Receptor/Transporter-independent Targeting of Functional Peptides across the Plasma Membrane*

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Targeting of peptides, proteins, and other functional cargo into living cells is contingent upon efficient transport across the plasma membrane barrier. We have harnessed the signal sequence hydrophobic region (SSHR) to deliver functional cargoes to cultured cells and to experimental animals. We now report evidence that two chirally distinct forms of SSHR composed of all L or all D amino acids showed similar membrane-translocating activity as assessed by confocal microscopy, flow cytometry, and direct fluorescence measurement. An attached nuclear localization sequence ferried by the SSHR enantiomers was rapidly translocated by SSHR-mediated endocytosis or transporter-based translocation. This mechanism of SSHR translocation is suitable for facile delivery of biologically active peptides for cell-based and animal-based functional proteomic studies.

The plasma membrane imposes tight control on the access of extracellular peptides and proteins to the cell interior. Through its mosaic structure of proteins and glycolipids embedded into the phospholipid bilayer, the plasma membrane provides a boundary for the 10,000–15,000 proteins expressed in a typical mammalian cell (1, 2). Despite this barrier, transfer of information across the membrane is essential for cell development, function, and survival. Membrane receptors and transporters sense the extracellular environment of growth-promoting or -inhibiting ligands, short peptides, ions, and nutrients. This recognition allows their cellular uptake through specific receptor-mediated endocytosis or transporter-based translocation. To bypass these inherent mainstays of the plasma membrane functional integrity, we harnessed a signal sequence-derived hydrophobic region to deliver functional cargoes composed of peptides and proteins to probe and modulate intracellular signaling (3, 4). However, the mechanism of SSHR1-directed translocation of functional cargo across the plasma membrane remains unexplained.

The overall structure of signal peptides is conserved in evolution between prokaryotes and eukaryotes, although the sequences of signal peptides are highly diverse (5). Their trirpartite structure comprises an NH2-terminal region (n region), and a hydrophobic h region of variable length; this is followed by a cleavage site (c region) for signal peptidase. The hydrophobic region, which usually forms a helix, is endowed with a membrane-translocating activity (6). Its primary function is to guide a nascent polypeptide chain from the ribosomal tunnel through a translocon pore that is open laterally toward the phospholipid bilayer and to the lumen of endoplasmic reticulum (7, 8). Moreover, the hydrophobicity of a signal sequence attached to the nascent protein specifies its co-translational or post-translational pathway of transport (8, 9).

Previously we reported that two SSHRs derived from human fibroblast growth factor 4 (Kaposi fibroblast growth factor) and human integrin β2 provided for the efficient outside-in translocation of attached cargo across the plasma membrane of human and murine cell lines (10, 11). Remarkably, primary cells studied ex vivo provide additional examples of the versatile use of the SSHR-based membrane-translocating motif (MTM) to ferry novel inhibitors for studying the function of calpain in human blood platelets (12) and for the functional ablation of tumor necrosis factor receptor-associated factor 6 in osteoclasts (13). Recently we unexpectedly found that a cell-permeant peptide inhibitor of nuclear import of proinflammatory transcription factors was rapidly (~20 min) delivered to mouse blood cells and organs following intraperitoneal injection (14). As a consequence of intracellular inhibition of signaling to the nucleus, expression of inflammatory cytokines was suppressed and animals were protected from death (14). These in vivo data imply that the SSHR-based peptide ferrying inhibitor of nuclear import has consecutively crossed into and out of plasma membranes from at least three cell types: mesothelial cells lining the peritoneum, endothelial cells lining blood vessels, and hemopoietic cells that circulate in blood and organs such as spleens. Similarly, in vitro delivery of functionally active Cre recombinase using SSHR-based MTM led to its

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1 The abbreviations used are: SSHR, signal sequence hydrophobic region; NFκB, nuclear factor κ B; FITC, fluorescein isothiocyanate; MTM, membrane-translocating motif; RAW, murine macrophage cell line RAW 264.7; DMEM, Dulbecco’s modified Eagle’s medium; LPS, lipopolysaccharide; RT, room temperature; FAUCS, fluorescence-activated cell sorter; ELUV, extruded large unilamellar vesicle(s); IL, interleukin; HIV, human immunodeficiency virus; Tat, transactivator of transcription.
distribution in multiple organs in mice, including the brain, which requires crossing the blood-brain barrier (15).

Despite this striking capacity for intercellular transfer, the mechanism of SSHR translocation through the plasma membrane remains unknown. To study this mechanism, we employed a number of approaches using chirally distinct forms of SSHR. They were analyzed by confocal laser scanning microscopy, flow cytometry of macrophages treated with peptide-containing vesicles, and direct fluorescence monitoring of SSHR-based translocation of the nuclear localization sequence with its positively charged cluster of amino acids across unilamellar phospholipid vesicles. These approaches were coupled with the ultimate test of translocating efficiency: functional measurements of the intracellular effect of the cargo on nuclear import of proinflammatory transcription factor NF-κB and on expression of cytokine genes regulated by this transactivator in macrophages. Using these approaches, we show that translocation of SSHR-linked cargo across the plasma membrane is based on temperature-sensitive diffusion through the phospholipid bilayer. These mechanistic findings will facilitate the rational design of a new generation of cell-permeant peptides for proteomic and drug delivery studies.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine macrophage RAW 264.7 (RAW) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum containing no detectable lipopolysaccharide (LPS, <0.006 ng/ml as determined by the manufacturer, Atlanta Biologicals, Norcross, GA), 2 mM l-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Cytotoxicity Assay—A freshly prepared solution of 10 μg/ml fluorescein diacetate and 100 μg/ml ethidium bromide in DMEM was added to an equal volume of peptide-treated or untreated cells and incubated at 37 °C for 20 min. Cells were observed by fluorescence microscopy, and orange-stained cells were counted as not viable.

Peptide Synthesis and Labeling—The L-SN50 and D-SN50 peptides were synthesized, purified, filter-sterilized, and analyzed as described previously (14, 16) using l or d amino acids as indicated (see Fig. 1A). Peptides were labeled with fluorescein isothiocyanate (FITC, Pierce) according to the manufacturer’s instructions. After extensive dialysis against water to remove free FITC, labeled peptides were lyophilized and stored at −20 °C. Before use the peptides were reconstituted in dimethyl sulfoxide (Me2SO) at 3.3 μM and then diluted to a 1 μM working stock solution with phosphate-buffered saline, pH 7.4. Relative fluorescence of 50 μM peptide solutions in HEPES-KCl (10 mM HEPES-KOH, pH 7.2, and 100 μM KCl) was measured in a Fusion Universal Microplate Analyzer (PerkinElmer Life Sciences) at 485 nm excitation, 535 nm emission, and 20 nm band pass.

Intracellular Detection of Peptides—The intracellular presence of peptides in RAW cells was demonstrated by confocal laser scanning microscopy using direct fluorescence. RAW cells in DMEM with heat-inactivated fetal bovine serum were transferred to 2-ml glass bottom microwell dishes (Mattek Corp.) at a concentration of 2 × 106 cells/ml and incubated at 37 °C in 5% CO2 for 20 h. The next day the medium was replaced with 0.5 ml of DMEM without serum. FMS-95, a fluorescein membrane dye (Molecular Probes, Eugene, OR), was added to cells at 5 μM for 5 min before the addition of 5 μM FITC-labeled l-SN50 or d-SN50 peptide or un conjugated FITC at room temperature for 10 min. Cells were then washed twice with ice cold DMEM followed by a final addition of 0.2 ml of ice-cold phosphate-buffered saline, pH 7.4. They were then immediately observed without fixation using a fluorescence confocal laser scanning microscope (Zeiss LSM510).

Flow Cytometry-based Protease Accessibility Assay—This assay was performed to ensure that cell-associated fluorescence was due to the pool of FITC-labeled peptide that was translocated across the plasma membrane to reach the cytoplasm. RAW cells in DMEM without serum (200 μl at 2 × 106/ml) were incubated with 5 μM FITC-labeled l-SN50 or d-SN50 peptide or un conjugated FITC at RT for 30 min in duplicate. One of each was then treated with proteinase K (5 μg/ml) at 37 °C for 10 min at 37 °C before adding to cells. After all incubations, cells were washed in phosphate-buffered saline, pH 7.4, two times and fixed with 1% paraformaldehyde. Cell fluorescence was measured in FACScalibur (BD Biosciences) using forward versus side light scatter, and green fluorescence was collected with a 535 ± 20-nm band pass filter.

Temperature Sensitivity Assay—RAW cells were incubated with 5 μM FITC-labeled peptides at 4 °C or RT for 30 min and then analyzed for cell fluorescence by FACS as above.

Cellular ATP Depletion—RAW cells were treated with antimycin A and 2-deoxyglucose or medium for 2 h as described previously (17), then incubated with 5 μM FITC-labeled peptides at RT for 30 min and analyzed for cell fluorescence by FACS as above.

Electrophoretic Mobility Shift Assay—80–90% confluent monolayers of RAW cells (60-mm plates with 3 ml of medium split 1:2 1 day before treatment) were treated with or without peptides at the indicated concentrations for 20 min at 37 °C. Cells were then further incubated with MITM based on SSHR but with an identical functional cargo. These approaches were coupled with the ultimate test of translocating efficiency: functional measurements of the intracellular effect of the cargo on nuclear import of proinflammatory transcription factor NF-κB and on expression of cytokine genes regulated by this transactivator in macrophages. Using these approaches, we show that translocation of SSHR-linked cargo across the plasma membrane is based on temperature-sensitive diffusion through the phospholipid bilayer. These mechanistic findings will facilitate the rational design of a new generation of cell-permeant peptides for proteomic and drug delivery studies.

RESULTS

Chirally Distinct SSHRs as Tools to Characterize the Plasma Membrane-translocating Mechanism—Receptor-mediated or transporter-based cellular uptake of peptides and polypeptides is usually dependent on their recognition in a chirally specific manner (20). In other words, if the SSHR composed of all L-amino acids is recognized by a receptor or transporter, then a “mirror image” of the SSHR made of all D-amino acids is not. By studying two chirally distinct forms of the same SSHR, we can deduce whether its plasma membrane-translocating activity is dependent on a chirally specific receptor/transporter. To characterize the plasma membrane-translocating mechanism of the SSHR, we designed two peptides, each with a chirally distinct MTM based on SSHR but with an identical functional cargo. Fig. 1A shows the sequence of the two peptides that contain MTM based on an SSHR made of either all l or all D amino acids and a cargo that comprises a nuclear localization sequence made of l-amino acids that form a positively charged...
RAW cells were incubated with 5 mM H9262 0.6-

cytoplasmic binder of L-SN50 (16). To establish whether the
line RAW comprised of L or D amino acids, respectively, whereas the functional cargo of the nuclear localization sequence derived from NFκB 1 is comprised of l amino acids in both peptides. B, top shows fluorescence confocal laser scanning microscopy. Bottom shows a Nomarski image of the same cells. RAW cells were incubated with 5 μM FITC-labeled peptides or an equimolar concentration of unconjugated FITC. The 0.6-μm section midcell demonstrates an apparent intracellular location of peptide. C, four images of one cell representing RAW cells preincubated 5 min with 5 μM FM5-95 followed by 10 min at RT with 5 μM FITC-l-SN50. Panels show FM5-95 labeled cell membrane and endosomes (red, upper left), FITC-l-SN50 (green, upper right), FM5-95 and FITC-l-SN50 images merged (lower left), and a Nomarski image (lower right). The colored panels represent the same 0.6-μm section midcell showing independent localization of the internalized peptide and plasma membrane/endosome probe. Pictures are representative of multiple unfixed cells from three independent experiments.

Two Chirally Distinct MTMs Translocate Peptide Cargo to Mammalian Cells—To ascertain whether l-SN50 and d-SN50 are equally translocated to mammalian cells, we monitored the transport of FITC-labeled peptides into murine macrophage RAW cells. Confocal microscopy studies indicated that the l-SN50 and d-SN50 peptides were similarly distributed in the cytoplasm of RAW cells (Fig. 1B). This pattern is consistent with the subcellular distribution of a nuclear import adaptor protein, importin/karyopherin α2, identified previously as a cytoplasmic binder of l-SN50 (16). To establish whether the distribution pattern of l-SN50 and d-SN50 is independent of the endosomal compartment, RAW cells were treated with a fluorescent lipophilic membrane probe FM5-95 known to stain the plasma membrane and early endosomes as described by the manufacturer (Molecular Probes). Subsequently, cells were pulsed with FITC-labeled l-SN50 peptide. The red fluorescent signal emitted by the FM5-95 probe was localized independently from the green fluorescent signal of l-SN50 (Fig. 1C). The merged image of both fluorescent reagents showed very little if any colocalization in multiple sections of unfixed RAW cells analyzed by confocal laser scanning microscopy (Fig. 1C). The divergence of fluorescent signals indicates an endosome-independent pathway of membrane translocation by the FITC-labeled l-SN50 peptide. A similar pattern of distinct fluorescent signals was observed with FITC-d-SN50 (not shown).

Because of the potential nonspecific binding of peptides to the cell surface, confocal microscopy does not provide definitive proof for intracellular location of the peptides studied (21). Therefore, we analyzed peptide translocation across the plasma membrane in RAW cells using flow cytometry coupled with a protease accessibility test. As shown in Fig. 2A, the broad spectrum protease, proteinase K, was used to distinguish between peptides not internalized and those translocated to the interior of the cell. For comparison, the control system contained peptides incubated with cells without proteinase K treatment. This analysis revealed that both peptides are susceptible to proteinase K, yet they escape proteolytic attack after being translocated to RAW cells where they remain inaccessible to proteinase K. The apparent additional gain in fluorescence in control cells that were not treated with proteinase K likely reflects a pool of peptides adsorbed on the cell surface and accessible to proteinase K. Translocation of both peptides across the plasma membrane of RAW cells was temperature-dependent, with the process inhibited at 4 °C (Fig. 2B). Consistent with the results showing peptide translocation independent of the endosomal compartment (Fig. 1C), translocation did not require ATP. Cells depleted of high energy stores demonstrated a similar gain in fluorescence because of peptide transduction as control cells that maintained a steady ATP level (Fig. 2C). These experiments lead us to conclude that both l-SN50 and d-SN50 move similarly across plasma membranes, bypassing the endosomal compartment to reach their cytoplasmic target.
Intracellular Function of the Cargo Delivered by Two Chirally Distinct MTMs Is Similar—Detection of a protease-inaccessible pool of L-SN50 and D-SN50 peptides provides a measure of their ability to translocate across the plasma membrane. However, the ultimate test of translocating efficiency is to establish that the cargoes ferried by chirally distinct MTMs display similar intracellular activity. Prior results have demonstrated an inhibitory function of the L-SN50 peptide toward nuclear import of NFκB and other proinflammatory transcription factors in T cells (16). Likewise, this peptide inhibited (in a concentration-dependent mode) inducible nuclear import of NFκB in LPS-stimulated RAW cells, demonstrated by use of electrophoretic mobility shift assay using 32P-labeled probes (Fig. 3A). Importantly, the D-SN50 peptide with chirally distinct MTM displayed a similar inhibitory potency.

Transcription factor NFκB plays a key role in the regulation of genes encoding inflammatory cytokines (22). Consistent with inhibition of nuclear import of NFκB, both peptides suppressed expression of inflammatory cytokines tumor necrosis factor α, IL-1β, and IL-6 in LPS-stimulated cells (Fig. 3B). The inhibition curves were almost identical for both peptides, suggesting that their inhibitory cargo was delivered with a similar efficiency by chirally distinct MTMs. Both peptides, in concentrations up to 150 μM, were not cytotoxic as determined by staining with fluorescein diacetate and ethidium bromide (23). Cumulatively, these functional studies of intracellular inhibitory activity of nuclear localization sequence cargo ferried by two chirally distinct MTMs reinforce fluorescence-based assays indicating that the mechanism of translocation across the plasma membrane of RAW cells is not due to a chirally specific receptor or transporter.

Two Peptides with Chirally Distinct MTMs Are Translocated across Phospholipid Bilayer—Model phospholipid membranes (“liposomes”) are impermeable to charged or hydrophilic molecules (24). To establish whether SSHR-based MTMs can ferry its positively charged cargo (i.e. nuclear localization sequence) through the phospholipid bilayer, we used ELUV composed of phosphatidylglycerol, phosphatidylcholine, and cholesterol (18). These uniformly sized liposomes were incubated with FITC-labeled L-SN50 and D-SN50. Proteinase K was used to destroy the pool of peptide not translocated across the phospholipid bilayer and thereby not protected from protease action. As shown in Fig. 4, both peptides diffused rapidly with similar kinetics of translocation to the protease-inaccessible interior of ELUV. Proteinase K-digested peptides did not produce any significant gain in fluorescence associated with ELUV, thereby attesting to complete digestion of both peptides under these experimental conditions. In striking contrast, proteinase K treatment of ELUV after peptide translocation revealed a similar gain in fluorescence as compared with control
Thus, there is very little residual binding of either peptide to the surface of ELUV. Taken together, our results strongly suggest that SSHR used as a membrane-translocating motif can ferry its positively charged cargo through a phospholipid bilayer without participation of proteinaceous receptors or transporters.

DISCUSSION

Here we provide four separate lines of evidence indicating that the signal sequence hydrophobic region ferries its functional cargo across the plasma membrane of mammalian cells through a mechanism other than a receptor/transporter-mediated pathway. (i) Peptides containing SSHR enantiomers of all L or all D amino acids gain entry into mammalian cells independently of the endosomal compartment. (ii) Peptides translocated by chirally distinct SSHR exert similar intracellular function by inhibiting nuclear import of a proinflammatory transcription factor and suppressing inflammatory cytokine gene expression. (iii) The SSHR-based translocation mechanism is operational in ATP-depleted cells. (iv) SSHR, with nuclear localization sequence as its positively charged cargo, crosses the phospholipid bilayer of unilamellar liposomal vesicles. The translocation process is also temperature-dependent, presumably due to the well known temperature-dependent lipid phase transition of the phospholipid bilayer (25).

Our data indicate that the SSHR-based translocation mechanism is not dependent on a chirally specific receptor or transporter and can proceed in ATP-depleted cells. Thus, an endocytosis-based uptake mechanism seems unlikely. In this regard, other groups have employed alternative MTMs derived from the fruit fly Antennapedia transcription factor and human immunodeficiency virus (HIV) transactivator of transcription (TAT) protein (26, 27). Although these MTMs are capable of ferrying attached cargo from the outside to the inside of mammalian cells, they enter cells via an endocytic pathway (28–31). Moreover, the

FIG. 3. Functional analysis of L-SN50 and D-SN50 peptides. A, concentration-dependent inhibition of inducible nuclear import of NFκB in RAW cells stimulated with LPS. L-SN50 (lanes 3–5) or D-SN50 (lanes 6–8) peptide was added to cells at concentrations shown 20 min before the addition of LPS. Control cells were treated with (lane 2) or without (lane 1) LPS. Nuclear extracts were analyzed by electrophoretic mobility shift assay using radiolabeled probes. The inhibitory effect of peptides on nuclear translocation of NFκB is concentration-dependent, whereas the constitutively expressed nuclear factor Y is not inhibited and indicates consistent loading of nuclear proteins in all lanes. The bands labeled ns represent constitutive nonspecific binding of probe. The gels are representative of three independent experiments. B, concentration-dependent inhibition of inflammatory cytokine expression in RAW cells stimulated with LPS. L-SN50 (■) or D-SN50 (▲) peptide was added to cells at concentrations shown 30 min before the addition of LPS. The medium was analyzed by enzyme-linked immunosorbent assays for levels of cytokines expressed. The bars represent mean ± S.D. from three independent experiments.

FIG. 4. Time-dependent translocation of FITC-labeled L-SN50 and D-SN50 peptides into ELUV. ELUV were incubated with 5 μM FITC-L-SN50 (left panel) or FITC-D-SN50 (right panel) at RT for the times indicated without proteinase K (●) or subsequently treated with 200 μg/ml proteinase K for 12 min at 37 °C (■). As a control, FITC-labeled peptides were pre-treated with 200 μg/ml proteinase K for 12 min at 37 °C before incubation with ELUV at RT for 30 min (▲). All samples were passed through Sephadex G-50 (fine) to remove non-ELUV-associated FITC peptides before measuring relative fluorescence. Each panel is representative of three independent experiments.

without proteinase K treatment. Thus, there is very little residual binding of either peptide to the surface of ELUV. Taken together, our results strongly suggest that SSHR used as a membrane-translocating motif can ferry its positively charged cargo through a phospholipid bilayer without participation of proteinaceous receptors or transporters.
TAT motif-based constructs are unable to translocate across liposomal phospholipid vesicles (32, 33). A temperature-dependent endocytic pathway based on cell membrane lipid rafts and caveolar endocytosis is proposed for internalization of the TAT motif-containing enhanced green fluorescent protein (34). Surprisingly, a similar TAT motif-based enhanced green fluorescent protein failed to demonstrate its capacity for intercellular transfer (35). Cumulatively, the mechanisms employed by Antennapedia or HIV TAT-based motifs for crossing the plasma membrane seem to differ from SSHR-directed plasma membrane phospholipid bilayer translocation.

How does SSHR, with its positively charged cargo, pass through the membrane phospholipid bilayer? The hydrophobic region of signal sequence we used contains proline as a helix-breaking residue (Fig. 1A). Consistent with the “helical hairpin” hypothesis of Engelman and Steitz (36), the presence of such a residue may allow SSHR to form, within a phospholipid bilayer, a hairpin-like loop that constitutes a leading edge for the attached cargo (Fig. 5). Thus, the translocation would proceed through looping and unlooping stages as proposed by de Vrije et al. (37). An alternative “tilted peptide” translocation mechanism envisages that the helix traverses a lipid bilayer after its insertion at a 45° angle (38). These two postulated “looping” and “tilted peptide” mechanisms are not mutually exclusive in their destabilizing effects on the phospholipid bilayer (Fig. 5). Such an effect may be attributed to the formation of non-bilayer lipid structures upon contact with the signal sequence hydrophobic region (39). Transient formation of these non-bilayer lipid structures reflects their ability to undergo topological transformation (40). Conversely, liposomes are known to destabilize the membrane of isolated neutrophil granules and induce a release of membrane-bound lysosomal enzymes (41). Nevertheless, the potential destabilization of the phospholipid bilayer by SSHR-based MTM has no apparent effect on the permeability of the plasma membrane. We did not observe any increase in plasma membrane permeability as evidenced by fluorescein diacetate/ethidium bromide staining with a peptide concentration of up to 150 μM. Thus, SSHR-directed movement of functional cargo through the phospholipid bilayer seems to be harmless in terms of its impact on the structural integrity of the plasma membrane at concentrations sufficient to inhibit intracellular signaling. The SSHR-based mechanism of translocation across the phospholipid bilayer is consistent with the membrane trigger hypothesis formulated by Wickner (42, 43) that postulated translocation of certain newly synthesized bacterial proteins (e.g. leader sequence-bearing M13 procoat protein) across a phospholipid bilayer without the aid of a proteinaceous pore or transport system.

The development of specific tools to analyze the 10,000–15,000 intracellular proteins and their pathways in a typical living cell (2) has been hampered by the lack of facile vehicles for delivery of peptides and large protein segments across a plasma membrane. Our work has demonstrated that the SSHR-based MTM possesses a number of desirable attributes. It is based on the hydrophilic region of a signal sequence that has been conserved through evolution. It translocates freely across a phospholipid bilayer, bypassing a more complex endocytic pathway apparently used by other MTMs such as Antennapedia-based or HIV TAT-based sequences (28–33). SSHR-based MTM allows development of specific probes to study intracellular protein networks, including functionally characterized new proteins. Hence, it provides a platform for the development of cell-based proteomic analytical tools and for intracellular delivery of novel drugs by enabling them to cross the plasma membrane barrier in multiple cell types (14). Altogether, our results elucidate a new outside-in cellular translocating mechanism of signal sequence hydrophobic region, underscoring its usefulness as an efficient vehicle for ferrying functionally diverse peptides and other cargos across the plasma membrane to the cell interior.

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Fig. 5. Two potential mechanisms for SSHR-based translocation of attached cargo across a phospholipid bilayer. Left, the looping-unlooping process involves the insertion of the bent helix into the outer phospholipid layer. The unloping of the helical “hairpin-like” form follows, allowing it to move the attached cargo (orange rectangle) through the inner phospholipid layer to the inside of the cell. Right, the “tilted peptide” mechanism involves the insertion of the leading helix into the phospholipid bilayer at a 45° angle. This allows the bent helix to carry its attached cargo (orange rectangle) to the interior of the cell (see text for details).
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