88. Investigation of fusion complex assembly from placental clathrin coated vesicle membranes

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Vectorial movement of macromolecules between compartments in the cell is mediated by membrane-bound transport vesicles. Fusion between transport vesicles and their appropriate target organelle requires the presence of soluble factors. NEM-sensitive factor (NSF) was one of the first components shown to be essential for vesicle fusion [1]. Later it was found that in order for NSF to function it required soluble factors from the cytosol, which were termed soluble NSF attachment proteins (SNAPs) [2].

SNAP binds to Golgi membranes in a specific and saturable manner [3], and using a detergent solubilised fraction from bovine brain, three proteins were isolated as putative SNAP receptors or SNAREs [4]. This work led to the formulation of the SNARE hypothesis [5], predicting that assembly of these factors into a "fusion complex" will only occur upon cognate pairing of appropriate vesicular (v) and target (t) SNAREs (SNAP receptors), through interaction of coiled-coil domains [6], hence contributing to the specificity of vesicular transport.

One of the central predictions of the SNARE hypothesis is that soluble membranes derived from transport vesicles should not be able to support formation of "fusion complexes", since they contain no t-SNAREs. We aim to test this prediction, using clathrin coated vesicles as a model.

Incubation of 35S-αSNAP and recombinant NSF-myc (tagged with 10 amino-acids from human c-myc), with detergent solubilised membranes derived from transport vesicles should not be able to support formation of "fusion complexes", since they contain no t-SNAREs. We aim to test this prediction, using clathrin coated vesicles as a model.

Incubation of 35S-αSNAP and recombinant NSF-myc (tagged with 10 amino-acids from human c-myc), with detergent solubilised membranes from purified human placental coated vesicles allowed formation of a complex containing 35S-αSNAP. Sedimentation of radiolabel to 20S was dependent on the presence of NSF (data not shown). This is consistent with the work of other investigators [7], who have formed 20S SNAP-SNARE-NSF complexes from rat liver Golgi membranes.

SNARE-SNAP-NSF complex formation was also followed by co-immunoprecipitation studies. In comparison with crude placental membranes, clathrin coated vesicles were enriched in their ability to form 20S complexes (figure 1).

Fig 1. Co-immunoprecipitation of 20S "fusion complexes"

Coated vesicles (ccvp) and crude membranes (cmp) from placentas were Na2CO3-washed and detergent solubilised. Co-immunoprecipitations typically contained 5μg of phospholipid, 500ng NSF-myc, 5ng 35S-αSNAP (7 x 10^4 dpm/ng) in IP buffer (20mM HEPES KOH, pH 7.4, 100mM KCl, 2mM DTT, 2mM EDTA, 1mM ATP, 1% PEG4000, 250μg/ml SBTI), in a final volume of 100μl. After incubation on ice for 5 min TX-100 was added to 1% (v/w). The mixture was solubilised on ice for 20 min, before adding 100μl of 10% slurry of Protein G-Sepharose in IP buffer saturated with 9E10 monoclonal Ab, which recognises the myc-epitope. Samples were incubated overnight and the beads recovered after washing, and bound 35S counted in the scintillation counter.

This competence to form 20S complexes was membrane and NSF dependant, and showed sensitivity to heat and protease treatment. 20S complexes isolated on the velocity sedimentation gradients could be precipitated using the monoclonal antibody raised against NSF-myc, confirming the direct interaction of NSF and αSNAP (data not shown).

Placental coated vesicles which have had all their peripheral soluble factors removed retain the ability to form the well characterised 20S fusion complexes, and this activity is enriched over crude membranes. One explanation for this could be that the placental coated vesicles contain more than one population of SNAREs, which under conditions of detergent solubilisation could pair to form a platform for recruitment of SNAPs and NSF. Alternatively, assembly of the "fusion complex" may not require interaction of cognate SNAREs and may take place before docking occurs.