Problem #1: Membrane remodeling in the endosome pathway

1) Dynamin constricts the vesicle neck from the outside, whereas ESCRT-III constricts from the inside.

2) Both events involve budding of lipid membrane away from the cytosol, so the membrane topology is the same.

3) p6 within Gag binds to Tsg101, an ESCRT-I component. This interaction is essential for HIV budding through the ESCRT pathway. If a fragment containing the Tsg101 binding site is exogenously expressed, it can act in a dominant-negative manner to inhibit the ability of gag polyprotein to recruit Tsg101. Such inhibition would prevent scission of buds, leading to accumulation of budding intermediates (and also multi-bud intermediates) similar to the EM shown in class.

4) Correct sorting into lumenal vesicles results in lumen localization of CPS-GFP. If CPS-GFP is not correctly sorted and left on the limiting membrane, the GFP fluorescence would appear as a ring. CPS-GFP can be mutated to change the lysines to arginine; the sorting of the mutants can be assessed by fluorescence imaging, to determine if it has a localization to the lumen or limiting membrane.

5) The region containing the lysine can be added to a protein that normally gets left on the limiting membrane. If the region is sufficient for sorting, the new protein will localize to the lumen.

Problem 2: Membrane fusion

1) In SNARE-mediated fusion, the ATP is used by NSF to disassembly SNARE complexes so that they can undergo future rounds of fusion. In gp41-mediated fusion, the gp41 can only mediate a single round of fusion; it is not recycled.

2) The "low pH" conformation is the most stable conformation. The "native" conformation is a metastable state (higher energy) that can only be generated by the normal biosynthesis pathway involving production of a precursor.

3) A trivial explanation is that Δ12 is defective for some feature other than membrane fusion. It would be important to show that Δ12 is expressed at normal levels (do Western blot), properly placed on the plasma membrane (could be analyzed by immunofluorescence or cell fractionation), is stable (Western blot), etc.

4) For an alpha helix, each amino acid has a translational distance of 1.5 angstroms. 24 residues would span 24x1.5=36 angstroms, which is approximately the thickness of the lipid bilayer. If the hydrophobic stretch were much less than 20 amino acids, it would be too short to fully span the membrane, at least as a helix.

5) Model: the TM segment of HA2 has to fully span the lipid bilayer to mediate full fusion. The GPI anchor and the Δ12 TM anchor do not fully span the membrane and mediate only hemifusion. When the cytosolic tail is added to Δ12, the short 15 amino acid TM segment is probably forced to span the bilayer (because the cytosolic tail has charged residues that cannot partition to the membrane). It would not be in an alpha-helical conformation.

Problem 3: ER translocation

1) For construct A:
lane1: Translation of yields full-length protein
lane 2: Because there are no microsomes to import into, the protein is digested by PK.
lane 3: Due to import into microsomes, the Myc tag and transmembrane region are protected. The topology is therefore: N-terminus out, C-terminus in.
lane 4: Detergent disrupts the microsomes and allows access of PK to protein.

For construct B: With the reverse orientation of the Myc tag, the tag is cleaved after import of the protein. This confirms the topology as N-terminus out, C-terminus in.

2) TM is essential: perform import studies of mutants having mutations in the TM.

TM is sufficient: transfer the TM to another non-imported protein (such as GFP) and test whether this hybrid protein is now imported.

3) The protein has the hallmarks of a TA protein. The import sequence is at the very C-terminus, so co-translational import is not possible. The ribosome shields ~40 amino acids in the exit tunnel. The C-terminal targeting sequence would be hidden until the polypeptide chain is released after translational termination.

4) Yes, the Get pathway requires ATP (due to Get3 ATPase).

5) SRP versus Get pathway: (A) co-translational versus post-translational. (B) binding to the protein: SRP versus the Get targeting complex (C) membrane targeting: SRP-SRP receptor interaction versus Get3-Get1/2 interaction (D) nucleotide requirements: GTP versus ATP (E) SRP is the major pathway and can handle more complex protein topologies; the Get pathway constitutes 3-5% of membrane proteins.

Problem #4: Protein quality control

1) Because X is misfolded, it will be unstable and subject to ERAD. The steady state levels of X, measured by Western blotting, should be increased in the presence of MG132. However, steady state levels do not directly indicate half-life.

To demonstrate half-life extension, one would have to do a pulse-chase type of experiment. A cohort of X would be labeled by pulsing cells with radioactive amino acids, followed by a chase with unlabeled media. The amount of radiolabeled X can be monitored (by SDS-PAGE) as a function of time to determine the half-life.

Alternatively, cells could be incubated with cycloheximide to stop new translation. The stability of the existing X could then be measured as a function of time by Western blotting.

2) A: X would be ubiquitylated and accumulate in cytosol. B: X would accumulate but not be ubiquitylated. In addition, it may not be retrotranslocated and therefore exist in the ER. C: Ubiquitylated X would accumulate but not be extracted from the ER membrane. Some of these features could be confirmed by cell fractionation experiments separating microsomes from cytosol, followed by Western blotting against X.

3) Cdc48 uses different adaptors for different substrates. It would be expected that depletion of some adaptors but not others would affect X degradation. Cdc48 uses many adaptors and functions on many substrates. NSF uses SNAP as an adaptor and seems to function primarily on SNAREs.

4) The 26S proteasome can unfold polypeptide chains, but p97 is required for more difficult substrates that need to be extracted, such as membrane- or DNA-associated ones.

5) Upon ligand stimulation, Ste2 is degraded in the vacuole via the MVB pathway. The UPS is not involved. The ubiquitylation is mono not poly.