Targeting of the Sendai Virus C Protein to the Plasma Membrane via a Peptide-Only Membrane Anchor

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Several cellular proteins are synthesized in the cytosol on free ribosomes and then associate with membranes due to the presence of short peptide sequences. These membrane-targeting sequences contain sites to which lipid chains are attached, which help direct the protein to a particular membrane domain and anchor it firmly in the bilayer. The intracellular concentration of these proteins in particular cellular compartments, where their interacting partners are also concentrated, is essential to their function. This paper reports that the apparently unmodified N-terminal sequence of the Sendai virus C protein (MPFLKKILKLRGR...; letters in italics represent hydrophobic residues; underlined letters represent basic residues, which has a strong propensity to form an amphipathic α-helix in a hydrophobic environment) also function as a membrane targeting signal and membrane anchor. Moreover, the intracellular localization of the C protein at the plasma membrane is essential for inducing the interferon-independent phosphorylation of Stat1 as part of the viral program to prevent the cellular antiviral response.

Several cellular proteins are synthesized in the cytosol on free ribosomes and secondarily associate with membranes as a consequence of posttranslational modification of their ends. A fatty acid chain (myristic acid) is added via an amide linkage to an N-terminal glycine of proteins, such as Src and human immunodeficiency virus type 1 gag, to anchor these proteins to the inside surface of the plasma membrane (PM). At the protein’s other end, a prenyl group is attached via a stable thioether linkage to a cysteine that is initially located 4 residues from the Ras protein’s carboxy terminus, in a so-called CAAX domain. After this prenylation, the terminal 3 amino acids (aa) are cleaved and the new carboxy terminus is hydroxyl-methylated, thus similarly remodeling a hydrophilic protein terminus to one that is hydrophobic (2, 26, 29, 34). The distinct nature of the lipid modification and the heterogeneity of the types of membrane subdomains determine the strength of the membrane interaction of the modified protein as well as the specificity of membrane targeting. Clusters of basic residues can also synergize with the lipophilic group to promote membrane binding and targeting. In each case, this membrane association is essential to the protein’s function. This paper reports that the apparently unmodified N-terminal sequences of the Sendai virus (SeV) C protein also function as a PM targeting signal and membrane anchor.

The SeV C proteins are expressed from the viral P gene, which maximizes its genetic expression via overlapping open reading frames (ORFs) (21) (Fig. 1). P gene mRNAs contain 5 start codons near their 5′ end, four of which are used for a nested set of “C” proteins, which initiate at ACG81 (C′, 215 aa), AUG114 (C, 204 aa), AUG183 (Y, or C24–204, 182 aa), and AUG201 (Y2, 176 aa) and terminate at UAA728. The second start codon, AUG104, initiates 3 proteins (P, V, and W) as a consequence of cotranscriptional mRNA editing (21). For SeV, ACG81, AUG114, and AUG183 are initiated by ribosomes which linearly scan from the mRNA 5′ end, whereas AUG183 and AUG201 are initiated by a form of ribosomal shunting (23). The SeV P gene thus expresses at least 7 primary translation products including the P protein, an essential subunit of the viral RNA polymerase. SeV that cannot express any of the C proteins are at the limit of viability, and those that can only express Y1 and Y2 are clearly viable but highly debilitated (they produce pinhole plaques and are avirulent in mice) (19, 22). This requirement for the C proteins in virus replication is due in part to their ability to regulate viral RNA synthesis in a promoter-specific fashion (39). The other important function of C is to counteract the innate immune response of the host cell, primarily by interacting with signal transducer and activator of transcription 1 (Stat1). Stat1 is activated via phosphorylation of Y701 at the PM by the Jak kinases associated with the interferon (IFN) receptors. This activation is essential for both IFN-α/β and IFN-γ signaling that induces an antiviral state (37).

SeV which specifically cannot express the longer C proteins cannot establish a respiratory infection in mice (22). Successful infection of mice requires multiple rounds of virus replication, which requires SeV to replicate in cells primed by paracrine IFN. SeV must thus not only prevent the establishment of an antiviral state in the infected cells but also must dismantle the preexisting antiviral state due to paracrine IFN. SeV which cannot express the longer C proteins appear to be defective in this latter property, as follows. Vesicular stomatitis virus (VSV), which is highly IFN sensitive, will not grow in mouse embryo fibroblasts, which constitutively secrete IFN. SeV, in contrast, is relatively IFN resistant and grows in these cells, and at 18 h post-SeV infection, these cells become increasingly permissive for VSV replication (12). SeV which cannot express the longer C proteins, however,
A hydrophobic environment. When C1–23 or C1–11 is fused to but has a strong propensity to form an amphipathic the major C protein is predicted to be intrinsically disordered bold represent hydrophobic residues) (11). This peptide within domains of the C1–204 protein (C1–23 and C24–204 or Y1), are indicated. A summary of the activities of the domains is included on the left. indep., which specifically destroy the the GFP fusion protein and that of Stat1 as well. Mutations also block IFN signaling like SeV wild type (wt) (Y1/Y2 are suf- cient to affect its IFN signaling, but they do not restore VSV replication. Moreover, the virulence of these mutant SeV is highly attenuated in mice (9). The longer C proteins thus appear to be specifically required to dismantle the preexisting IFN-induced antiviral state.

The N-terminal halves of the longer C proteins are predicted to be intrinsically disordered (PONDRI) and presumably conformationally adaptable (11). C is known to interact with other viral and cellular proteins in addition to Stat1, and its interaction with Stat1 itself is also complex. (i) All four SeV C proteins bind Stat1 in pull-down assays, and their cellular expression inhibits IFN-induced Stat1 activation (i.e., phosphorylation, measured 45 min post-IFN treatment). Activated Stat1 is phosphorylated on tyrosine701; for simplicity, this is referred to as p-Stat1. (ii) The longer C proteins (but not the shorter Y proteins) paradoxically induce p-Stat1 formation (i.e., phosphorylation) in an IFN-independent manner throughout the course of the infection (p-Stat1 is formed in the absence of IFN, and even in cells that cannot respond to IFN) (10). (iii) The longer C proteins (but not the shorter Y proteins) also induce bulk Stat1 loss. This loss does not require phosphorylation of Tyr701 of Stat1 (10). C1–23 alone (fused to the N terminus of green fluorescent protein [GFP]) is also sufficient for this activity.

The ability of the longer C protein to induce Stat1 loss maps to the first 11 residues of C1–204 (MPSFLKILKL . . . ; letters in bold represent hydrophobic residues) (11). This peptide within the major C protein is predicted to be intrinsically disordered but has a strong propensity to form an amphipathic α-helix in a hydrophobic environment. When C1–23 or C1–11 is fused to the N terminus of GFP, these peptides induce the instability of the GFP fusion protein and that of Stat1 as well. Mutations which specifically destroy the α-helical potential (MPSFLKKPKL . . . [P PP]) or reduce the hydrophobicity of the peptide without affecting its α-helical potential (MPSaAkkAAkA . . . [45]) eliminate the ability of these N-terminal sequences to induce Stat1 loss in trans. This paper presents evidence that (i) the N-terminal 23 residues of C1–204 also act as a membrane tar-
For luciferase assays, 2TGH cells were transfected with 1 μg of the various constructions, 1 μg of plSRE(luc), 0.3 μg of pTK(luc), and 6 μl of Fast Green (Roche). At 30 h posttransfection (hpt), the cells were treated with 1,000 IU of IFN-β (Serono) per ml or left untreated. At 18 h post-IFN treatment, the cells were harvested and assayed for firefly and Renilla luciferase activity (Promega). Relative expression levels were calculated by dividing the firefly luciferase values by the Renilla luciferase values.

**Immunoblotting.** Cytoplasmic extracts were prepared using 0.5% NP-40. Equal amounts of total proteins (Bradford) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes by semidry transfer. The primary antibodies used included a rabbit anti-RED and -TOM (A32316; Chemicon), mouse monoclonal anti-Stat1 (C-terminus, 22120; Transduction Laboratories), rabbit anti-phospho-Stat1 (Y701, 06-657; Upstate Biotechnology), rabbit anti-phospho-Stat2 (Y869, 07-242; Upstate Biotechnology), rabbit anti-activin (provided by G. Gabbiani, Geneva, Switzerland), anti-GFP (Clontech), and rabbit anti-transferrin receptor (ZYMED). The secondary antibodies used were alkaline phosphatase-conjugated goat anti-rabbit (or mouse) immunoglobulin G (Bio-Rad). The immobilized proteins were detected by light-enhanced chemiluminescence (Pierce), and the results were quantified in a Bio-Rad light detector using Quantity One software (Bio-Rad).

**Confocal microscopy of TOM and GFP proteins.** Six-well plates containing glass coverslips were seeded with 10^4 2fTGH cells and transfected the following day with 2 μg of plasmid DNAs expressing TOM and GFP fluorescent proteins. At 24 hpt, the cells were fixed (3% formaldehyde, 0.3% Triton X-100), and their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were then mounted on glass slides. Images were acquired using a 100× objective lens in a Zeiss LSM 510 Meta confocal microscope.

**Membrane flotation gradient centrifugation.** Ten-centimeter plates of transfected 2TGH cells were harvested 48 hpt and washed in cold phosphate-buffered saline. Intact cells were pelleted at 500 g for 5 min (4°C) and barely resuspended with a Pasteur pipette in 2.5 ml of HB (8.5% sucrose, 0.02% imidazole). The cells were pelleted at 1,200 g for 10 min (4°C), resuspended, and lysed in 300 μl of HB by repeated passages in a 1-ml syringe and a 22-gauge needle (10 times). Cell lysates were centrifuged at 1,000 g for 10 min (4°C) to remove unbroken cells and nuclei. The postnuclear supernatant (PNS) was then mixed with 300 μl of 60% (wt/wt) sucrose and placed at the bottom of a centrifuge tube, which was then filled with HB. The gradients were centrifuged at 55,000 rpm for 2 h at 4°C in a Sorvall SS35 rotor (Sorvall RC M150 GX centrifuge). Three fractions were collected from the top of the centrifuge tube, and their proteins were precipitated with 6% trichloroacetic acid, 0.02% deoxycholate. The amount of RED, actin, and transferrin receptor present in each fraction was determined by immunoblotting.

**RESULTS**

**Biological activity of tagged C proteins.** C^{1–204}, the major C protein, appears to be composed of two domains (C1–23 and C24–204) that can also act independently. We therefore investigated whether a fluorescent reporter protein could be placed between these two domains without affecting their anti-IFN activity. The anti-IFN effects of the SeV C proteins can be seen by transfecting highly IFN-competent 2TGH cells with various C protein constructs, along with either 3× FLAG-Stat1 and -2 (to follow transfected Stat activation), or IFN-stimulated response element (ISRE)-promoted luciferase reporter plasmids to follow IFN-stimulated gene (ISG) expression. The cells were treated (or not) with 1,000 IU of IFN-β for 18 h at 30 hpt, and then the expression of the reporter gene was determined. Alternatively, the cells were similarly IFN treated at 48 hpt, and the levels of p-Stat1 and p-Stat2 (phospho-Tyr^{695}-Stat2) were determined 45 min later. The C protein constructs used were based on a red fluorescent protein carrier (tdTomato [36], or TOM) on which C^{1–23} was fused to the N terminus of TOM and C^{24–204} (or Y_{1,2}) was fused to its carboxy terminus. As shown in Fig. 2, fusion of C^{24–204} to the carboxy terminus of TOM (TOM-C^{24–204}) reduces IFN-induced p-Stat1 and p-Stat2 formation to ca. 15% or less of the unmodified TOM control and similarly reduces signaling to plSRE-luc. The additional presence of C^{1–23} fused to the N terminus of TOM-C^{24–204} enhances the inhibition of IFN-induced p-Stat2 and IFN signaling, such that only background levels of both were induced by IFN-β treatment. The placement of TOM between the two domains of C^{1–204} thus does not inhibit its anti-IFN activity. This is consistent with the notion that these two domains act independently, and tethering them to TOM rather than to each other has little effect.

The additional presence of C^{1–23} fused to the N terminus of TOM-C^{24–204} also recapitulates the paradoxical formation of p-Stat1 induced by C^{1–204} expression in the absence of IFN treatment, which reaches 40% of the level induced by a saturating amount of IFN-β (Fig. 2, top). In the normal course of events, IFN-α/β binding to its cell surface receptor leads to p-Stat2 formation, followed by the recruitment and phosphorylation of Stat1 at Tyr^{701}. A p-Stat1/p-Stat2 dimer then forms at the PM, which migrates to the nucleus and activates expression of ISGs (35, 37). During SeV infection, however, p-Stat1 levels increase throughout the infection even though IFN-induced
p-Stat1 formation is inhibited (when measured 45 min after IFN treatment) (10). SeV C-induced p-Stat1 formation does not lead to ISG expression, at least in part because IFN-induced p-Stat2 formation is also inhibited by C (13). IFN-independent p-Stat1 formation is the only activity of the three listed above that requires both the Y module that can bind Stat1 and C1–23 (Fig. 1).

C1–23 targets the plasma membrane of yeast and animal cells. While investigating whether C 1–23 fused to the N terminus of GFP also induced GFP instability in S. cerevisiae (the opposite result was found) (Fig. 3A), we noticed that C1–23 caused GFP to localize at what appears to be the inside surface of the PM (Fig. 3A, middle). The outside surface of these cells could be removed without disturbing the ring-like appearance of the yeast (data not shown). GFP carrying the φ5 mutant peptide (F4L5I8L9L11 all mutated to A), were expressed in S. cerevisiae (A) or 2fTGH cells (B). 2fTGH cells were also infected with either SeV in which a GFP-C1–204 fusion protein is the only C protein expressed (its normal C gene is closed) (panel c, right) or with otherwise wt SeV expressing an unmodified GFP transgene similarly placed between the M and F genes (panel c, left). The yeast cells were photographed in a simple fluorescent microscope. The 2fTGH cells were examined in a confocal microscope (see Materials and Methods). The photos of the different GFP fusion proteins were exposed for different times to compensate for the relative stabilities of the various fusion proteins (11).

\[ \text{FIG. 3. Intracellular location of C}^{1-23}\text{-GFP in yeast and animal cells. GFP, GFP with C}^{1-23}\text{fused to its N terminus (C}^{1-23}\text{-GFP), or GFP carrying the φ5 mutant peptide (F}^{4L}^{4L}^{5L}^{5L}^{8L}^{11} \text{all mutated to A), were expressed in S. cerevisiae (A) or 2fTGH cells (B). 2fTGH cells were also infected with either SeV in which a GFP-C}^{1-204}\text{fusion protein is the only C protein expressed (its normal C gene is closed) (panel c, right) or with otherwise wt SeV expressing an unmodified GFP transgene similarly placed between the M and F genes (panel c, left). The yeast cells were photographed in a simple fluorescent microscope. The 2fTGH cells were examined in a confocal microscope (see Materials and Methods). The photos of the different GFP fusion proteins were exposed for different times to compensate for the relative stabilities of the various fusion proteins (11).} \]

Biochemical evidence for C1–23 association with cellular membranes. Several cellular proteins are targeted to the inner surface of the PM due to the presence of short peptide sequences which act as targeting signals (30). H-Ras and K-Ras, for example, are highly homologous PM-associated proteins involved in cell signaling, which generate distinct signaling outputs in vivo. This difference in signaling is due to their different carboxy-terminal CAAX domains that mediate PM association (26). H-Ras and K-Ras are both farnesylated at
their processed carboxy-terminal cysteine, creating a highly hydrophobic carboxyl end. However, the complete membrane anchor of H-Ras includes two palmitoyl groups, whereas that of K-Ras has a polybasic domain that includes six contiguous lysines. The Ras isoforms are thought to generate distinct signals because they are localized to distinct domains of the PM. When fused to the carboxy terminus of GFP (GFP-tH and GFP-tK, respectively), the H-Ras and K-Ras CAAX domains target GFP to distinct PM surface domains (32).

We presume that the targeting of the C protein to the PM is required for its function(s) and that C1–23 serves essentially to concentrate the Stat1-interacting Y module at this intracellular location. We therefore (i) compared the ability of C1–23 fused to the N terminus of DsRed (RED) to associate with cellular...
membranes with that of the K-Ras CAAX domain (tK) fused to the carboxy terminus of RED (C1–23-RED versus RED-tK) and (ii) examined the ability of the K-Ras CAAX domain to retarget the mutant H9021/5 construct to the PM (by cell fractionation and centrifugation) (42). Unlike TOM, which is a tandem dimer with one set of termini, RED forms a stable tetramer with 4 amino and carboxy termini (36) and should associate more stably with membranes. The transferrin receptor was monitored as a marker for integral membrane proteins (Fig. 4). When unmodified RED (data not shown) or one carrying the inactive H9021/5 peptide (H9021/5-RED) is expressed in 2fTGH cells, 60% of RED is found in the pellet fraction and only 10% is found at the lightest (membrane) positions of the flotation gradient (Fig. 4A and B). The addition of wt C1–23 to RED (C1–23-RED) (Fig. 4) or tK to RED (data not shown) led to only 10% of the protein being present in the pellet fraction, and 60% or more was now found in the flotation fraction. The simultaneous addition of both C1–23 and tK (C1–23-RED-tK) (Fig. 4) did not improve the membrane association. However, addition of tK to H5-RED (H5-RED-tK) (Fig. 4) almost fully restored the membrane association. In all cases, expression of the various RED constructs did not affect the membrane association of the transferrin receptor (80% of which was routinely found in the flotation fraction) (Fig. 4B), and this serves as an internal control for the fractionation. Thus, C1–23 fused to the N termini of RED is as effective in associating the marker protein with cellular membranes by this biochemical test as the membrane anchor of K-Ras. Mutation of the 5 large hydrophobic residues of MPSFLKminoK (letters in bold represent hydrophobic residues) to Ala (H5) eliminates this membrane association.

We note that C1–23 does not contain an N-terminal glycine or any cysteines, and the free N terminus itself is unlikely to play a role, as GFP-C1–204 is also concentrated at the PM (Fig. 3C); hence, C1–23 apparently functions without the aid of a lipid group.

**C/Stat1 colocalization at the plasma membrane.** C-induced pY-Stat1 formation requires both C1–23 that targets the PM, and the Y module which interacts with Stat1. As TOM fluorescence matures 10 times more quickly and is more intense than that of RED (36), we examined whether C1–23-TOM-C24–204 can also induce Stat1 colocalization at the PM. TOM-C24–204 constructs carrying various N-terminal peptides were transfected into 2TGH cells along with either unmodified GFP as a negative control (Fig. 5, panels a and c) or GFP fused to the carboxy terminus of Stat1 (panels b and d). Their intracellular
Localization was then examined by confocal microscopy 24 h post-transfection (hpt).

As shown in Fig. 5a, when GFP and (N-terminally empty) TOM-C24–204 were coexpressed, both proteins were found throughout the cell and neither was concentrated at the cell periphery. Similar results were found when TOM-C24–204 was coexpressed with Stat1-GFP (Fig. 5b), except that Stat1-GFP is less concentrated in the nucleus than unmodified GFP, presumably because this much larger fusion protein does not contain a nuclear localization signal. When C1–23-TOM-C24–204 and GFP are coexpressed (panel c), TOM is now localized at the cell periphery and at perinuclear endomembranes, whereas GFP remains distributed throughout the cell, but GFP is not concentrated at the periphery. However, when C1–23-TOM-C24–204 and Stat1-GFP are coexpressed (Fig. 5d), both fusion proteins are concentrated at the cell periphery.

**FIG. 6.** C1–23 of C1–23-TOM-C24–204 can be replaced with carboxy-terminal H- or K-Ras CAAX domains for colocalization with Stat1-GFP at the PM. Plasmids expressing P8P9-TOM-C24–204 (a and b) or P8P9-TOM-C24–204 in which the H- or K-Ras CAAX domains were fused to the carboxy termini of C24–204 (P8P9-TOM-C24–204-tH [c and d] and P8P9-TOM-C24–204-tK [e and f]) were transfected into 2TG cells along with either GFP or Stat1-GFP as indicated on the left. The transfected cells were examined by confocal microscopy 24 h post-transfection.
proteins are clearly colocalized at the cell periphery. This colocalization does not occur when either the Δ5A or P8P9 peptide is fused to TOM-C24–204 (Fig. 6a and b; also data not shown). The addition of wild-type (wt) C1–23 to TOM-C24–204 also appears to concentrate the red marker protein in perinuclear, punctate structures which may be endomembranes, but Stat1-GFP is not colocalized with C1–23-TOM-C24–204 in these perinuclear structures (Fig. 5d).

We also examined whether P8P9-TOM-C24–204, which is unable to target the PM, can be retargeted to the PM by the addition of wild-type (wt) C1–23 to TOM-C24–204. This was done by transfecting 2F-TG cells with plasmids expressing 3X-flag-Stat1 in combination with TOM, TOM-C24–204, C1–23-TOM-C24–204, or P8P9-TOM-C24–204. The addition of C1–23 to TOM-C24–204 resulted in the formation of a new band corresponding to Stat1, indicating that the Stat1 protein is now targeted to the PM. This result is consistent with the hypothesis that the addition of C1–23 to TOM-C24–204 allows the protein to gain PM targeting capability.

![Graph and images showing colocalization and targetting results](attachment:image.png)

**FIG. 7.** C1–23 of C1–23-TOM-Y1 can be replaced with the H- or K-Ras CAAX domains for IFN-independent pY701-Stat1 formation. (A) Plasmids expressing TOM, TOM-C24–204, C1–23-TOM-C24–204, or P8P9-TOM-C24–204 or the same constructs in which the H- or K-Ras CAAX domains (H/K) were fused to the carboxy termini of TOM or C24–204 (as indicated) were transfected into 2F-TG cells along with 3X-flag-Stat1. Cell extracts were prepared at 48 hpt, and equal amounts of total cell proteins were examined by Western blotting for their levels of bulk 3X-flag-Stat1 (Stat1), p-3X-flag-Stat1 (p-Stat1), actin, and TOM proteins. The p-Stat1 levels (normalized to actin) are reported as a bar graph below. As a positive control, TOM-transfected cells were treated with 1,000 IU of IFN-γ at 48 hpt and harvested 45 min later (IFN Ctrl); the endogenous p-Stat1, which is visible here, is indicated. This level of p-3X-FLAG-Stat1 was set to 100. The results shown are representative of three separate experiments. All the immunoblots were exposed for the same time. (B) Plasmids expressing TOM, C1–23-TOM-C24–204, C1–23-TOM, TOM-C24–204, or a mixture of the latter two constructs, as indicated, were transfected into 2F-TG cells along with 3X-flag-Stat1. For further details, see legend to panel A. The symbols beside the various TOM bands (lower panel) refer to the constructs listed below the lane numbers.
addition of tH or tK to the carboxy terminus of this double-fusion protein. As shown in Fig. 6a, when PpP9-TOM-C24–204 and GFP are coexpressed, both proteins were found throughout the cell, but neither was concentrated at the cell periphery. Similar results were found when PpP9-TOM-C24–204 was coexpressed with Stat1-GFP (Fig. 6b). When PpP9-TOM-C24–204tH (Fig. 6c) or PpP9-TOM-C24–204tK and GFP are coexpressed (Fig. 6e), TOM is now localized at the cell periphery, whereas GFP remains distributed throughout the cell. PpP9-TOM-C24–204 can thus be retargeted to the PM by adding either the H-Ras or K-Ras CAAX domain to its carboxy terminus. More importantly, when PpP9-TOM-C24–204tH or -tK and Stat1-GFP are coexpressed (Fig. 6d and f), both fusion proteins are again colocalized at the cell periphery. The Y module of PpP9-TOM-C24–204tK can thus interact with Stat1 and lead to its concentration at the PM even when this localization is due to the membrane anchor of K-Ras. These results suggest that C1–23 functions essentially as a membrane anchor that tethers the Y module to the PM.

The C1–23 PM targeting signal of the C protein can be functionally replaced by that of H- or K-Ras. We also examined whether C1–23 could be replaced by tH or tK for IFN-independent p-Stat1 formation. Various TOM constructs were transfected into 2TGH cells along with 3× FLAG-Stat1 (to distinguish the transfected from the endogenous Stat1), and their p-Stat1 levels were determined at 48 hpt (Fig. 7). As a positive control, these levels were compared to that induced by treatment with 1,000 IU of IFN-β of cells transfected with unmodified TOM, 45 min post-IFN treatment, and this value was set to 100 (IFN ctrl). As shown in Fig. 7A, neither TOM (lane 1), TOM-C24–204 (lane 4), nor 5p-TOM-C24–204 (lane 10) induced transfected p-Stat1 formation above background levels, whereas C1–23-TOM-C24–204 (lane 7) induced p-Stat1 levels to ca 60% of the reference maximum (IFN ctrl). This p-Stat1 formation required the presence of C1–23 and C24–204 on the same TOM polypeptide, as p-Stat1 formation remained essentially at background levels when these two domains were expressed on separate TOM proteins (Fig. 7B). When tH and tK were fused to the carboxy termini of the inactive TOM, these carboxy-terminal Ras protein anchors were unable to rescue the activity of unmodified TOM (lanes 2 and 3). However, these Ras protein anchors clearly rescued in part the activity of TOM-C24–204 (lanes 5 and 6) and 5p-TOM-C24–204 (lanes 11 and 12). The Y module (C24–204) can thus function in IFN-independent p-Stat1 formation when targeted to the PM by either C1–23 or tH and tK. This suggests that C1–23 acts essentially as a PM targeting signal for this activity of C1–204. In contrast, when tH and tK are fused to the carboxy terminus of C1–23-TOM-C24–204, both Ras membrane anchors now reduce the activity of the pseudo-wt protein (lanes 8 and 9). Although we cannot exclude that in some cases the reduced activity is the result of decreased TOM expression, this apparent competition between the C1–23 and the Ras anchors is consistent with the notion that the various PM targeting signals, tH, tK, and C1–23, all localize to distinct PM domains.

**DISCUSSION**

Several peripheral membrane proteins contain regions that are intrinsically disordered and conformationally adaptable, with clusters of hydrophobic residues (Φ) whose side chains can insert into the lipid core of the bilayer and basic residues that interact electrostatically with the acidic phospholipids of the inner leaflet of the PM (8, 24). For cellular proteins, this cluster acts in conjunction with a lipid group that has been added to the sequence, e.g., an N-terminal myristate for Src and MARCKS (7), an acylated N-terminal Ala in the case of myelin basic protein (25), or a carboxy-terminal prenyl group for Ras (14). The addition of the lipid group to the protein not only provides a way of tethering it to the membrane, it is also presumably an integral part of the mechanism that directs the membrane-bound protein to its final intracellular location. For example, the discovery that some of the enzymes that modify CAAX proteins are restricted to the endoplasmic reticulum has led to the recognition that all nascent Ras proteins transit endomembranes en route to the PM (28). Ras protein prenylation, however, is insufficient for Ras trafficking to the PM, which requires a second signal, palmitoylation of cysteines for N- and H-Ras, or a polybasic stretch for K-Ras (15). Moreover, Ras proteins can also move from the PM back to the Golgi, via depalmitoylation of N- and H-Ras and protein kinase C phosphorylation of the hypervariable domain of K-Ras (28, 31). N- and H-Ras are highly expressed on both the Golgi and PM, and this may also be so for C1–23-bearing proteins (Fig. 5c and d).

C1–15 (MPFLKKKILKLRGR...), letters in bold represent hydrophobic residues; underlined letters represent basic residues) does contain a polybasic stretch, like K-Ras. However, as far as we know, this peptide is not modified by lipidation, nor does it appear to require a lipid group elsewhere for its activity, as neither the C protein, GFP, RED, or TOM is known to be lipidated. The SeV peptide associates with membranes even when its N terminus carries GFP (Fig. 3c), and extensive Ala scanning mutation of C1–23 has not uncovered any residues (other than the 5Φ residues) that are strictly required for membrane association (11). Thus, how C1–23 directs proteins to the PM remains largely unknown. Nevertheless, C1–23 fused to the N terminus of TOM or RED leads to the concentration of the fusion protein at the PM as efficiently as the carboxy-terminal membrane anchor of K-Ras (Fig. 5). In cell fractionation studies (Fig. 4), C1–23 fused to RED also directed the fusion protein to membrane fractions as efficiently as tK.

Although cellular proteins that traffic to the PM do so in conjunction with a lipid group, other regions of these same proteins that are not lipidated also interact with the membrane, and these latter interactions serve as models for C1–23, directed PM association. The scaffolding region of caveolin (aa 92 to 101, with 5 Φ and 3 positive charges) and the effector region of MARCKS (aa 151 to 175, with 5 Φ and 13 positive charges) (3) exist in a nonhelical, extended conformation both in solution and when bound to liposomes. Their binding energy is derived from the electrostatic interactions with the polar head groups, plus hydrophobic interactions of the aromatic side chains with the bilayer. For MARCKS, EPS studies have shown that the 5 Φ penetrate the hydrocarbon core or acyl chain region of the bilayer (3). Region 83 to 93 of the intrinsically disordered myelin basic protein (NPYVHIHEKNIV; letters in bold represent hydrophobic residues; underlined letters represent basic residues) is a closer example to SeV C1–23, as this peptide forms an amphipathic α-helix upon binding to
lipid vesicles and the helix is embedded in the lipid bilayer (4, 5). There are also two examples of viral proteins that are not known to be lipidated that associate with intracellular membranes. A characteristic feature of plus-strand RNA viruses is that their nonstructural proteins form a membrane-associated replication complex, together with viral RNA and altered cellular membranes. For hepatitis C virus and bovine viral diarrhea virus, this membrane association is due to the N-terminal 11 residues of C1–204 to participate in an induced-fit interaction with a hydrophobic surface of a cellular protein which would somehow lead to bulk activity minimally required the first 11 residues of C1–204 to form an amphipathic α-helix that interacts in-plane with the membrane interface (27, 33). There are thus several examples of peptide-only membrane association, although in many cases, the basis for the particular membrane targeting remains unknown.

The critical SeV peptide region MPSKKILKLRGR . . . (letters in bold represent hydrophobic residues; underlined letters represent basic residues) is predicted to be intrinsically disordered within the C protein and contains 5 Φ and 6 positively charged residues. The fact that it apparently functions as a membrane anchor independent of a lipid group may in part be due to its propensity to form an amphipathic α-helix. Membrane interfaces have a potent ability to induce secondary structure in membrane-active peptides such as hormones, toxins, and those with antimicrobial activity (40). The partitioning of small peptides into membrane interfaces is dominated by the very unfavorable free energy cost of partitioning the peptide bonds. Hydrogen bonding of the peptide bonds reduces this high free energy cost and thereby promotes the formation of secondary structure (41). Furthermore, peptides which change from a random coil to an α-helix upon binding to a lipid bilayer derive a substantial fraction of their binding energy from the conformational change (20). The postulated helical transition of C1–23 upon membrane association should kinetically favor this association.

The unusual properties of C1–23 were uncovered because (i) this peptide alone fused to GFP could mimic the activity of the wt C1–204 protein in inducing bulk STAT1 loss and (ii) only C proteins that contain C1–23 induce IFN-independent p-Stat1 formation (11). Mutational analysis of C1–23 indicated that this activity minimally required the first 11 residues of C1–204 to form an amphipathic α-helix, in which the 5 large hydrophobic residues played an essential role (MPSKKILKLRGR . . .; letters in bold represent hydrophobic residues; underlined letters represent basic residues) (11). We hypothesized that C1–23 could participate in an induced-fit interaction with a hydrophobic surface of a cellular protein which somehow would lead to bulk Stat1 loss. While this may still be true, it is now clear that C1–23 and its ability to form an amphipathic α-helix is also required to target this peptide to the inside surface of the PM. These properties of C1–23 are also required for IFN-independent p-Stat1 formation that requires the Stat1-interacting Y module as well. Moreover, although C1–23 directs C24–204Y, to both the PM and to what appears to be perinuclear endomembranes, Stat1 colocalizes only with the C protein that is present at the PM. This suggests that other cellular proteins that are specifically present at the PM (e.g., the IFN receptors and Jak kinases) are required for this colocalization. The simplest explanation for our results is that Y acts as an adaptor that connects Stat1 to the Jak kinases associated with the type I and II IFN receptors and which normally phosphorylate Tyr701 of Stat1 in response to IFN-α/β and IFN-γ, respectively (37).

REFERENCES


