to determine the orientation of BM2 in membranes. (A) Schematic diagram to illustrate the recombinant DNA molecules constructed. M2gBM2 has the first four residues of BM2 (MLEP) substituted with 25 residues (MSNLTE-VETPIRNEWGCRCNDSSNP) derived from the ectodomain of a modified influenza A virus M_2 protein (M2g). The M2g domain provides a site for addition of N-linked carbohydrate (NLT) and provides the epitope for the

(DYKDDDDK) for the Flag MAb was added to the C-terminus of BM2 (BM2Flag) (Fig. 3A). The orientation of virtually all natural integral membrane proteins is absolute. However, for the artificial M2gBM2 protein a caveat that has to be considered is that because the region bordering the BM2 TM domain has been altered the chimeric molecule might adopt more than one orientation as observed in other artificial membrane proteins (Parks and Lamb, 1991, 1993). The addition of the Flag tag to the BM2 C-terminus is considered most unlikely to alter the BM2 orientation in membranes because it is located 84 residues distal from the TM domain. It is interactions of the TM domain with the endoplasmic reticulum translocation machinery that specify orientations.

BM2, M2gBM2, and BM2Flag were transiently expressed using a pCAGGS expression vector (Niwa et al., 1991) in 293T cells and cell lysates immunoprecipitated with anti-BM2 sera, MAb 14C2 (Zebedee et al., 1985) (anti-M2tag), or a MAb to the Flag epitope (anti-Flag). As shown in Fig. 3B the BM2Flag protein had a gel mobility consistent with addition of eight residues to its C terminus ($M_r \sim 17$ kDa). Three major species of M2gBM2 were observed after precipitation with either the BM2 sera or the M2tag sera (Fig. 3). The fastest migrating band had a gel mobility consistent with an unglycosylated M2gBM2 species ($M_r \sim 18$ kDa); the middle band (designated gM2gBM2) had a mobility consistent with addition of an N-linked carbohydrate chain to the protein ($M_r \sim 22$ kDa) and the third species was a "swarm" of heterogeneously migrating protein, designated pL ($M_r \sim 22$–30 kDa). We have shown previously for the related gM_2 protein which contains the M_2 ectodomain and glycosylation site that it migrates on SDS–PAGE as a heterogeneously migrating

14C2 M_2-specific monoclonal antibody (MAb) (M2Tag). BM2Flag contains the epitope (DYKDDDDK) for the Flag MAb added to the C-terminus of BM2. TMD = transmembrane domain; CHO = N-linked carbohydrate chain. (B) BM2, M2gBM2, and BM2-Flag were transiently expressed using the pCAGGS expression vector in 293T cells. At 18 h posttransfection cells were metabolically labeled with Pro-mix L[^{35}S] in vitro cell labeling mix (150 μCi/ml) in DMEM Met$^-$Cys$^-$ for 2 h. Cells were lysed and protein was immunoprecipitated with anti-BM2 sera, anti-M2Tag, or anti-Flag sera; polypeptides were subjected to electrophoresis on SDS–PAGE. Un = mock empty vector transfected cell lysate; pL = heterogeneously migrating gM2gBM2 species. (C) Protease treatment of microsomes. M2gBM2, BM2-Flag, and influenza A virus M_2 protein were transiently expressed in HeLa-CD4-LTR-β-gal cells and metabolically labeled as described above. A crude microsomal fraction was prepared and aliquots treated with trypsin or 1% Nonidet P-40 + trypsin as described under Materials and Methods. Microsomes were pelleted through a sucrose cushion, lysed, and immunoprecipitated with appropriate antisera as indicated. U = untreated microsomes; T = microsomes digested with trypsin; D = microsomes digested with trypsin in the presence of 1% Nonidet P-40; N-tM_2 = protease protected ectodomain and TM domain of influenza A virus M_2 protein; N-tg$M2gBM2$ = protease protected ectodomain and TM domain of gM2gBM2; N-t$M2gBM2$ = protease protected ectodomain and TM domain of M2gBM2; Asterisk = protease protected cytoplasmic tail and TM domain of M2gBM2; pL = heterogeneously migrating gM2gBM2 species; M = marker polypeptides.

protein and this is due to a polylactosaminoglycan addition to the high mannose carbohydrate chain (Sakaguchi et al., 1996), a modification which occurs in the Golgi apparatus. Species of greater mass (indicated by asterisks; $M_r \sim 44$–70 kDa) were also observed on occasion and these species may represent an SDS-resistant oligomer (dimer) of gM2gBM2 (Fig. 3B). A pulse-label and chase protocol indicated that the M2gBM2 species largely converted to gM2gBM2 and after a 90-min chase period, 80% of M2gBM2 was observed as the heterogeneously migrating protein (data not shown). These data suggest that the N-terminal domain that is modified by carbohydrate addition and recognized by the N-terminal specific M2tag antibody is lumenal.

To provide evidence that BM2 protein has a cytoplasmic tail and to further corroborate the orientation of BM2 in membranes, a crude preparation of microsomes from [^{35}S]metabolically labeled BM2Flag, M2gBM2, and influenza A virus M$_2$-expressing HeLa-CD4-LTR-β-gal cells was isolated, treated with trypsin, and pelleted through a sucrose gradient. A caveat that has to be added concerning the interpretation of these experiments is that due to the method of preparing microsomal membranes, a small percentage of vesicles reseal in their non-native orientation (Zebedee, 1988). As shown in Fig. 3C digestion with trypsin of microsomes expressing influenza A virus M$_2$ protein and immunoprecipitation with the M2tag antisera yielded a protease protected N-terminal fragment of 7.5 kDa as demonstrated previously (Zebedee et al., 1985). Essentially all M$_2$-related species were lost on trypsin treatment in the presence of 1% Nonidet P-40. Digestion of microsomes expressing BM2Flag with trypsin caused a loss of 91% of the radioactivity in the BM2Flag band when immunoprecipitated with either the anti-Flag or the anti-BM2 sera, a finding consistent with the large majority of BM2Flag molecules having ~84 C-terminal residues residing in the cytoplasm. Digestion of microsomes expressing M2gBM2 with trypsin and immunoprecipitation with the N-terminal-specific M2 tag antisera yielded protease-resistant fragments of 13 kDa (N-tgM2gBM2) and 8 kDa (N-tM2gBM2), masses consistent with these fragments containing the glycosylated and unglycosylated gM$_2$ ectodomain and the BM2 TM domain. Immunoprecipitation of trypsin-treated M2gBM2-expressing microsomes with anti-BM2 sera yielded a small amount of a fragment (Fig. 3C, asterisk) that is consistent with loss of the gM$_2$ domain and protection of the BM2 TM domain and cytoplasmic tail and this species most likely represents vesicles that resealed in the nonnative orientation. In all cases essentially all BM2-related species were lost on trypsin treatment in the presence of 1% Nonidet P-40. Taken together the biochemical data indicated that the bulk of the BM2 molecules are oriented in membranes in an N$_{out}$C$_{in}$ orientation.

BM2 is expressed at the cell surface

To examine the subcellular distribution of BM2 immunofluorescence microscopy was performed on Vero and HeLa CD4-LTR-β-gal cells that transiently expressed BM2,

M2gBM2, and BM2-Flag. The biochemical data shown above indicates that BM2 largely adopts an N$_{out}$C$_{in}$ orientation. Thus it can be predicted that fluorescence at the cell surface would only be detected for cells expressing M2gBM2 and staining with the M2tag antisera, whereas neither BM2-Flag nor BM2 would be detected by surface staining with the Flag-antisera or with the BM2 antisera, respectively. Lack of detection of BM2 at the cell surface with the BM2 antisera is predicted because an ectodomain of four to seven residues is likely too small a region to which an antibody would gain accessibility at the cell surface even should such a region be an antigenic epitope in the original SDS-denatured β-galactosidase–BM2 fusion protein antigen. In contrast the M2tag antisera, the Flag antisera, and the BM2 antisera would all be expected to show fluorescent staining after permeabilization of the plasma membrane with detergent. As shown in Fig. 4A for Vero cells, M2gBM2 was detected at the cell surface after staining with M2tag antisera, whereas neither BM2-Flag nor BM2 could be detected at the cell surface using anti-Flag sera or anti-BM2 sera, respectively. In permeabilized cells all three proteins could be detected with the antisera unique for the expressed protein. In permeabilized cells, particularly after staining with the M2tag or Flag antisera, the BM2 proteins showed extensive staining in a perinuclear region of the cell, characteristic of Golgi staining (see, for example, Hiebert et al., 1988; Sakaguchi et al., 1996; Zebedee et al., 1985). It is also interesting to note that when the anti-BM2 sera was used for intracellular staining, the characteristic Golgi staining pattern found when using the anti-M2Tag or anti-Flag antibodies was not observed. This antisera was raised against an SDS-denatured fusion protein. Although it could be argued that BM2 is the native protein whereas M2gBM2 and BM2-Flag are not native, the explanation for the subtle staining difference that we prefer is that the anti-BM2 sera only recognizes forms of BM2 in the endoplasmic reticulum and that the oligomerized (see below) and properly folded oligomer present in the Golgi apparatus is not well recognized by the BM2 antisera. Transient expression of M2gBM2 and BM2-Flag in the highly transfectable HeLa CD4-LTR-β-gal cells (Fig. 4B) showed similar staining patterns as found in Vero cells. The BM2 sera was found to give a high background in HeLa CD4-LTR-β-gal cells and thus was not used. To provide positive data for the expression of BM2-Flag at the cell surface, serial confocal images in the X-Y plane of HeLa CD4-LTR-β-gal cells transiently expressing BM2-Flag and M2gBM2 and, as a control for a known surface protein, the influenza A virus M$_2$ protein (A/M2), were reconstructed in the Z-plane (top to bottom of cell). To minimize the amount of intracellular BM2 related proteins in subcellular compartments, transfected cells were treated with cycloheximide (100 μg/ml) for 2 h prior to fixation to "chase" the cohort of newly synthesized molecules through the exocytic pathway to the cell surface. The M2gBM2 and A/M2 molecules were stained at the cell surface using the M2tag antibody,
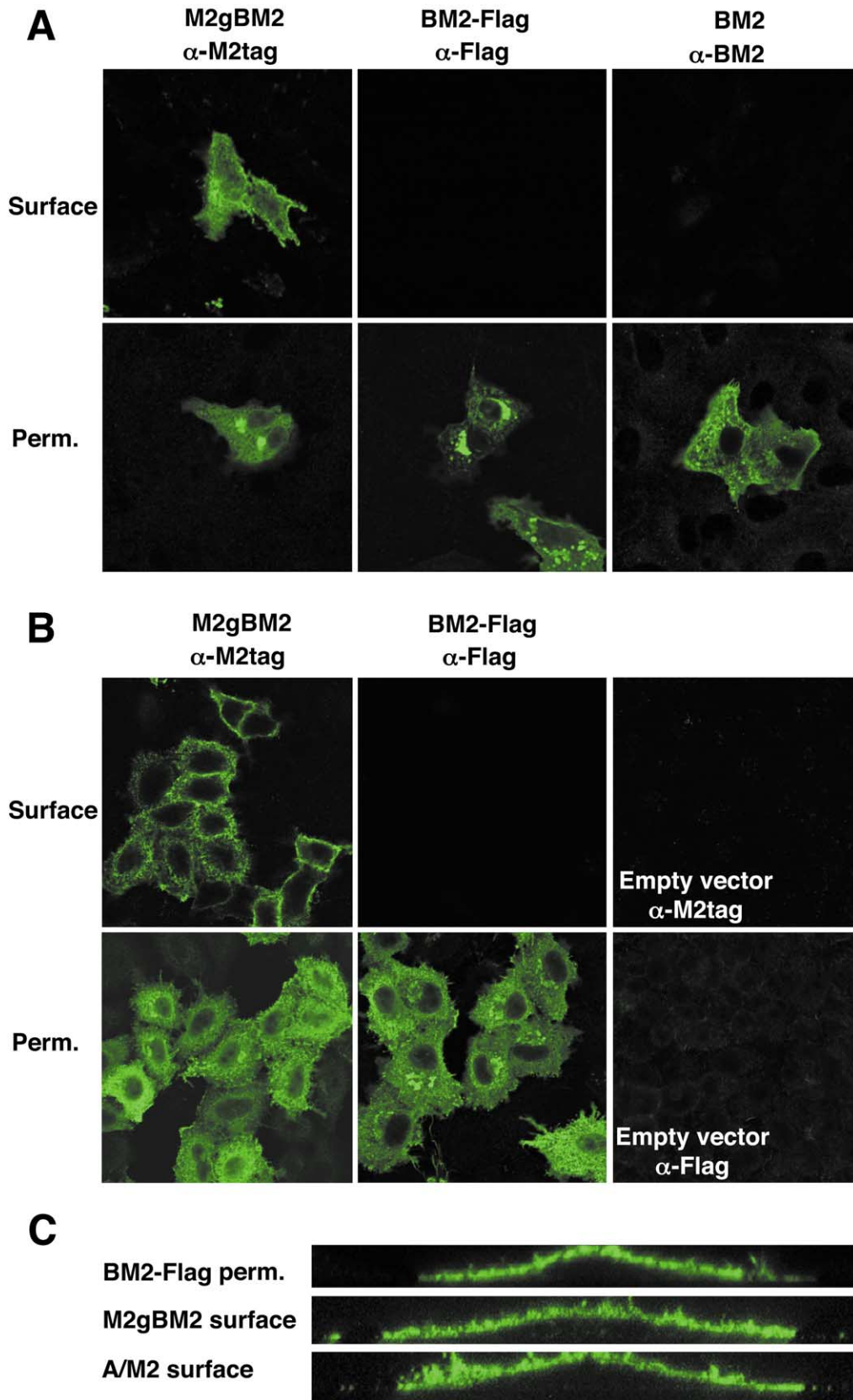
Fig. 4. Specific immunofluorescent staining of BM2, M2gBM2, and BM2-Flag indicates BM2 adopts an $N_{out}C_{in}$ orientation in membranes. Eukaryotic expression vectors expressing M2gBM2, BM2-Flag, BM2, and influenza A virus M_2 protein were transfected into cells and at 18 h posttransfection cells were fixed with 2% formaldehyde. For permeabilization (Perm.) cells were treated with 0.1% saponin. Cells were stained with primary antibodies as indicated. (A) Vero cells expressing M2gBM2, BM2-Flag, or BM2. The secondary antibody used was FITC-conjugated donkey anti-mouse IgG secondary antibody or FITC-conjugated goat anti-rabbit IgG secondary antibody as appropriate. (B) HeLa-CD4-LTR-β-gal cells expressing M2gBM2 or BM2-Flag. The secondary antibody used was Alexa Fluor 488 conjugated goat anti-mouse IgG secondary antibody. (C) HeLa-CD4-LTR-β gal cells expressing M2gBM2, BM2-Flag, or A/M_2 as in (B). Z-section reconstructions on serial 7 micron sections in the X-Y plane were calculated to show surface staining of the proteins.
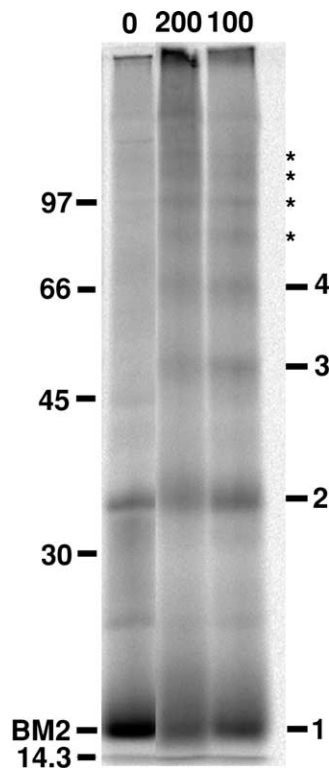
Fig. 5. BM2 is an oligomer. HeLa-CD4-LTR-β-gal cells transiently expressing BM2Flag were ^{35}S-metabolically labeled, removed from the culture dish, and treated with 0.2% Nonidet P-40 containing the bifunctional crosslinker 3,3′-dithiobis(sulfosuccinimidyl-propionate) (DTSSP) and polypeptides were examined by SDS–PAGE on 15% gels. 0 = no added crosslinker; 200 and 100 = 200 and 100 μM DTSSP.

whereas BM2-Flag was stained using the Flag antibody on detergent permeabilized cells. As shown in Fig. 4C, the Z-plane reconstructions showed that BM2-Flag was expressed at the cell surface.

Oligomeric form of BM2

Many integral membrane proteins form oligomers. Thus, to examine if BM2 formed oligomers, cells transiently expressing BM2Flag were ^{35}S-metabolically-labeled, removed from the culture dish, and treated with 0.2% Nonidet P-40 containing various concentrations of the bifunctional crosslinker 3,3′-dithiobis(sulfosuccinimidyl-propionate) (DTSSP) and polypeptides were examined by SDS–PAGE on 15% gels. As shown in Fig. 5, with no added cross-linker BM2 migrated as an ∼17-kDa species and as observed in Fig. 3 a small amount of a ∼34 kDa was observed and the mass of this species is consistent with that of an SDS-resistant dimer. On treatment with DTSSP higher oligomers of BM2 were observed (species 2–4) which are of mass of ∼34, 51, and 68 kDa and thus these data suggest the monomeric BM2 species forms covalent adducts that are dimers, trimers, and tetramers of BM2. Therefore, the most likely minimal native oligomeric form of BM2 is a tetramer.

It was also observed that crosslinking with DTSSP yielded a ladder of four minor species of higher masses (85–136 kDa) (indicated by asterisks) consistent with cross-linking of two tetramers (Fig. 5).

Discussion

The data reported here provide evidence that the influenza B virus BM2 is an integral membrane protein that is expressed at the cell surface. Similar to the influenza A virus M_2 protein (Lamb et al., 1985) and the influenza B virus NB glycoprotein (Williams and Lamb, 1986), the available evidence indicates that the vast majority, if not all, of the BM2 protein molecules adopt an $N_{out}C_{in}$ orientation. The M_2, NB, and BM2 proteins possess only a single hydrophobic domain and lack a cleavable signal sequence and proteins with these characteristics are classified as Type III integral membrane proteins (von Heijne, 1988; von Heijne and Gavel, 1988). Cross-linking studies suggest the most likely minimal native oligomeric form of BM2 is a tetramer.

The influenza A virus M_2 protein has a proton selective ion channel activity that is the target of the antiviral drug amantadine (Chizhmakov et al., 1996; Duff and Ashley, 1992; Lin and Schroeder, 2001; Mould et al., 2000a,b; Pinto et al., 1992; Sugrue and Hay, 1991). The M_2 proton channel functions during uncoating of virions in endosomes permitting protons to enter the virion and bringing about dissociation of protein–protein interactions, principally weakening those between the M_1 and nucleocapsid NP protein. In addition, the M_2 protein functions during its transport through the exocytic pathway. There the M_2 protein ion channel activity causes equilibration of pH of the lumen of the *trans*-Golgi network (TGN) with the cytoplasm (for reviews, see Hay, 1992; Lamb et al., 1994). The function of the BM2 protein in the life cycle of influenza B virus is currently unknown. Because the M_2, NB, and CM2 proteins of influenza A, B, and C viruses, respectively, are all small integral membrane proteins with the same orientation in membranes (reviewed in Lamb and Krug, 2001), it has been speculated by analogy to the known function of the influenza A virus M_2 protein that all these proteins have ion channel activity. The data in this article show that BM2 is the fourth integral membrane protein of influenza B virus, in addition to HA, NA, and NB. Furthermore, BM2 has the same membrane orientation as M_2, NB, and CM2.

The mature influenza A virus M_2 protein is a homotetramer (Holsinger and Lamb, 1991; Sugrue and Hay, 1991) of 96 residues and is oriented in membranes such that it has 23 N-terminal extracellular (or lumenal) residues, a 19 residue TM domain, and a 54 residue cytoplasmic tail (Lamb et al., 1985; Tobler et al., 1999). A considerable body of experimental evidence indicates that this TM domain constitutes the proteinaceous core (the channel pore) that allows a flux of protons across the membrane. M_2 protein TM domain histidine 37 is essential for both ion selectivity and
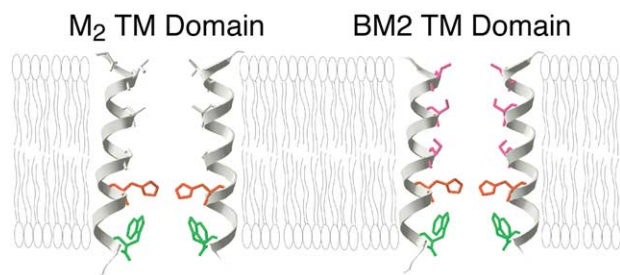
M$_2$ TM Domain   BM2 TM Domain

Fig. 6. Schematic diagram to indicate the conservation of the BM2 TM domain His and Trp residues as compared to residues in the influenza A virus M$_2$ proton-selective ion channel protein. Histidine residues are colored red; tryptophan residues are colored green and in BM2 the serine residues are colored magenta.

activation, the latter being caused by a low pH environment (pH$_{out}$) at the M$_2$ N-terminal ectodomain residues (Pinto et al., 1992; Wang et al., 1995). Furthermore, recent data indicate that gating of the M$_2$ ion channel is governed by a single side chain at residue 41 of the TM domain and that this property is mediated by an indole moiety (Tang et al., 2002). Unlike many ion channels where the gate is formed by a whole segment of a protein, the data suggested a model of striking simplicity for the M$_2$ ion channel protein, with the side chain of tryptophan 41 blocking the pore of the M$_2$ channel when pH$_{out}$ is high and with this side chain leaving the pore when pH$_{out}$ is low. Thus, the Trp[41] side chain acts as the gate that opens and closes the pore (Tang et al., 2002).

If the amino acid sequence of the BM2 TM domain region [7]ILSICSFILSALHFMAWTI[25] is modeled as an α-helix, then S[9], S[12], S[16], H[19], and W[23] could all form the same face of an α-helix and thus these residues could form the aqueous pore of an oligomeric ion channel. Remarkably, the two key residues for activation and gating (H[37] and W[41]) in the M$_2$ ion channel are found at the same spacing HXXXW (H[19] and W[23]) in the BM2 TM domain (Fig. 6). The demonstration that NB of influenza B virus when expressed in bacteria and reconstituted into planar membranes has an ion channel activity (Fischer et al., 2001; Sunstrom et al., 1996) is subject to artifacts (reviewed in Lamb and Pinto, 1997). Furthermore, the ion channel activity reported by Fischer and co-workers (2001) was reported to be blocked by amantadine (Fischer et al., 2001), yet amantadine does not inhibit the replication of influenza B virus. Thus, it seems possible that the BM2 protein will have an ion channel activity and this awaits to be determined using electrophysiological recordings. The very recently developed influenza B virus-specific reverse genetics methodology (Hoffman et al., 2002; Jackon et al., 2002) should help toward determining if the BM2 protein is essential for the replicative cycle of the virus.

Materials and methods

Cells and viruses

Vero, 293T, and MDCK cells were maintained in DMEM with 10% fetal calf serum (FCS). HeLa CD4-LTR-β-gal cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum, 200 μg/ml G418 (Genetecin, Invitrogen Inc., San Diego, CA), and 100 μg/ml hygromycin B (Invitrogen). These cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (HeLa-CD4-LTR-β-gal from Dr. Michael Emerman). Stocks of influenza B virus (strains B/MD/59, B/Singapore/79, B/Texas/84, and B/Ann Arbor/86) were grown in 10-day-old embryonated chicken eggs.

Plasmid construction

All plasmid constructs were based on the cDNA to RNA segment 7 of influenza B/Lee/40 virus whose cloning and sequencing has been previously described (Briedis et al., 1982; Horvath et al., 1990). The BM2 coding region was cloned from the entire segment 7 coding region by the polymerase chain reaction (PCR), using Vent DNA polymerase (New England Biolabs, Beverley, MA) or Tgo DNA polymerase (Roche Applied Sciences, Indianapolis, IN). The modified M2gBM2 and BM2-Flag molecules were made by standard 4-primer PCR reactions. The eukaryotic expression vector pCAGGS (Niwa et al., 1991) or pALTER (Promega Corp., Madison, WI) was used for transient expression of cDNAs. Details of all cloning steps are available upon request.

Infection of cells and metabolic radiolabeling

For biochemical experiments MDCK cells in 10-cm dishes were infected with influenza B/MD/59 virus at a multiplicity of infection of 5 PFU/ cell. At 7 h postinfection the cells were washed with DMEM without methionine and cysteine (DMEM, met$^-$cys$^-$), incubated in DMEM, met$^-$cys$^-$ for 30 min, and labeled for 2 h with 150 μCi/ml Pro-mix L[^{35}S] in vitro cell labeling mix (Amersham Biosciences, Piscataway, NJ). Following the labeling period the cells were washed with phosphate-buffered saline (PBS) and scraped off the dishes in 2 ml PBS and pelleted at 466 g for 5 min and stored at $-80°$C.

Antibodies

Antisera specific for influenza B/Lee/40 BM2 protein was raised in rabbits using as antigen a SDS-denatured BM2-β-galactosidase fusion protein (Horvath et al., 1990). Antibody to influenza B virus HA was obtained from the National Institutes of Allergy and Infectious Diseases Influenza Reagent Repository. The MAb 14C2 (M2tag) has been

described previously (Zebedee and Lamb, 1988). Anti-Flag MAb (M2) was obtained from Sigma-Aldrich (St. Louis, MO).

NaCl and Triton X-100 treatment of cell lysates

The cells from one 10-cm plate of B/MD/59-infected MDCK cells were resuspended in DHB (20 mM Tris–HCl pH 7.5, 1 mM MgCl$_2$), Dounce homogenized, and pelleted at 1000 g. The postnuclear supernatant was divided into 4 and either left untreated, adjusted to 0.5 M NaCl, 2% Triton X-100, or 0.5 M NaCl + 2% Triton X-100 incubated at 25°C for 10 min, and centrifuged at 150,000 g for 30 min at 4°C. The pellet was solubilized in RIPA buffer (0.15 M NaCl, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 0.01 M Tris–HCl pH 7.4). An equal volume of 2× RIPA buffer was added to the supernatant and the solubilized pellet and supernatant were centrifuged at 132,000 g for 10 min and immunoprecipitated with anti-BM2 or anti-B/HA sera.

Flotation gradient analysis of membrane fractions

A cell homogenate from one 10-cm plate of [^{35}S]methionine/cysteine metabolically labeled influenza B virus-infected MDCK cells was subjected to flotation centrifugation on Optiprep gradients (density 1.058 to 1.278) essentially as described (Lindwasser and Resh, 2001) using a Beckman SW60 rotor (4 h, 4°C, 170,000 g).

Preparation of microsomes and alkali treatment

Isolation of microsomes and treatment of microsomal membranes with alkali (pH 11.5) was performed as described previously (Parks et al., 1989; Paterson and Lamb, 1987). Trypsin treatment of microsomes was performed as described previously (Paterson and Lamb, 1987).

Transfections

293T, Vero, or HeLa-CD4-LTR-β-gal cells were transfected with pCAGGS BM2, pCAGGS M2gBM2, pCAGGS BM2-Flag, or pAlter A/M$_2$ using transfection reagent Trans IT LT-1 (Panvera, Madison, WI), Effectene (Qiagen, Valencia, CA), or Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturers' protocols. For metabolical labeling 6-cm-diameter dishes of 293T cells or HeLa CD4-LTR-β-gal cells were used. For immunofluorescence Vero cells or HeLa-CD4-LTR-β-gal cells were grown on glass coverslips.

Chemical cross-linking

HeLa-CD4-LTR-β-gal cells transiently expressing BM2Flag were ^{35}S-metabolically labeled, removed from the culture dish, and treated with 0.2% Nonidet P 40 containing various concentrations of the bifunctional crosslinker (DTSSP) as described previously (Russell et al., 1994). Polypeptides were examined by SDS–PAGE on 15% gels.

Immunofluorescence

At 18 h post transfection cell monolayers on coverslips were fixed with 2% methanol-free formaldehyde for 15 min and blocked with 1% bovine serum albumin (BSA) or 3% egg albumin in PBS for 30 min. For intracellular protein staining, all solutions contained 0.1% saponin (Sigma-Aldrich) to permeabilize cells. For surface staining cells were left unpermeabilized. Cells were incubated for 30 min with appropriate antibodies. Cells were washed four times in PBS and incubated for 30 min with Alexa Fluor 488 conjugated goat anti-mouse IgG secondary antibody (Molecular Probes Inc., Eugene, OR), FITC-conjugated donkey anti-mouse IgG secondary antibody, or FITC-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Fluorescence was observed using a Zeiss LSM 410 confocal microscope (Zeiss, Inc., Thornwood, NY) and Zeiss software.

Immunoprecipitation of polypeptides and SDS–PAGE

For immunoprecipitation cell pellets or gradient fractions were adjusted to 1× RIPA buffer containing 0.15 M NaCl, 50 mM iodoacetamide (IAA), 1 mM phenyl-methylsulfonyl fluoride (PMSF), and protease inhibitors (Paterson and Lamb, 1993). Lysates were clarified by centrifugation at 132,000 g for 15 min in a Beckman TLA 100.2 rotor and appropriate antibody aliquots were added to the supernatants. After 1–3 h incubation at 4°C immune complexes were then adsorbed to Protein A–Sepharose beads (Protein G beads for mouse MAbs) for 30 min at 4°C. The beads were then washed three times with RIPA + 0.3 M NaCl, twice with RIPA + 0.15 M NaCl, and once with 50 mM Tris (pH 7.4), 0.25 mM EDTA, 0.15 M NaCl. Samples were then resuspended in SDS–PAGE sample loading buffer [10% SDS, 1 M Tris (pH 6.8)] containing 2.5% dithiothreitol (w/v), boiled, and fractionated by SDS–PAGE on 17.5% gels containing 4 M urea (Lamb and Choppin, 1976). Visualization and quantification of radiolabeled protein bands were done using a Fuji BioImager 1000 and Image Quant v3.3 software (Fuji Medical System, Stamford, CT).

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