5.2 Assays for translocation

A. Proteolysis

The gold standard for translocation over the years has been to demonstrate that a protein domain is protected from proteolysis and that this protection is abolished by the addition of low concentrations of non-denaturing detergents, which presumably solubilize the protecting lipid bilayer without altering protein folding. This is typically carried out using either proteinase K or trypsin, or trypsin and chymotrypsin.

In RRL it is performed as follows: after completion of translation (1 hr at 24øC), tubes are transferred to ice, samples are aliquoted for each condition (e.g. minus protease, plus protease and plus protease, plus detergent) and adjusted to approximately 10 mM CaCl2 (e.g. addition of 1 ul of 0.1 M to a 9 ul aliquot. Proteinase K is prepared at a concentration of 2 or 4 mg/ml in 10 M Tris pH 8 and incubated at 37øC for 30 minutes (to destroy any contaminating lipases) then flash frozen. The predigested proteinase K can be aliquoted and stored at -80. I usually thaw an aliquot (50 ul), use it and discard it rather than refreeze. Add Proteinase K to a final concentration of approx 0.2 mg/ml (e.g. 1 ul 2 mg/ml added directly to a 10 ul volume already adjusted as above to 10 mM CaCl2), vortex gently and digest ON ICE and IN THE COLD ROOM for 45 min. Kill the digestion by transferring the entire sample into 10 volumes (100 ul) of 0.1 M Tris pH 8, 1.0% SDS in a boiling water bath. In addition, use a pipette person tip dipped in 0.1 M PMSF in ethanol - make fresh - to do this transfer (capillary action pulls in a half ul of PMSF which mixes with your sample, helping to kill the protease). Cap the aliquots for no protease before using the protease and cap the no detergent tubes before adding a detergent, to prevent screw-ups. You can run a 5 ul aliquot (= 0.5 ul of original translation reaction) on a gel to check total products before proceeding to immunoprecipitation. Just mix it with 10 ul of loading buffer with DTT, incubate, reboil for 2 minutes and load onto gels as usual. If your protein is particularly resistant to digestion (e.g. as evidenced by protection even in the detergent-treated protease lanes), you may need to increase the protease concentration or the time. I would not mess with the temperature. If you are proteolyzing membranes that have been reisolated from RRL cell-free translations by centrifugation or gel filtration, there will be a lot less total protein and less proteinase K should be used. In the case of WG you can try doing everything at 24øC. In this case, do not transfer tubes to ice, simply aliquot, add CaCl2, protease, etc. The protocol for trypsin instead of proteinase K is essentially the same.

An alternate method for proteolysis of membrane-targeted molecules follows:

1. Separate membrane-targeted molecules by gel filtration. A 20 or 30 ml tranlation reaction works well for this method.
2. Measure void volume total and split into three equal aliquots.
3. Treat each aliquot as follows:
    1. no protease
    2. add proteinase k (the amount to add is tricky; the literature quotes anywhere from0.02 to 1 mg/ml final; generally you have to titrate to see which concentrations give you the best protection of your control molecules. For

preprolactin, I have found that 0.03mg/ml final for 15 to 30 min on ice gives good resutls.)

4. add proteinase k (as above) and Triton X-100 to 1% final.
5. Incubate tubes on ice for required time.
6. Kill protease by adding 1/2 volume of 50% trichloroacetic acid (TCA). (This will also precipitate your proteins.)
7. Spin full speed in microfuge for 15 minutes.
8. . Wash with ethanol:ether (1:1 v/v). Spin again full speed for 15 minutes.
9. Dry pellets on bench or in 37degrees incubator for 10 or 15 minutes.
10. Resuspend in loading buffer and run of SDS-PAGE gels.