UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
KATEDRA FARMACEUTICKÉ CHEMIE A KONTROLY LÉČIV

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CARDIFF UNIVERSITY

VÝVOJ METODY PRO *IN VITRO* HODNOCENÍ FÚZE ČASNÝCH ENDOSOMŮ

STEPS TOWARDS THE DEVELOPMENT OF AN *IN VITRO* ASSAY OF EARLY ENDOSOME FUSION

Supervisors: Mgr. Marta Chlupáčová
Dr. Arwyn T. Jones

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Lucie Švédová

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ABBREVIATIONS

AP	adapter protein, assembly protein
ATP	adenosine 5´-triphosphate
av-β-gal	avidin-β-galactosidase
BB	breaking buffer
β-gal	β-galactosidase
bHRP	biotin-horseradish peroxidase
bi-Tf	biotin-transferrin
BSA	bovine serum albumin
CCV	clathrin-coated vesicle(s)
ci-MPR	cation-independent mannose-6-phosphate receptor
CP	creatine phosphate
CPK	creatine phosphokinase
CPP	cell penetrating peptide(s)
CPT	Center for Polymer Therapeutics
CTR	cocktail of reagents
DNA	deoxyribonuceic acid
DTT	dithiothreitol
ECV	endosomal carrier vesicle(s)
EDTA	ethylenediaminetetraacetic acid
EE	early endosome(s)
EEA1	early endosome antigen
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N´*,*N´*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
GTPγS	guanosine-5´-(3-thiotriphosphate)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonate
Hsp70	heat shock protein 70
K	lysine
LDL	low-density lipoprotein
LE	late endosome(s)

LY	lysosome(s)
MBG	methylumbelliferyl-β-D-galactopyranoside
MU	7-hydroxy-4-methylcoumarin, methylumbelliferone
NEM	*N*-ethylmaleimide
NSF	*N*-ethylmaleimide-sensitive factor
PBS	phosphate buffered saline
PCI	photochemical internalization
PEG	polyethylene glycol
PI-3-kinase	phosphatidylinositol-3-kinase
PI-3-P	phosphatidylinositol-3-phosphate
PNS	postnuclear supernatant
PTD	protein transduction domain
Q	glutamine
R	arginine
RNA	ribonucleic acid
RV	recycling vesicle(s)
SDS	sodium dodecyl sulphate
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptor
Tf	transferrin
WB	washing buffer
Y	tyrosine

1. INTRODUCTION

There is an increasing number of macromolecular drugs (proteins, oligonucleotides and synthetic polymers), macromolecular conjugates and nanosized drug carriers (liposomes, nanoparticles) in research and development (Jones A. T. *et al.*, 2003). Drug design consists of two aspects: the ability to induce desired response, which is studied by pharmacodynamics, and the transport of the drug to the place of action, analysed by pharmacokinetics. No matter how potent the drug is *in vitro*, it is ineffective if it can not reach the appropriate subcellular compartment (Kearsey J., 2004). Efficient intracellular drug delivery can avoid non-specific effects, reduce toxicity, allow reduction in dosage levels and lead to a better patient compliance. In the 1960's polymers began to be used in drug-delivery systems, and scientists were forced to combine understanding of pharmacokinetics, biological interface and biocompati-bility (Rosen H. *et al*., 2005).

Some hydrophobic molecules can pass directly through the cell membrane by a process of simple diffusion. Most hydrophilic substrates need a means of facilitating their passage into the cell, *e.g.* protein channels controlled by ion concentration or chemical messengers. Endocytosis is an energy-dependent mechanism of uptake that is utilised by various substances without direct passage through the cell membrane (Kearsey J., 2004). This pathway will be discussed in more detail later in this project.

Particle bombardment, microinjection or electroporation are some mechanical (physical) methods, that have been investigated in drug delivery to introduce macromolecules into cells. Viruses are used as vectors and as models for cytosolic and nuclear targeting. Cell penetrating peptides (CPP) containing short basic protein transduction domains (PTD) and their conjugates with various cargos are intensively studied for endosomal escape into cytosol after association with the plasma membrane and subsequent internalization by endocytosis. Transferrin receptor, which is over-expressed in malignant cells, can also be exploited. 'Smart polymers', sometimes also called 'encrypted polymers', are promising drug carriers into cytosol. Bio-inspired, pH-responsive polymeric carriers can disrupt endosomal membrane in a pH-dependent fashion (Murthy N. *et al.*, 2003). On the endocytic pathway there is a decrease in pH from pH 7.4 to approximately pH 4.5. This pH drop serves as a trigger for a conformational change of the polymer leading to its conversion from a hydrophilic coil to a hydrophobic helix, which is membrane active and therefore can disrupt the endosomal border. Another way is masking of disruptive part of molecule by

polyethylene glycol (PEG) chains conjugated *via* acid-labile acetal linkage and the low pH in the endosome will then allow release of the therapeutic cargo (Hoffman S. A. *et al*., 2003). The final therapeutic agent contains pH-sensitive polymer (or masked membrane-disruptive polymer), targeting ligand for receptor-mediated endocytosis and the drug.

Another approach uses the features of light. Photochemical internalization (PCI) can improve cytosolic delivery of endocytosed drugs. Photosensitizing compounds, such as aluminium phthalocyanine, localized in endocytic vesicles induce photochemical damage of the vesicular membranes upon light exposure and therefore intact macromolecules can be released into the cytosol. Only areas exposed to light are subjected to the drug effect. It is interesting that the illumination can be performed even before the drug uptake. This pre-permeabilisation of endocytic vesicles is suitable especially for the light-unstable drugs (Prasmickaite L. *et al*., 2002).

Liposomes are able to provide protection and targeting of the encapsulated macromolecule (Fretz M. M. *et al*., 2005). The disadvantage of such a lipid-based compound is a rapid removal from blood circulation by the reticuloendothelial system.

The science and medicine can not rely only on small compounds and therefore need to find the ways how to make large molecules efficient in therapy. Drug-delivery technologies come into play earlier in the development cycle, they can even rescue failed compounds, such as those with low solubility (Rosen H. *et al*., 2005). Diseases associated with intracellular compartments, strategies for delivering drugs into those compartments and crossing the numerous biological barriers lying between drug administration and effect are on the top list of today's research (Jones A. T., 2001).

2. THEORETICAL BACKGROUND

2.1. Endocytosis

It is estimated that more than 10% of human genome is implicated in controlling membrane trafficking pathways. Macromolecular drugs are candidates for cellular uptake by the mechanism of endocytosis (Jones A. T. *et al*., 2003). Endocytosis is a fundamental process performed by all eukaryotic cells. It is characterised by the continuous and regulated formation of prolific numbers of membrane vesicles at the plasma membrane (Mellman I., 1996). The basic mechanism is the reverse of what occurs during exocytosis or cellular secretion. Endocytosis is essential for cell homeostasis and plays a key role in membrane dynamics and response to extracellular stimuli by recycling, down-regulating and desensitizing of receptors and membrane proteins (D'Hondt K. *et al*., 2000). Cells use endocytosis for a number of cellular processes including uptake of nutrients, for transmission of metabolic, neuronal and proliferative signals, for defence against microorganisms. Many infectious agents hijack this pathway as their entry route to the host cell (Mellman I., 1996).

The process involves invagination, or folding inward of the plasma membrane, surrounding the external space, pinching off (budding) and releasing the vesicle to the cytoplasm. The vesicles deliver their content into a number of intracellular organelles including lysosomes for degradation (lysosomotropic delivery, 30 - 60 min). Membrane components of endocytic vesicles are subject to highly complex and iterative molecular sorting events resulting in their targeting to specific destinations such as Golgi network or relatively rapid recycling out of the cell (5 - 10 min) or at the opposite part of plasma membrane in the case of transcytosis. The entire cell surface is internalized at least once or twice per hour (Mellman I., 1996, Jones A. T. *et al*., 2003).

There are two general types of endocytosis: phagocytosis, where large particles ($\geq$ 500 nm) are internalized into phagosomes, and pinocytosis, the uptake of particles (usually $\leq$ 200 nm) dissolved in a fluid phase or receptor-mediated internalization into endosomes.

<u>Phagocytosis</u>

Phagocytosis is generally restricted to motile cells. Some species of protozoa use phagocytosis for a nutritional reasons and distinct cell types of mammalian immune

system (neutrophiles and macrophages) use pseudopodia extensions for a defence against invading pathogens or for collecting defective or old cells. This receptor-mediated process is often facilitated by opsonins, that bind to a foreign particle and make it distinguishable for the receptors of motile blood cells. When the phagosome is formed, it matures by a series of vesicle fusion and fission reactions and finally combines with lysosome to create a phagolysosome, in which the ingested particles are degraded (Pollard T. D. *et al*., 2002). Phagocytosis is a very complex process involving specialized plasma membrane receptors, rearrangement of cytoskeletal elements and dynamic exchange of phagosomal components with several intracellular compartments (Berón W. *et al*., 1995).

Pinocytosis

Pinocytosis can be generally split into clathrin-dependent and clathrin-independent endocytosis. At least four distinct processes have been suggested to provide endocytic activity in the absence of clathrin, these are caveolae mediated uptake, adsorbtive endocytosis, fluid-phase uptake and macropinocytosis.

Caveolae (~ 60 nm) are flask-shaped plasma membrane invaginations, characterised by a coat consisting of caveolins. These are able to internalize *e.g*. cholera toxin or ricin (Mineo Ch. *et al*., 2001). Caveolin-1 in non-muscle cells and caveolin-3 in muscle cells are integral membrane proteins, that serve as markers of caveolae. Caveolae have been considered to be a specialised form of lipid raft domains with a unique morphology generated by caveolin. Lipid rafts are highly ordered lipid microdomains in exoplasmatic leaflet of the plasma membrane caused by the interaction of sterols (cholesterol, ergosterol) and sphingolipids. Lipid rafts may selectively incorporate proteins, therefore act as dynamic organizers of protein interactions during membrane transport or signal transduction (Ikonen E., 2001). Caveolae endocytosis appears to be a constitutive dynamin-dependent process formed by the interaction between caveolin and cholesterol (Ikonen E. *et al*., 2005).

Adsorbtive endocytosis is an uptake process based on the interactions between positively charged particles and the negatively charged plasma membrane.

Cells continuously internalize fluid from their surroundings to ensure the sampling of the environment. Fluid-phase endocytosis could be a type of surveillance in mammalian cells. Alternatively it could be a mechanism of washing the bulk of plasma membrane by passing through acidic endosomes to remove nonspecifically adsorbed

molecules. In lower organisms it plays an essential role in nutrient uptake (Pollard T. D. *et al*., 2002).

Macropinocytosis is a term used to describe the internalization of large areas of plasma membrane together with significant amounts of fluid. Large irregular macropinosomes (sometimes greater than 1 μm) are formed during extensive actin rearrangement. Finding that this process is inhibited by cholesterol depletion suggests that macropinocytosis is another lipid raft-dependent pathway.

Clathrin-dependent endocytosis provides the uptake of receptor-bound ligand complexes and extracellular fluid. Receptor-mediated means taking up specific ligands that bind to complementary cell-surface receptors. The process is typically initiated by formation of clathrin-coated vesicles (CCV) at the plasma membrane (Fig. 1). After losing the coat by the process of depolymerisation, these vesicles (~ 120 nm) are able to fuse with early endosomes (EE). EE form a tubulovesicular compartment in peripheral cytoplasm and are crucial for sorting events. Due to an ATP-dependent proton pump the pH gradually decreases through the endocytic pathway. Slightly acidic pH of EE causes dissociation of receptor-ligand complexes (Mellman I., 1996). Receptors accumulate in tubular part of EE and can be returned to the plasma membrane in recycling vesicles (RV). Ligands and other soluble molecules are stored in vesicular part of EE and after fission of tubular extensions are moved as endosomal carrier vesicles (ECV) to late endosomes (LE). Degradative enzymes are active in this compartment, but are more concentrated in lysosomes. Lysosomes are the final compartment of the endocytic pathway and can be distinguished from LE by higher density (Clague J. M., 1998). An ability to inhibit or alter traffic to later endocytic compartments has distinct advantages for drug-delivery strategies (Jones A. T. , 2001).

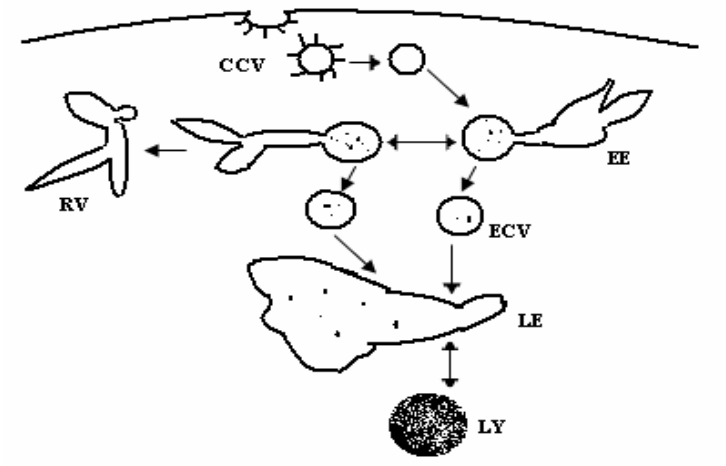

Figure 1. Clathrin-dependent endocytosis. CCV – clathrin coated vesicle, EE – early endosome, ECV – endosomal carrier vesicle, RV – recycling vesicle, LE – late endosome, LY – lysosome.

Recycling of transferrin

A well-described example of receptor-mediated endocytosis is the internalization and recycling of transferrin. Transferrin (Tf) is a major mammalian serum glycoprotein and transports iron from sites of absorbtion and storage to tissue cells. Receptors for Tf are found on all growing cells (Dautry-Varsat A. *et al*., 1983). Transferrin receptors have long been explored as a target for receptor-mediated delivery of anticancer drugs and genes and also as a means of transfer drugs across epithelial barriers *via* transcytotic pathways. Not-surprisingly these are upregulated in rapidly dividing cells as cancer cells usually are (Jones A. T. *et al*., 2003). Diferric transferrin (also called ferrotransferrin) binds to cell surface receptors and the complex is internalized (endocytosed). The pH of the early endocytic vesicles (5.9 – 6.0) causes dissociation of iron from the transferrin-receptor complex. The iron remains in the cell and is released from EE to the cytosol. Iron-free transferrin called apotransferrin remains bound to its membrane receptor and is recycled within RV to the cell surface. At the neutral pH of extracellular fluid apotransferrin dissociates from the receptor and is then available for binding other two iron atoms. This cycling is caused by binding activity of Tf and its iron-free analogue. At pH 5.9 apotransferrin binds to membrane transferrin receptors to the same extent and with the same affinity as does diferric transferrin at pH 7.0. Apotransferrin is quickly dissociated from this receptor when the pH is raised to 7.0 (Dautry-Varsat A. *et al*., 1983).

Rab proteins

There are various characteristic proteins (biochemical markers) for distinct endocytic compartments. Rab proteins are small GTPases belonging to ras superfamily, that regulates vesicular transport during endocytosis. They have been implicated principally in the control of vesicle docking and fusion. Rab activity is partly controlled through membrane association and the status of bound nucleotides (Rodman S. J. *et al*., 2000).

Compartment	pH	Marker proteins
Early (sorting) endosomes - EE	5.9 – 6.0	rab4, rab5, transferrin receptor, EEA1
Recycling vesicles - RV	6.4 – 6.5	rab4, rab11, transferrin receptor
Endosomal carrier vesicles - ECV	< 6	
Late endosomes - LE	5.6 - 6.0	rab7, rab9, ci-MPR, lgps, lamps
Lysosomes - LY	5.0 – 5.5	lgps, lamps

Table 1. Characteristics of organelles on the endocytic pathway (Reproduced from Clague J. M., 1998). Cation-independent mannose-6-phosphate receptor (ci-MPR), highly glycosylated and conserved members of protein family enriched in late endosomal and mainly lysosomal compartment (lgps, lamps).

2.2. Clathrin-dependent endocytosis

Internalization

Receptors determined for this type of endocytosis selectively accumulate at plasma membrane coated pits. Most of them are transmembrane proteins and they need sorting signals to be internalized (D'Hondt K., 2000). So-called internalization motifs encoded in cytoplasmic domains of these receptors are crucial for a coat assembly (Pollard D. T. *et al*, 2002). The best characterised is a YXXØ motif, where Y is tyrosine, X represents any aminoacid and Ø represents a bulky hydrophobic amino acid. Alternatively dileucine, dilysine motif, acidic cluster of amino acids or ubiquitination can serve as an internalization signal. Receptors can be endocytosed constitutively (LDL or transferrin receptor) or only after ligand binding and conformational change of cytosolic domain such as in the case of EGF (epidermal growth factor) receptor (Clague J. M., 1998). Transmembrane proteins lacking internalization signals are not internalized in these pits (Lodish H. *et al*., 2000). The pits must function as molecular filters collecting certain plasma membrane proteins (receptors) and excluding others. Many kinds of receptors cluster in the same coated pit (Alberts B. *et al*., 1994).

The targeting and transport specificity of the clathrin coat is derived from adapter or assembly protein (AP) components of the coat. The plasma membrane AP-2 complex consists of two large chains (α2 and β2 adaptins), an intermediate μ2 chain and a small chain σ2. The intermediate chain binds to a cytoplasmic domain of transmembrane

receptor and both large chains contain clathrin binding sites. Moreover there are other additional proteins linking AP complex to plasma membrane. Synaptojanin, amphyphisin and dynamin are the most famous ones. Amphyphisin binds to AP and recruits dynamin to the neck of budding vesicles. Synaptojanin binds to amphyphisin and also to dynamin (Lodish H. *et al*., 2000). The APs are proposed to link cargo selection to a clathrin coat (D'Hondt *et al*., 2000). On the whole, major components of the endocytic clathrin coated vesicle are AP-2 complex, clathrin triskelions and dynamin. Another class of proteins that may function in a manner similar to AP complexes are arrestins.

Purified clathrin molecules are called triskelions and contain three heavy chains and three light chains. After binding to AP-2 complex clathrin molecules polymerize and form polygonal lattice with an intrinsic curvature shaped as a cage (Lodish H. *et al*., 2000). An essential protein required for budding of CCV is dynamin. Dynamin is a large GTPase which works as a mechanochemical enzyme (D'Hondt *et al*., 2000). After dynamin subunits polymerize around the neck of a pit and form a collar (a ring-like structure), hydrolysis of GTP is thought to regulate contraction of the polymeric dynamin until the vesicle pinches off (Lodish H. *et al*., 2000).

CCV's

Even though CCV's are stable in the microenvironment of cytosol they lose their coat and the assembly particles just after their formation. A cytosolic chaperon protein Hsp70 catalyzes depolymerization of clathrin into triskelions, which can be reused (Lodish H. *et al*., 2000). Uncoated vesicles are able to fuse with early endosomes. The fusion conditions are assumed to be similar to EE homotypic fusion discussed later.

Early endosomes

Although the selective inclusion of receptors at plasma membrane coated pits is the initial sorting event during endocytosis, early endosomal compartment serves as a more specific level of sorting and quality control (Jones A. T. *et al*., 2003). Many proteins and lipid microdomains establishing the destination of endocytosed material have been already described. EE form dynamic tubulovesicular structures that interchange components with other endosomes, the Golgi complex and the plasma membrane through budding and fusion events (Berón W. *et al.*, 1995). The endosomal lumen is acidified through the activity of a proton pump, the vacuolar ATPase (Pollard D. T. *et*

al., 2002). Therefore EE host the initial dissociation of receptor-ligand complexes. Receptors and other membrane proteins accumulate in the tubular extensions and soluble contents accumulate in vesicular regions. The tubular parts pinch off to form recycling vesicles and the vesicular elements proceed to LE and lysosomes (Mellman I., 1996). Transit through EE is very rapid (2 - 3 min).

Early endosome fusion

The most widely studied fusion event on endocytic pathway of mammalian cells is homotypic fusion between early endosomes. *In vitro* assays that reconstitute this event have been constructed (Clague J. M., 1998) and will be discussed later.

Homotypic fusion is a fusion between vesicles that can not be distinguished using cell markers such as those described in Table 1. The reason for this fusion rises from the need of membrane for recycling tubular elements of EE. Because a sphere has the smallest ratio of surface area to volume, when two vesicles of equal diameter fuse with each other to form a larger sphere containing twice the volume, excess membrane is generated. Multiple fusion events generate large amounts of excess membrane. The excess membrane can be removed in a form of tubules, which have a high surface-to-volume ratio (Pollard D. T. *et al*., 2002).

There are thousands of shuttle vesicles in the cytosol at any one time and their task is to specifically find their target organelle, dock with it and deliver their cargo by fusing with the target membrane. This is aided by a number of cytosolic proteins that bind to membranes to mediate a traffic effect. Early endosome antigen (EEA1) is an example of one of these proteins and was discovered as an autoantigen associated with subacute cutaneous systematic lupus erythematodus. This protein resides in cytosol and specifically associates with the cytoplasmic face of the EE membrane. It has not been detected on other membrane compartments. EEA1 contains a double zinc finger on C-terminus, that is a cysteine rich domain also called FYVE domain. This part of EEA1 molecule interacts with phosphatidylinositol-3-phosphate (PI-3-P) and is a determination for endosomal targeting. PI-3-P is created from phosphatidylinositol in energy dependent process catalysed by phosphatidylinositol-3-kinase (PI-3-kinase). Other two binding sites in EEA1 molecule were found to be effectors of active form of rab5 (GTP form). The interaction between EEA1 of one endosome and rab5 of the second one acts as a docking/tethering signal necessary before EE fusion event (Wilson M. J. *et al*., 2000). A fungal metabolite wortmannin, a specific inhibitor of PI-3-kinase,

has multiple effects on endocytic processes including inhibition of fluid-phase uptake and early endosome fusion. Wortmannin causes dissociation of EEA1 from endosomal membrane and therefore inhibits fusion of EE (Jones A. T. *et al*., 1995). A protein called rabex 5, that associates with rabaptin 5, catalyzes the exchange of GDP bound to cytosolic rab5 for GTP inducing a conformational change in rab5, allowing it to bind to a surface protein on a particular endosome (Lodish H. *et al*., 2000).

After the docking of vesicles, fusion can proceed. Each vesicle has a pair of SNAREs (soluble NSF attachment protein receptors). Every v-SNARE (originally for donor vesicle) specifically binds to a cognate t-SNARE (a target membrane in the case of heterotypic fusion). SNAREs fit together like a key and a lock. *N*-ethylmaleimide-sensitive factor (NSF, NEM sensitive factor) and SNAP (soluble NSF attachment protein) bind to the t-SNARE/v-SNARE complex to stabilize perfusion complex. Fusion of the two membranes immediately follows prefusion. Finally the t-SNARE/v-SNARE complex must dissociate, probably in the presence of NSF and SNAP (Lodish H. *et al*., 2000). After untangling of SNAREs the new vesicle is capable of another fusion or step.

Late endosomes and lysosomes

After the detachment of recycling tubules, the vacuolar portions of EE move as endosomal carrier vesicles (ECV), sometimes also called multivesicular bodies, along microtubules toward the perinuclear region. Here the vesicles can fuse with LE, or according to another theory they maturate by processes of recycling and gradual gaining lysosomal hydrolases (Pollard D. T. *et al*., 2002). LE contain constituents of the fluid phase with very few receptors. LE also communicate extensively with Golgi network. LE are enriched in lgps/lamps, rab7, rab9 and ci-MPR. There is an active exchange of material between LE and lysosomes, where hydrolytically active enzymes initiate the degradative process. Lysosomes are distinguished from LE by higher density and the absence of ci-MPR, rab7 and rab9 (Mellman I., 1996).

2.3. *In vitro* assays of vesicular transport

The understanding of the molecular mechanism governing movement of proteins and lipids between the various compartments of eukaryotic cells is a key goal of cell biological research. This aim has been greatly facilitated by the development of assays

that reconstitute specific traffic events *in vitro*. The primary requirement for such experiments is a method for preparing membranes containing suitable markers. Mainly fusion events in various stages of endocytic pathway are studied. Assays of trafficking within the endocytic pathway have mainly focused on mixing of two complementary probes separately introduced into the donor and acceptor organelles. It is essential to ensure that the reaction occurs in sealed organelles and is prevented if either compartment is leaky (Cook R. N. *et al*., 2001).

All the assays can be subdivided into two groups according to the way of preparation of membranes. Cell free assays, using postnuclear supernatants (PNS) or purified subcellular fractions, and broken cell assays, in which the plasma membrane is disrupted by mechanical or chemical means, but the organisation of the internal organelles remains largely intact. The single most crucial parameter is to find optimal conditions for cell breakage (Cook R. N. *et al*., 2001).

Cell free assays

All the homotypic and most of heterotypic fusion assays use cell free systems. The major advantage is that individual membrane preparations can be stored as frozen aliquots. Moreover the absence of nucleus permits more extreme treatments. Conversely, the major disadvantage is the reduced overall efficiency of the reaction. The techniques of homogenisation comprise a ball homogeniser (cell cracker), a passage through a narrow gauge needle, freezing-thawing with violent mixing and sonication (Cook R. N. *et al*., 2001).

Broken cell assays

The primary advantage of this methods is the increased efficiency of the assay (may exceed 80%). The disadvantage is the need to prepare fresh membranes on each occasion. Broken cells can be prepared from adherent cells by selective disruption of the mammalian plasma membrane. Mechanical shear of adherent cells or scraping with a ‘rubber policeman’ can be used. Large holes are created allowing release and capture of transport vesicles, although the subcellular architecture is largely maintained. Selective disruption of the plasma membrane in suspensed cells can be achieved by gentle homogenisation in ball homogeniser or by freezing-thawing. Both types of cells (adherent and suspensed) can be permeabilised by treatment with mild detergents such as digitonin or pore-forming toxins such as streptolysin O (Cook R. N. *et al*., 2001).

Avidin-biotin interaction

The most widely used endocytic assay systems are based on mixing of an avidin-enzyme conjugate with a biotinylated probe (Cook R. N. *et al*., 2001). There is an exceptionally high affinity constant and low dissociation constant of the egg-white glycoprotein avidin (or its bacterial counterpart streptavidin) and the vitamin biotin. The general idea of this approach is that biotin, coupled to low- or high-molecular-weight molecules, can still be recognized by avidin, either as the native protein or bound to *e.g*. enzyme or fluorescent group. Avidin-biotin system serves as a universal tool in the research. The major applications are in immunoassays, affinity chromatography, flow cytometry or fluorescent microscopy. The disadvantage, which has to be solved, is the presence of certain 'undesirable' structural properties in the avidin molecule, which may lead to high levels of 'non-specific' binding (Bayer A. E. *et al*., 1990).

Performance of *in vitro* fusion assays

Gruenberg and Gorvel used an *in vitro* assay for reconstitution of early endosome fusion. Avidin and biotinylated horseradish peroxidase (bHRP) were chosen as markers. After the internalization step, when the markers were separately introduced into EE, the cells were homogenised and fractions containing EE were combined in the presence of ATP and cytosol. Following fusion a complex between avidin and bHRP was formed. Finally the membranes were broken by detergents in the presence of a quenching agent, biotinylated insulin, that binds free avidin. The avidin-bHRP was then immunoprecipitated with anti-avidin antibody and the enzymatic activity of bHRP was measured. The substrates of HRP (*o*-dianisidine and H_2O_2) were used and the brown-coloured product was quantified in a spectrophotometer at 455 nm (Graham J. M. *et al*., 1997).

Another assay was performed by Davey, Hurtley and Warren. The reaction between enzyme and substrate endocytosed separately into EE was used. Both the enzyme and the substrate were provided by enveloped animal viruses that use endocytic pathway as a means of entering cells during infection. Fowl plague virus with its own enzyme neuraminidase and Semliki Forest virus possesing tritium-labeled sialic acid residues were endocytosed. PNSs were prepared. The cells were first broken (homogenised) by several passages through homogeniser and the homogenate was centrifuged. The supernatant was stored at -80°C and after thawing, it was used for

fusion. The fusion enabled the neuraminidase of Fowl plague virus to cleave the tritium-labeled sialic acid residues. The fusion was monitored by determination of the amount of tritium-labeled sialic acid (Davey J. *et al*., 1985).

Woodman and Warren studied fusion between endocytic vesicles containing ^{125}I-transferrin and ones containing anti-transferrin antibody. The immune complex was quantified (Woodman G. P. *et al*., 1988).

All the assays use high potassium isoosmotic solutions buffered to approximate neutrality and contain an ATP regenerating system (creatine phosphate and creatine phosphokinase). Typically the mixtures of HEPES, KCl and sucrose are used (Cook R. N. *et al*., 2001).

Outcomes of *in vitro* assays

The major application of *in vitro* assays is the identification of the factors that are required for the reconstitued event. They can be also used to investigate the effects of drugs/macromolecules on the events studied. Now it is possible to reconstitue some steps using purified proteins instead of cytosol (Cook R. N. *et al*., 2001).

EE fusion is a time, temperature, cytosol and energy dependent event. NEM inhibits this effect and this led to the discovery of NSF as a fusogenic factor. It was elucidated that fusion was not dependent on the acidic microenvironment of EE, because NH_4Cl did not inhibit the assay (Wessling-Resnick M. *et al*., January 1990). When the fusion assay was carried out in the presence of mitotic cytosol, the fusion activity was reduced. This finding indicated that EE fusion was arrested during mitosis because of the cell-cycle control protein kinase cdc2 (Tuomikoski T. *et al*., 1989). The nonhydrolyzable nucleotide analog guanosine-5´-(3-thiotriphosphate) GTPγS was found to inhibit fusion and does this before the NEM sensitive step. The factor inhibited by GTPγS was identified as a rab5. Other functions of rab5 were later discovered. The addition of the fungal metabolite wortmannin, known to be a specific inhibitor of PI-3-kinase, was found to inhibit EE fusion (Jones A. T. *et al*., 1995). The comparison of yeast and mammalian cells led to the finding that some of the proteins governing the traffic events are highly conserved. This is demonstrated by the fact that fusion between vesicles originating from different cell types can occur. PNS prepared from K562 cells (human leukaemia cell line) containing avidin-linked probe can be employed in cell free fusion assays in conjunction with PNS with biotinylated probe originated either from

chinese hamster ovary cells or from K562 cells (Wessling-Resnick M. *et al*., October 1990). This was the assay studied in this project at Cardiff university.

2.4. Development of *in vitro* assays of early endosome fusion

The method used was developed by Marianne Wessling-Resnick and William Braell in 1990 (Wessling-Resnick M. *et al*., January 1990). A cell free system in this work utilizes a fluid phase probe (avidin coupled to β-galactosidase (av-β-gal)) and a modified ligand of the transferrin receptor (biotin-transferrin (bi-Tf)). K562, a highly undifferentiated cell line of the granulocytic series, is used for cell extracts preparation. The fusion of EE was monitored through the association reaction between the internalized avidin- and biotin-linked probes. The complex of β-gal-av-bi-Tf is formed after a successful fusion event. When the vesicles are lysed, the complex is released and can be captured in the wells coated with anti-transferrin antibody. The fusion can be quantified through β-gal, which converts the non-fluorescent substrate (methylumbelliferyl-β-D-galactopyranoside, MBG) to a fluorescent product (methylumbelliferone, MU).

Internalization and cell extracts preparation

K562 cells need to be harvested, washed with phosphate buffered saline (PBS) and resuspended in uptake buffer containing either av-β-gal or bi-Tf as EE probes. The probes can then be internalized under specific conditions. All the steps following internalization must be performed on ice. For PNS preparation the cells are resuspended in breaking buffer (BB) and the sample is passed several times through stainless steel ball homogenizer up to 90% cell breakage. PNS (containing EE and other membranes) are finally separated from heavier nuclei and intact cells. After protein concentration determination are the aliquots stored at -80°C.

In vitro vesicles fusion

PNS preparations containing internalized av-β-gal and bi-Tf are mixed on ice in a mixture including 1 mM ATP, 50 μg/ml creatine phosphokinase (CPK), 8 mM creatine phosphate (CP), 10 μg/ml biotin-insulin and 1 mM dithiothreitol (DTT). The final recommended protein concentration is 2 – 4 mg/ml. The addition of biotin-insulin into

the mixture serves as a scavenger of any avidin-linked probe, which may be present due to lysed vesicles. After the incubation of the reaction mixture at 37°C for 30 min the EE fusion is quenched by addition of membrane-disrupting lysis buffer.

ELISA procedure (MBG assay)

The fusion between EE is monitored by enzyme-linked immunosorbent assay (ELISA) procedure. Rabbit anti-transferrin antibody in a coupling buffer is incubated in wells at 4°C overnight (alternatively at 37°C for 3 hours). Coated wells are washed and filled with lysed sample. The samples are incubated for three hours and after washing the wells the β-gal activity as 'anti-Tf antibody – Tf-bi – av-β-gal' is measured. A detailed description of the method can be found in the experimental section.

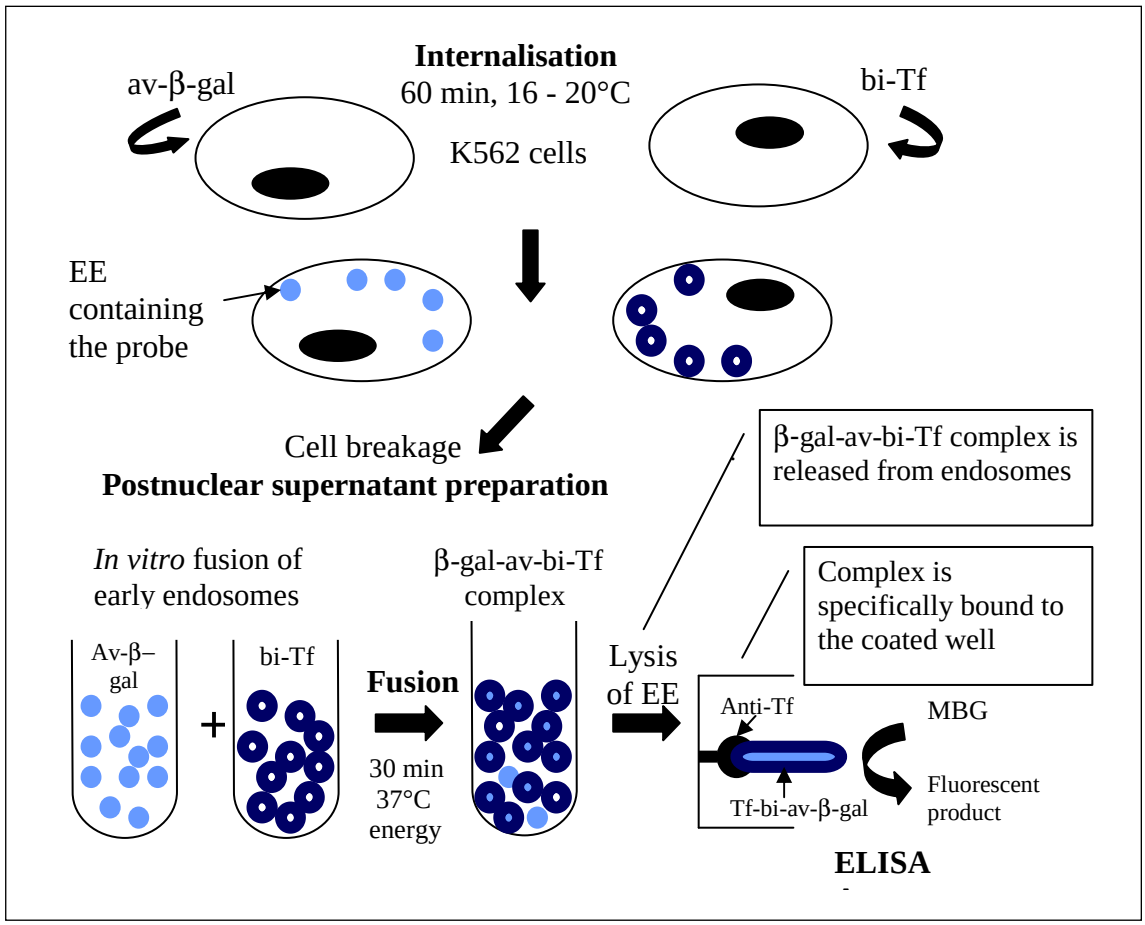

Figure 2. *In vitro* assay of early endosome fusion.

2.5. Cell penetrating peptides

Plasma membrane effectively prevents the uptake of macromolecules by limiting their passive entry. Traditional approaches to modulate protein function have relied on the discovery of specific drugs and small molecules, which could be easily delivered into the cell. However unlike 'information-rich' macromolecules, they often suffer from poor target specificity, unwanted side-effects and toxicity.

Remarkably the identification of a particular group of proteins with enhanced ability to cross the plasma membrane in a receptor-independent fashion has led to the discovery of a class of protein domains with cell membrane penetrating properties (Wadia S. J. *et al*., 2005). Not only do these proteins pass through the plasma membrane, but they are also able to act as vectors for small molecule drugs (doxorubicin), DNA (tumor suppressor gene p53), antisense oligonucleotides, peptides, proteins (β-galactosidase, horseradish peroxidase, RNAase A, Cre recombinase, green fluorescent protein), liposomes and even inorganic 40 nm iron particles (Wadia S. J. *et al.*, 2005).

Protein transduction domains

The best characterised peptides are those derived from HIV-1 transcriptional activator Tat protein, herpes simplex virus structural protein VP22 and *Drosophilla melanogaster* homeoprotein antennapedia transcription protein. The study of HIV has provided striking insights into the molecular biology of eukaryotic cells including macromolecular transport into and out of the nucleus, intracellular trafficking of proteins or fusion of lipid bilayers (Greene C. W. *et al*., 2002). HIV-1 Tat protein plays a critical role in viral replication. It is organised into three functional domains, one of them, called basic region, is thought to be required for nuclear import (Wadia S. J. *et al*., 2005). This sequence has been intensively studied and shorter domains have been characterized. The part extending from residues 49 to 57 (RKKRRQRRR) is generally considered to be the minimal sequence responsible for the cell internalization (Caron J. N. *et al*., 2004). Because arginine is a main component of this domain, synthetic peptides were prepared and tested both *in vitro* and *in vivo*. Octaarginine (R8) showed internalization characteristics similar to those of the Tat peptide (Nakase I. *et al*., 2004). There is no homology between the primary and secondary structure of these PTD and the rate of cellular uptake has been found to correlate to the number of basic arginine

residues present (Wadia S. J. *et al*., 2004). Even peptides synthesized from either D- or L-amino acids and reversed amino acid sequences are internalized (Lundberg M. *et al*., 2003).

Mechanism of internalization

Cellular mechanisms of Tat peptide uptake and internalization are still largely unexplored. The binding of Tat peptide to the plasma membrane does not involve any specific receptors, is not affected by low temperature and is saturable at very high protein concentration. The basic character of PTDs indicates the dependence on non-specific interactions between the charged side groups of arginine residues (guanidine head group is a critical structural feature) and sulfated-membrane-bound glycoproteins such as heparan sulfate proteoglycans (Tyagi M. *et al*., 2001). Each arginine is highly charged at physiological pH and can donate a hydrogen allowing the formation of stable hydrogen bonds with anions such as sulfate (Wadia S. J. *et al*., 2005). It has been estimated that internalization occurs by a constitutive lipid- and temperature-dependent macropinocytosis and that binding of the Tat PTD to the cell surface is sufficient to stimulate macropinocytotic uptake (Wadia S. J. *et al*., 2005). Possibly more than one pathway is involved in internalization. Although macropinosomes are thought to be inherently leaky vesicles compared to other types of endosomes, the majority of the Tat proteins remains trapped within these compartments where they are non-functional. Tat protein transduction domain does not appear to involve any distuption of the plasma membrane (Wadia S. J. *et al*., 2005). It has been shown that endosome disruption with choroquine can significantly increase Tat-fusion protein access to the cytosol and nucleus (Caron J. N. *et al*., 2004). The positive charge of PTDs allows these proteins in case of escape from endosomes to bind to negatively charged structures within the cells, such as DNA (Lundberg M. *et al*., 2003).

Discrepancies in findings

Many different results have been published regarding PTDs, however some artifacts concerning the cellular localization of various entities have been reported. A cellular redistribution with nuclear targeting of these proteins following cell fixation was demonstrated (methanol, ethanol, acetone and paraformaldehyde cause membrane disruption). FACS (flow cytometry analysis) quantification appeared to have been overestimated by extensive signal from the non-internalized peptide, which adheres

very strongly to the cell surface. Trypsin treatment has to be used prior to FACS analysis. Nevertherles there are over 50 demonstrations of biological responses obtained with cargos vectorized by various PTDs (Vives E., 2003). There are several explanations for this phenomenon: (1) PTD proteins exert effects on the cell surface, probably by affecting the receptors of plasma membrane, (2) the proteins exert their effect within endosomes, where the effect does not depend on cytosolic or nuclear localisation (β-gal, magnetic nanoparticles), or (3) the PTDs are released from the endosomes into the cytosol by endosomolysis. It is likely that endosomolysis is an infrequent event and only a few of the endosomes release their contents into the cytosol.

It is very difficult to make any conclusion regarding PTDs because of wide variety of the PTDs sequences used as cell penetrating peptides, large differences within the cargos (size, composition, physicochemical properties), different types and lengths of linkage between peptide and cargo, variable concentration of PTDs fusion chimeras, large heterogenity of cell types and protocols used in experiments (Brooks H. *et al*., 2005).

Factors which affect a particular proteins function by mutation, deletions in the amino acid sequence, changes in expression (overexpression, suppression) lead to alterations in normal cellular function and often underlie a wide variety of genetic and acquired disorders. The ability to manipulate cell biology at the protein level would provide us with a powerful tool for treatment of variety human diseases including cancer (Wadia S. J. *et al*., 2004).

3. AIMS OF THE PROJECT

The main goal of this project was to set up an *in vitro* assay of early endosome fusion. Antibodies against cytosolic proteins required for fusion and fusion inhibitors such as wortmannin, NEM and local anaesthetic could then have been studied to verify that the assay conforms with published literature. To date there are no studies on the effects of CPP on early endosome fusion and this could be easily tested provided an in-house assay was available. This knowledge would have given important information regarding the peptides putative effects on the integrity of the endosomal system.

4. MATERIALS AND METHODS

4.1. Materials

General chemicals

Phosphate buffered saline tablets (PBS), potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium hydroxide (NaOH), L,D-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-*N,N,N´,N´*-tetraacetic acid (EGTA), trometamol (Tris, TRIZMA base), octylphenol ethylene oxide condensate (Triton X-100), sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES sodium salt), bovine serum albumin (BSA), heparine sodium salt from porcine intestinal mucosa, 4-methylumbelliferyl-β-D-galactopyranoside (MBG) and adenosine 5´-triphosphate disodium salt (ATP) were purchased from Sigma (Dorset, UK). Glucose, sucrose, ethanol, sodium chloride (NaCl), sodium carbonate (Na_2CO_3) and hydrochloric acid (HCl) were purchased from Fisher Scientific Laboratories (Leicestershire, UK), creatine phosphate disodium salt (CP) and creatine phosphokinase (CPK) from porcine heart were purchased from Calbiochem (Nottingham, UK)

Protein conjugates

Insulin-biotin labelled from bovine pancreas, β-galactosidase (lactase, β-gal) and avidin-β-galactosidase (av-β-gal) were purchased from Sigma (Dorset, UK), biotin-transferrin (bi-Tf) was purchased from Molecular Probes (Leiden, Netherlands) and rabbit anti-human transferrin (anti-transferrin antibody) was purchased from Research Diagnostics, Inc.(Concord MA, USA).

Cell culture

K562 cells were purchased from ECACC (Wiltshire, UK), RPMI 1640 media containing L-glutamine, foetal bovine serum (FBS) and trypan blue solution 0.4% were purchased from Gibco BRL (Paisley, UK).

Buffers and solutions

Buffer A – 150 mM NaCl, 25 mM HEPES, pH 7.4

Bradford dye stock – 100 mg of Coomassie Brilliant Blue G-250, 50 ml of 95% ethanol, 100 ml of 85% H_3PO_4 made up to 1 liter with distilled water.

Breaking buffer (BB) - 0.1 M KCl, 85 mM sucrose, 20 μM EGTA, 20 mM HEPES, pH 7.4

Coupling buffer – 50 mM Na_2CO_3, pH 9.6

Developing buffer – 133 mM glycine, 83 mM Na_2CO_3, pH 10.7

CTR cocktail - 10 mM ATP, 80 mM CP, 0.5 mg/ml CPK, 0.1 mg/ml biotin-insulin, 10 mM $MgCl_2$, 40 mM NaCl, 10 mM DDT and 50 mM HEPES, pH 7.5

Dilution buffer – 0.05% Triton-X 100, 50 mM NaCl, 1 mg/ml heparin, 10 mM Tris, pH 7.5

Incubation solution - av-β-gal PNS, equal protein concentration of bi-Tf PNS,

30 μl of solution X and BB up to 50 μl per well

Lysis buffer – 10% Triton-X 100, 1% sodium dodecyl sulphate (SDS), 50 μg/ml biotin-insulin, 10 mM Tris, pH 7.5

MBG buffer - 0.3 mM MBG, 1.5 mM $MgCl_2$, 0.1 M NaCl, 19 mM 2-mercaptoethanol, 25 mM Tris, pH 7.5

Phosphate buffered saline (PBS) – 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4

Solution X - 16.7% of CTR cocktail (Figure 11) and 83.3% of BB

Uptake buffer – 150 mM NaCl, 1 mg/ml glucose, 1 mg/ml bovine serum albumine (BSA), 25 mM HEPES, pH 7.4

Washing buffer (WB) – 1% Triton-X 100, 0,1% SDS, 50 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5

4.2. Equipment

General laboratory equipment

Weighing scale Precisa 180A was purchased from Sartorius (Hanover, Germany), Varifuge 3.0 RS centrifuge was obtained from Heraeus Instruments (Germany), Eppendorf centrifuge 5417R was from Eppendorf (Hamburg, Germany), Optima^{TM} LE-80K ultracentrifuge and Beckman type 50.4 Ti ultracentrifuge rotor was from Beckman Coulter (USA), Sunrise absorbance reader was from Tecan (Austria) and cell cracker (stainless steel ball homogeniser) was from European Molecular Biology Laboratories (Heidelberg, Germany). Medium binding strips 6301 (wells) were purchased from

Thermo Electron Bioscience (Basingstoke, UK) and second type of wells was from Jencons-PLS (Leighton Buzzard, UK).

Cell culture

Neubauer haemocytometer was obtained from Weber Scientific (Sussex, UK).

Fluorescence measurement

FLUOstar OPTIMA machine was purchased from BMG LabTechnologies (Offenburg, Germany) and 96 wells plate COSTAR 3695 96 was purchased from Fisher (Loughborouhg, UK)

4.3. Methods

Maintenance of cells

K562 cells were grown in RPMI 1640 medium containing L-glutamine enriched with 10% FBS, 100 U/ml penicilin and 100 μl/ml streptomycin. The cells were maintained in a CO_2 incubator (5% CO_2 atmosphere, 37°C). The cells were split in a laminar flow cabinet at a cell density of 1×10^6 cells per ml. Usually 8 ml of confluent cells were added into 12 ml of pre-warmed medium in a sterile 75 ml flask. Passage numbers, total volume and date of splitting were recorded. The maximum passage number used was 50.

Cell number evaluation

A Neubauer haemocytometer was cleaned with ethanol and firmly covered with a coverslip. Cells in a flask were resuspended gently, 20 μl was removed and mixed with the same volume of 2% (v/v) trypan blue solution. Approximately 10 μl were placed at the edge of a coverslip. The haemocytometer was situated under the microscope and the living cells in one quadrant were counted. Trypan blue dye can permeate only a dead cell membrane, therefore blue stained cells were not counted in a cell number evaluation. The result was determined by the following formula:

$$\text{cells/ml} = \text{number of viable cells per quadrant} \times \text{dilution factor} \times 10^4$$

Bradford protein assay

Bradford protein assay is a spectroscopic method used for determination of a protein concentration of a solution. A protein-dependent colour change is measured. The colour of a Coomassie Brilliand Blue G-250 dye is pH dependent. Under the acidic conditions a doubly-protonated red form is most stable. However when binding to a protein with certain basic amino acids the unprotonated blue form becomes the more stable one. This method can not be used in the presence of detergents. Protein standards (most often BSA) are necessary for this assay. The Bradford dye stock, which is stable in a dark bottle at 4°C, contains 100 mg of Coomassie Brilliant Blue G-250, 50 ml of 95% ethanol and 100 ml of 85% H_3PO_4 made up to 1 l with distilled water. The non-linear calibration curve was usually measured for 0, 2, 5, 10 and 20 μg of BSA. The BSA was dissolved in a distilled water and finally 800 μl of the Bradford dye stock was added up to 1000 μl. The measured samples were diluted 1/100 and 1/10 with water and 5 μl of each was added into separate tubes containing 195 μl of water and 800 μl of dye stock. After adding 200 μl of each sample into 96 well plate the absorbance was measured using 595 nm filter. The protein concentration of the samples was estimated by comparison of the absorbance with the calibration curve.

MBG assay

MBG buffer was prepared and stored in 50 ml tube at – 20°C. It was pre-warmed to 37°C in a water bath and centrifuged at 1000 rcf for 5 min before each assay. Purchased av-β-gal was diluted with sterile PBS to final concentration 1 unit per 1 μl, divided into aliquots of 10 μl and stored at – 20°C. The stock of β-galactosidase was prepared similarly with the final concentration 5 units per 1 μl. A calibration of increasing amount of enzyme in MBG buffer was prepared on ice in 1.5 ml eppendorf tubes and incubated for 60 min at 37°C. The total volume of samples was usually 200 μl. Developing buffer (1 ml) was added into each sample to block av-β-gal (or β-gal) activity. Finally 200 μl of each sample were removed and added into 96 Costar well plate. The fluorescence was measured on FLUOstar OPTIMA reader with 360 nm excitation and 460 nm emission filter. Gain 1000 or 2000 were used for most of the experiments.

This assay was adapted to fusion assay when the captured av-β-gal complex was quantified rather than the free enzyme in a tube.

Postnuclear supernatant (PNS) preparation

Confluent K562 cells (1 million cells/ml) were collected by centrifugation (5 min, 1000 rcf), washed 3 × with PBS and were resuspended in twice pellet volume of uptake buffer containing either av-β-gal (0.5 mg/ml) or bi-Tf (1 μM). The cells were left to internalize the probe for 1 hour at 16 - 20°C with intermitent mixing. Av-β-gal probe had to be dialysed in uptake buffer against buffer A with 10 000 dialysis tube for 2 hours at 4°C before internalization to remove possible inhibitors of fusion. The temperature of internalization step is crucial to let the cells accumulate the probes in early endosomes. At 16 – 20°C the probes accumulate in EE and do not traffic to the next stages of endocytic pathway or to recycling vesicles in case of bi-Tf nor towards lysosomes in case of av-β-gal. After the internalization the cells were prepared for breakage. The cells were washed 5 × with ice cold PBS and resuspended in twice the pellet volume of breaking buffer (BB). A stainless steel ball homogeniser (cell cracker) was placed on ice with two 5 ml syringes and was used for homogenisation (cell breakage). The breakage and all the steps after internalization were performed on ice to prevent probes from moving into next compartments of endocytic pathway. About 6 plunges with 8.004 size ball of cell cracker were made. The breakage was observed under the microscope. Trypan blue was used for staining of dead cells. For next experiments 90% of cell breakage was required. Postnuclear supernatant (PNS) containing endosomes and other membranes was separated from heavier nuclei and intact cells by a centrifugation step (1000 rcf, 5 - 10 min, 4°C). PNS was harvested (upper layer) and dialysed for 2 hours against ice cold breaking buffer (10 000 dialysis tube). The concentration of proteins was determined with Bradford protein assay and aliquots of 100 μl were made, snap frozen in liquid nitrogen and stored at -80°C. It has been shown that samples retain fusion activity when stored under these conditions. PNS containing either av-β-gal or bi-Tf inside early endosomes were prepared in the same way.

Cytosol from K562 cells preparation

The process is very similar to PNS preparation. K562 cells were harvested, washed with PBS and put on ice. BB was prepared and pre-cooled. The cells were resuspended in twice the pellet volume of BB and let to swell on ice for 5 min. The ball homogeniser was set up and placed on ice. The cells were broken as described above and centrifuged

for 10 minutes at 1500 rcf. The supernatant was dialysed against ice cold BB for 2 hours at 4°C. The sample was loaded into microfuge tubes and centrifuged at 4°C for 5 min at 90000 rcf. The upper layer was collected, protein concentration was measured and aliquots were snap frozen in liquid nitrogen and stored at -80°C.

Preparation of wells

Anti-transferrin antibody (usually 4 μg per well) was diluted with coupling buffer and the solution (350 μl) was added into wells. Binding was performed in parafilm covered wells overnight at 4°C. Alternatively, binding was performed for 3 hours at 37°C. The wells were then washed three times with PBS, twice with washing buffer (WB) and incubated 15 min at 37°C with WB. One more washing with WB and three more with PBS followed. The wells were filled with PBS and stored at room temperature until loading with samples.

5. RESULTS AND DISCUSSION

5.1. Fluorometric method for measuring β-galactosidase activity (MBG assay)

β-Gal is an enzyme biosynthetised by the bacterium *Escherichia coli*. For maintenance of the active conformation the SH groups in the catalytic site are important and their presence makes the enzyme quite succeptible to oxidation. Magnesium and sodium are activators, but heavy metals, which complex sulfhydryl groups, and EDTA, which chelates magnesium, are inhibitors. This tetrameric protein can hydrolyse natural disacharide lactose and many synthetic substances. Fluorogenic substrates, such as methylumbelliferyl-β-D-galactopyranoside (MBG, Fig. 3a), are used in various detecting systems for the presence of this enzyme. MBG is a highly sensitive substrate for determination of glucuronidase levels when the released methylumbelliferone (7-hydroxy-4methylcoumarin, MU, Fig. 3b) is monitored. MU fluoresces strongly in ultraviolet light at pH 9-10, its conjugates show little or no fluorescence (Strachan R. *et al*., 1962). Wavelenghts 360 nm for excitation and 460 nm for emission were used. The fluorescence is approximately 100 × more intense at pH 10 than at pH 7 and the stability of fluorescence is increasing with the pH. The alkalisation of the system with developing buffer not only stabilizes MU, but also stops the reaction, because pH optimum of β-galactosidase lies between 6 - 8.

(a) (b)

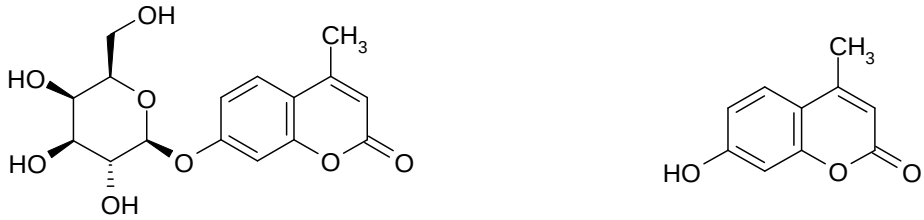

Figure 3. (a) Structural formula of MBG, which is non-fluorescent and can be converted by β-gal to MU (b), which fluoresces in ultraviolet light at higher pH.

The initial goal of this study was to generate a calibration curve for β-gal and av-β-gal concentration, the latter is used as a signal-making probe in *in vitro* fusion assay of EE. The fluorescence was measured after 1 hour incubation of samples with increasing concentration of β-gal in MBG buffer (Fig. 4). The stock of MBG buffer was stored

at -20°C and heated to 37°C and centrifuged before use. The graph shows the concentration dependent increase of the β-gal activity, the detection limit is 10^{-5} units.

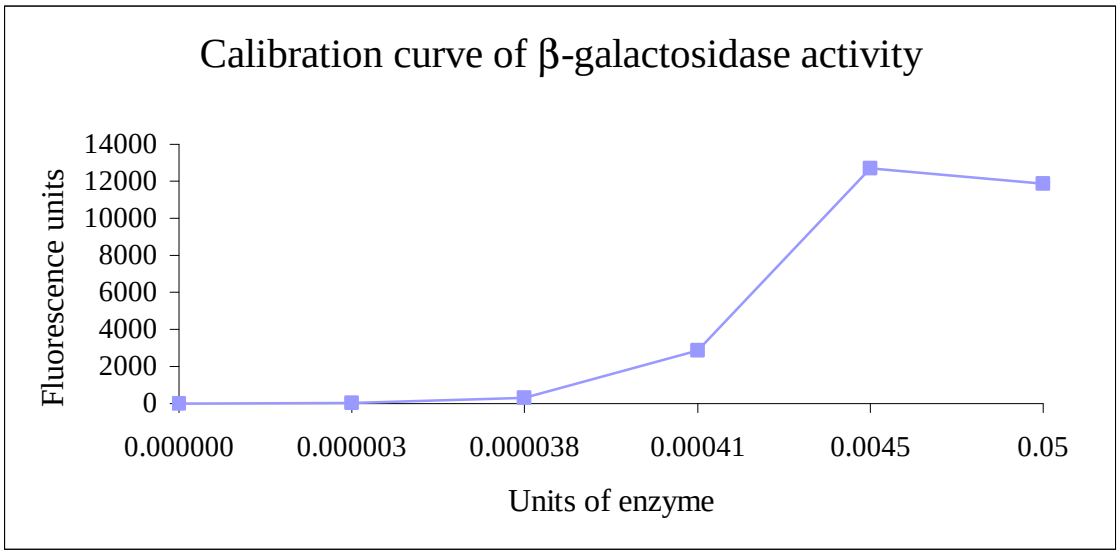

Figure 4. Calibration curve of β-gal (MBG assay). Solutions (200 µl) of enzyme in MBG buffer were prepared and incubated for 1 hour at 37°C in plastic eppendorfs. The reaction was stopped by dilution with developing buffer (1 ml). Final solution (200 µl) was added into each well of a Costar 96 well plate and the fluorescence was measured (gain 1000).

5.2. Avidin-β-galactosidase characteristics

MBG assay was repeated with increasing amounts of av-β-gal and a calibration curve was determined (Fig. 5). The possible change in the activity of modified enzyme and the increase of detection limit were investigated. More concentrated solutions of enzyme in MBG buffer were used, because lower activity of modified enzyme was assumed.

Experiments were also performed to investigate the effects of time (Fig. 6), temperature (Fig. 7) and the amount of substrate.

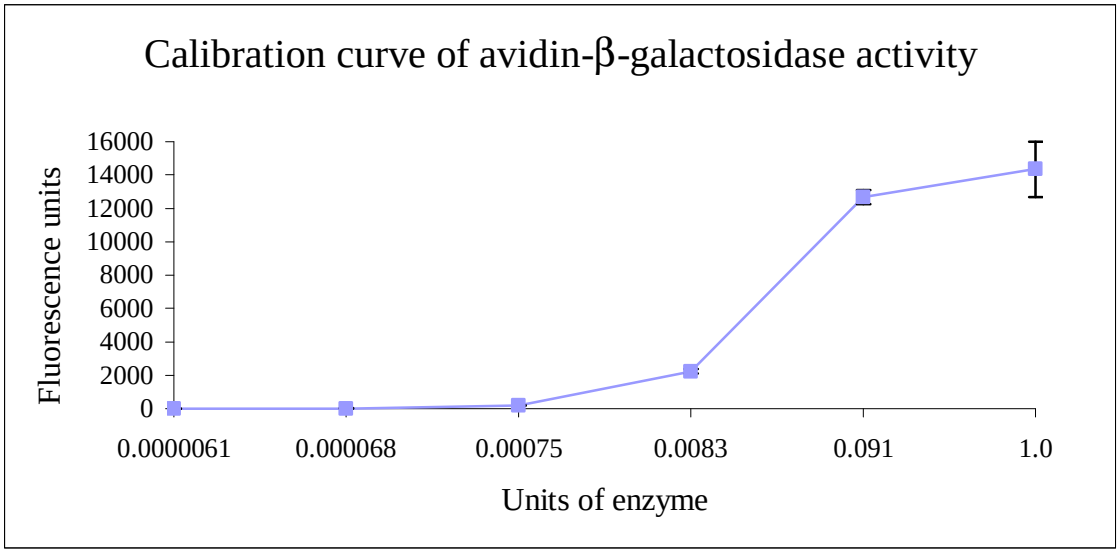

Figure 5. Calibration curve of av-β-gal (MBG assay). The detection limit is approximately 10 × higher than the one of β-gal. Error bars represent the mean and the standard deviation.

Time dependence with two different concentrations of enzyme in MBG buffer was measured. As expected there was a time-dependent increase in fluorescence at both concentrations.

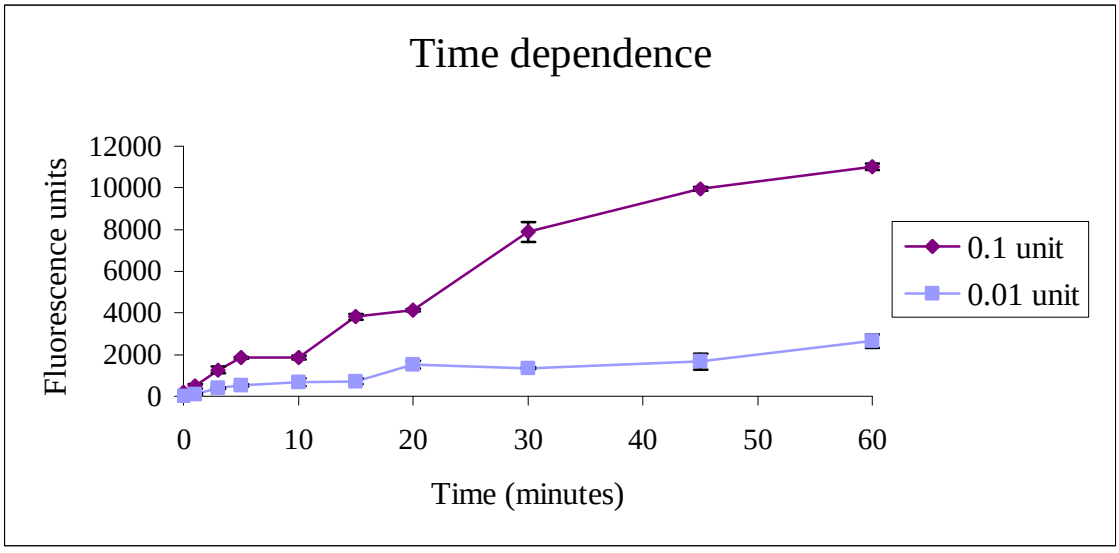

Figure 6. Solutions containing 0.1 and 0.01 units of av-β-gal in MBG buffer (200 µl) were prepared and incubated from 10 to 60 min at 37°C. The preparations were

made on ice and one sample kept on ice was used as a control (blank) value. All the samples were diluted at the same time with 1 ml of developing buffer and fluorescence of 200 µl of the final solution was determined.

A similar assay was performed at different temperatures. The optimal temperature for most of human-body enzymes is close to 37°C. There is a significant decrease in activity when incubated at 4°C (on ice).

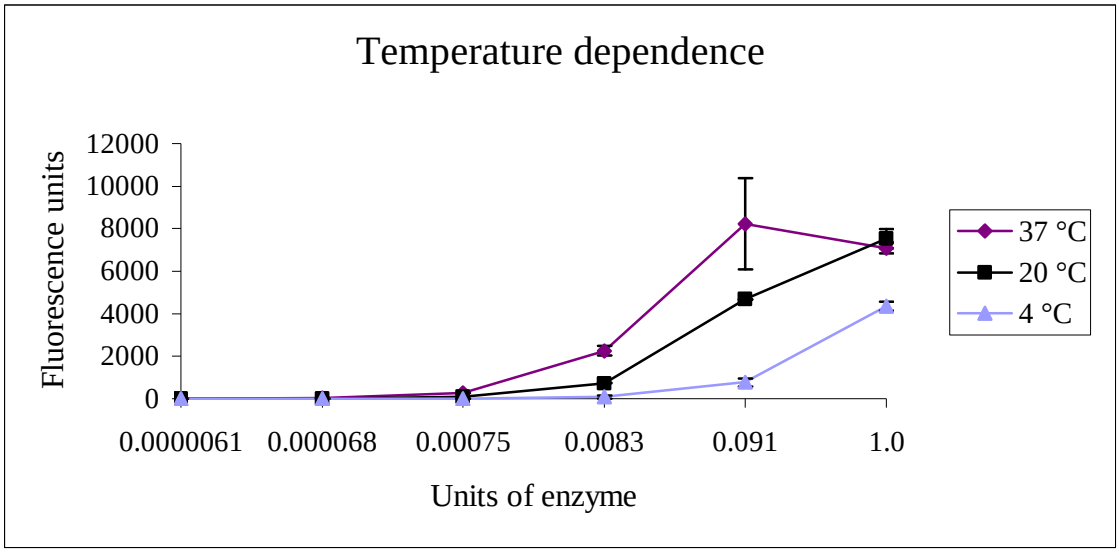

Figure 7. The samples were prepared the same way as for the calibration curve measurement (Fig. 4) and incubated for 60 minutes at different temperatures.

The last experiment testing the av-β-gal activity clarified the amount of substrate that can be converted after 1 hour incubation at 37°C. The volume of substrate was increased and therefore the concentration of enzyme was decreased. The solution of MBG used until now (200 µl of 0.3 mM MBG buffer) contains 60 nmol of substrate. This amount was not enough to saturate 0.1 unit of enzyme. When the volume of MBG buffer was increased to 300 µl (90 nmol) and 400 µl (120 nmol) the fluorescences of 0.1 unit of av-β-gal were equal, therefore the conclusion is that the saturation of 0.1 unit of av-β-gal lies between 60 and 90 nmol of MBG (data not shown).

5.3. Determination of anti-transferrin antibody specifity

Bi-Tf (a receptor-bound marker) is used as a complementary probe to av-β-gal in the fusion assay. Avidin has an exceptionally high affinity to biotin. Dissociation constant of avidin-biotin complex is 10^{-15}, which is extremely high and higher than 10^{-11} for antibodies-antigens or 10^{-5} for enzyme-substrate complex (Bayer A. E. *et al*, 1990). When these markers are separately introduced into EE, that are afterwards allowed to fuse, the probes are expected to associate and form β-gal-av-bi-Tf complex. The efficiency of fusion can be measured through β-gal, which can convert non-fluorescent MBG to a fluorescent product (MBG assay). Before fluorimetry the complex has to be released from EE and specifically captured in the well coated with anti-transferrin antibody. The specifity of anti-Tf antibody had to be therefore verified before fusion assay could be performed.

The wells were prepared as described in Materials and Methods and an increasing amount of anti-Tf antibody was placed in the individual wells. The solution containing av-β-gal (2.63 nM), bi-Tf (2.63 nM) and cytosol (2 - 4 mg of proteins per ml) was prepared. The amount of av-β-gal used for this experiment was chosen to correspond with 0.1 unit of enzyme. A control solution with av-β-gal and cytosol only for a measurement of non-specific binding of avidin to the surface of the wells was also prepared. Cytosol provided proteins for possible competition with exogenous transferrin for the anti-transferrin antibody. Water rather than breaking buffer (BB) was used as a solvent to dilute the samples up to 50 μl per well before incubation. After 30 minutes of incubation at 37°C the probes were expected to be totally bound to form β-gal-av-bi-Tf complexes. Non-complete lysis buffer (no biotin-insulin inside, 10 μl per well) and dilution buffer (300 μl per well) were added. The samples were centrifuged at 10000 rcf for 10 min at 4°C and pre-prepared wells containing PBS were re-filled with 350 μl of centrifuged sample. The samples were incubated for 3 hours and then the wells were washed as described. Substrate (350 μl of MBG buffer) was added to each well and these were incubated for 1.5 hour at 37°C. As already described MBG provides a substrate for av-β-gal, which is bound to bi-Tf-anti-Tf antibody-well, and a fluorescent product is released. The fluorimetry (300 μl of final solution) was read after diluting 300 μl of content of each well with 1 ml of developing buffer (Fig. 8). In this

experiment lysis buffer and dilution buffer were used to simulate the conditions of fusion assay.

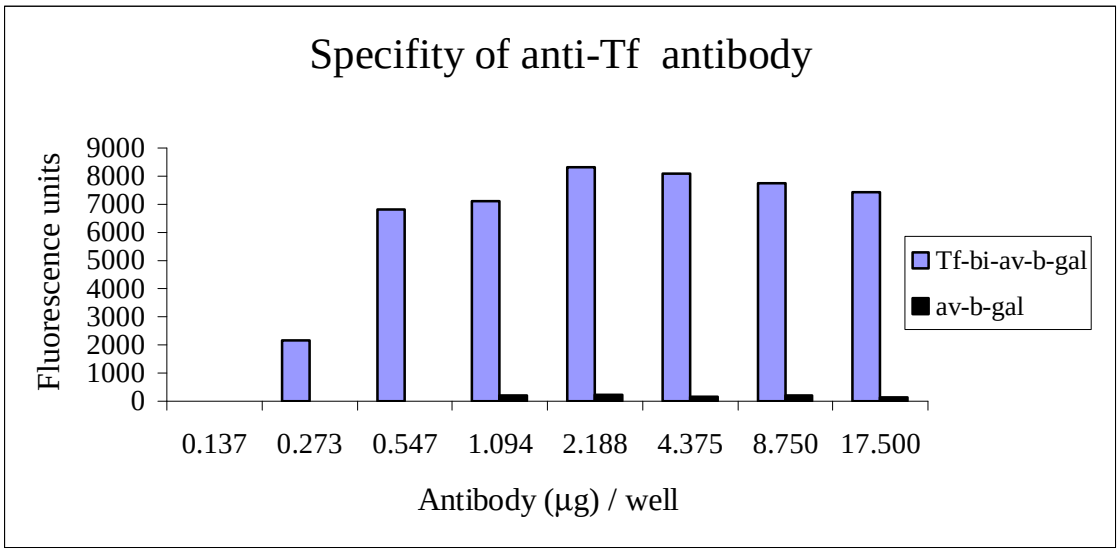

Figure 8. The sample (350 μl) was placed in each well. In the control samples biotin-transferrin was omitted for measuring non-specific binding of av-β-gal to anti-Tf antibody. There was a clear difference between complete (β-gal-av-bi-Tf) and control (av-β-gal only) sample. As the av-β-gal is not bound to anti-transferrin antibody, it does not bind to the well, and therefore the fluorescence is close to the blank value.

Another experiment was carried out to assess the specificity of the av-β-gal activity on anti-Tf antibody coated wells. An excess of biotin-transferrin was added into individual samples to investigate whether this would compete with the β-gal-av-bi-Tf complex for the antibody. Up to 500 times excess of bi-Tf was used. The symbol R is used to express the relationship between the amounts of markers inside the well.

$$R = [\text{bi-Tf}]/[\text{av-}\beta\text{-gal}]$$

This single probe was supposed to compete with β-gal-av-bi-Tf complex for anti-Tf antibody bound to the well surface and it was predicted that this would decrease the activity (Fig. 9).

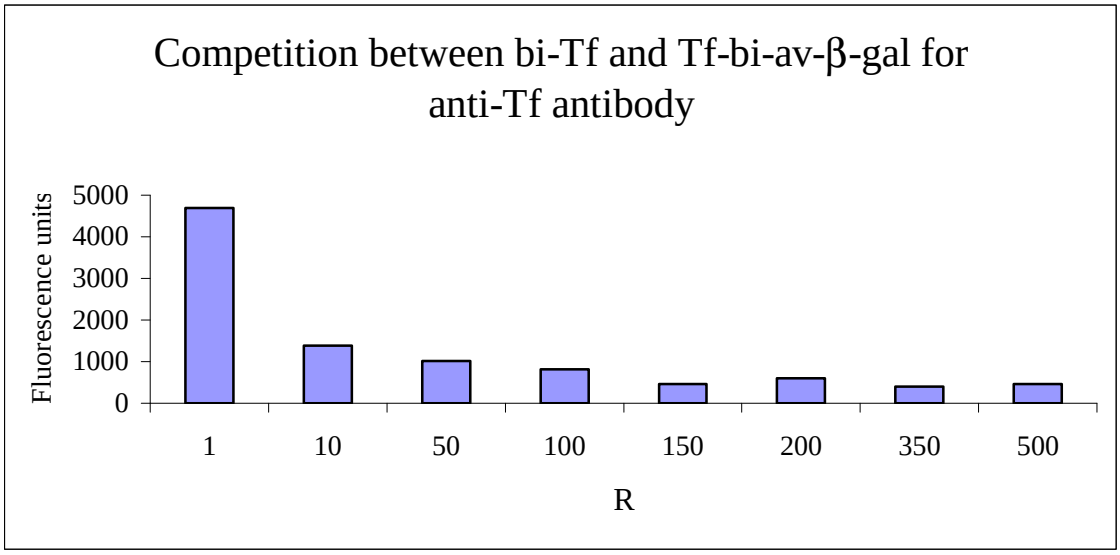

Figure 9. Different volumes of bi-Tf were added into individual samples before 30 min incubation at 37°C. The X axis values shows the amount of [bi-Tf]/[av-β-gal] in each well. The results confirm the theory of decreasing signal with increasing amount of bi-Tf.

5.4. Localisation of probes in postnuclear supernatant

The procedure for PNS preparation is described in Materials and Methods. The aim of this experiment was to prove that av-β-gal is localised inside of EE within the PNS fraction, the probe was quantified with MBG assay. PNS containing biotin-transferrin inside endosomes served as a control. MBG assay was performed on particular fractions after the PNS was centrifuged at 25000 rcf and 4°C for 10 minutes. The upper fraction (cytosol) was isolated and the pellet (membranes including endosomes) was resuspended in BB and centrifuged again to remove any residual cytosol. The pellet was finally resuspended in BB up to the same volume as the cytosolic fraction. This fraction was called membranes and the protein concentration of all the samples was measured. β-Galactosidase activities in the cytosol, membranes and intact PNS was then performed using the MBG assay. Prior to this, non-complete lysis buffer (10 µl, lacking biotin-insulin) was added into each sample and mixed gently. This will lyse the endosomes and the enclosed probes will be released. The samples were diluted to certain protein concentration with necessary volume of BB up to 50 µl. MBG buffer (300 µl) was added and the samples were incubated for 1 hour at 37°C. Developing

buffer (1 ml) alkalised the samples and inactivated av-β-gal. Finally the fluorescence was measured (Fig. 10). The experiment was carried out in 1.5 ml eppendorfs.

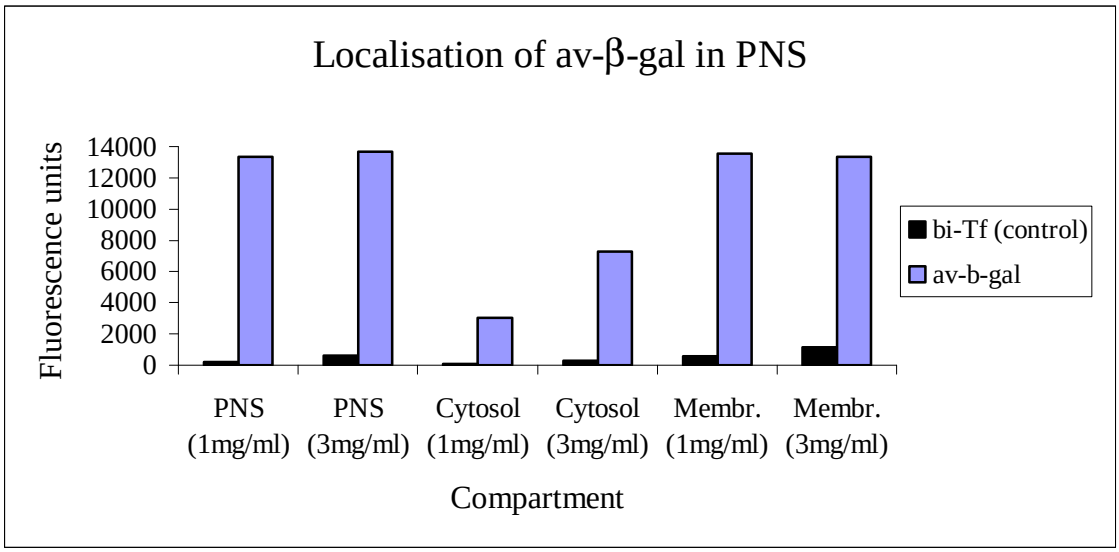

Figure 10. The numbers in brackets represent the protein concentration of samples. The fractions designated as Membr. represent fractions enriched in early endosomes. The graph shows that marker is mostly localised inside this fraction. The possibility exists that the fluorescence limit lies at 14000, thus the real values may be much higher. The same experiment was repeated with lower protein concentration (0.2 mg/ml and 0.5 mg/ml) to address this issue and the difference between membrane fraction and cytosol was 13000 fluorescence units (data not shown) with the membrane fractions having higher fluorescence than PNS fractions.

This experiment required adjustment of protein concentration of individual fractions to the same value. Adding different volumes of samples and BB could have been a source of some inaccuracy. Also measuring the protein concentration was dependent on the quality of pellet dissolving.

5.5. Fusion assay

These preliminary experiments then allowed for investigation of the full fusion assay. Two types of EE containing complementary probes (two types of PNS) were incubated for 30 - 60 min at 37°C in the presence of energy, biotin-insulin for

scavanging av-β-gal outside the endosomes, Na^+, Mg^{2+} and the reducing agent DDT. EE are assumed to fuse and fusion allows the probes to combine and form β-gal-av-bi-Tf complex. When the membranes are lysed with detergent, the complex is released and can be captured in the wells coated with anti-transferrin antibody. Finally the complex can be detected by MBG assay.

Fusion assay reagents

Reagent	**Stock solution**	**Comment**
ATP	0.1 M	pH 7.0 (247 mM NaOH) stored at -80°C
CP	0.8 M	stored at -80°C
CPK	10 mg/ml	in 10 mM Tris, pH 7.5, 0.1% BSA stored at -80°C
Biotin-Insulin	1 mg/ml	in 10 mM Tris, pH 7.5 stored at -20°C
DDT	1 M	
$MgCl_2$	0.1 M	
NaCl	4 M	
HEPES sodium salt	0.5 M	pH 7.4

Table 2. The stocks of fusion assay reagents.

Preparation of wells

This step is described in Material and Methods.

In vitro fusion of early endosomes

Incubation solutions (50 µl per well) were prepared and were composed of PNS with endocytosed av-β-gal, equal protein concentration of PNS with endocytosed bi-Tf, 30 µl of solution X (described in Materials and Methods) and BB to give a final volume of 50 µl per well. The amount of both postnuclear supernatants is determined by final protein concentration of incubation solution, which optimately should be between 2 - 4 mg/ml (Wessling-Resnick M. *et al*., January 1990). When the protein concentration is lower than this, there may be an insufficient amount of proteins required to support fusion. Too high protein concentration predisposes the assay to be inhibited by

inhibitory factors. Solution X is composed of 16.7% of CTR cocktail (Fig. 11) and 83.3% of BB. Non-complete CTR cocktail without ATP, CP and CPK can be prepared as a stock, that is stored at -20°C. ATP and energy regenerating system completes the CTR cocktail when the fusion assay is carried out. All the preparations must be performed on ice. The final concentrations of reagents in incubation buffer correspond to conditions used by Braell (Wessling-Resnick M. *et al.*, January 1990): 1 mM ATP, 8 mM CP, 50 µg/ml CPK, 10 µg/ml biotin-insulin, 1 mM $MgCl_2$, 4 mM NaCl, 1 mM DDT and 5 mM HEPES. Samples containing no av-β-gal PNS and samples containing no bi-Tf PNS were used as controls. The samples were incubated for 30 min at 37°C in plastic eppendorf tubes, a sample containing both marked types of endosomes incubated on ice was utilised as a control. No fusion should occur at this temperature. Fusion was terminated by addition of lysis buffer (10 µl per well). That contains strong detergents destroying biological membranes. The samples were vortexed gently and 300 µl of dilution buffer was added per well. Samples were vortexed again and centrifuged at 4°C at 10000 rpm for 10 min. All the steps excluding incubation were performed on ice.

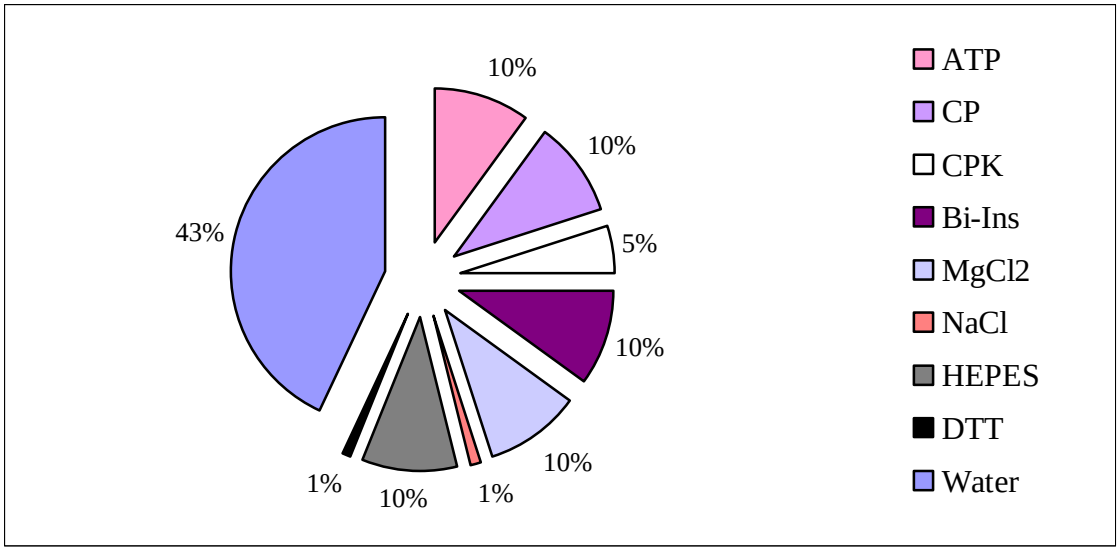

Figure 11. CTR cocktail. For the preparation the stocks of reagents described in Table 2 were used.

Binding to the wells

After the centrifugation step the samples (350 µl per well) were carefully added into pre-prepared wells and incubated at 37°C for 3 hours. Subsequently the wells were

washed to remove all the non-bound substances. The process of subsequent washing has already been described (3 × PBS, 3 × WB, 15 min incubation with WB, 1 × WB and 3 × PBS).

MBG assay

The wells were filled with 350 µl of MBG buffer and incubated for 90 - 120 min at 37°C. Finally 300 µl was carefully removed from each well (it was important to avoid touching the well surface) and mixed in sterile plastic 1.5 ml eppendorf with 1 ml of developing buffer. 300 µl of this solution was removed into a well of a Costar 96 well plate and the fluorescence of the sample was determined (Fig. 12).

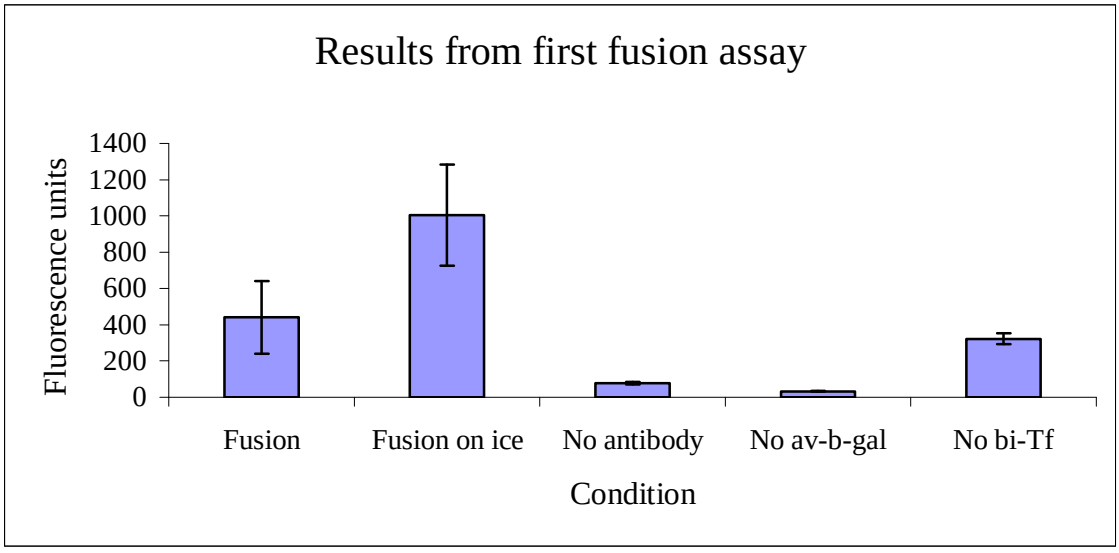

Figure 12. First attempt at the fusion assay. The protein concentration of incubation solution was 3.6 mg/ml and gain 2000 was used for fluorescence measurement.

Unexpectedly, the signal from tubes kept at 4°C was higher than those kept at 37°C. However no signal was detected in wells incubated in the absence of antibody. The fluorescence of sample without PNS containing bi-Tf was expected to be lower and this was confirmed in this experiment.

The experiment was repeated in a cold room, where the temperature did not exceed 4°C (Fig. 13). The results from this experiment show the expected higher values at 37°C, but the fluorescence values were almost 10 × higher in comparison with the

previous experiment. This was surprising as the same samples were used for both experiments. When these experiments were repeated we were unable to observe a temperature dependent fusion as shown in Figure 13.

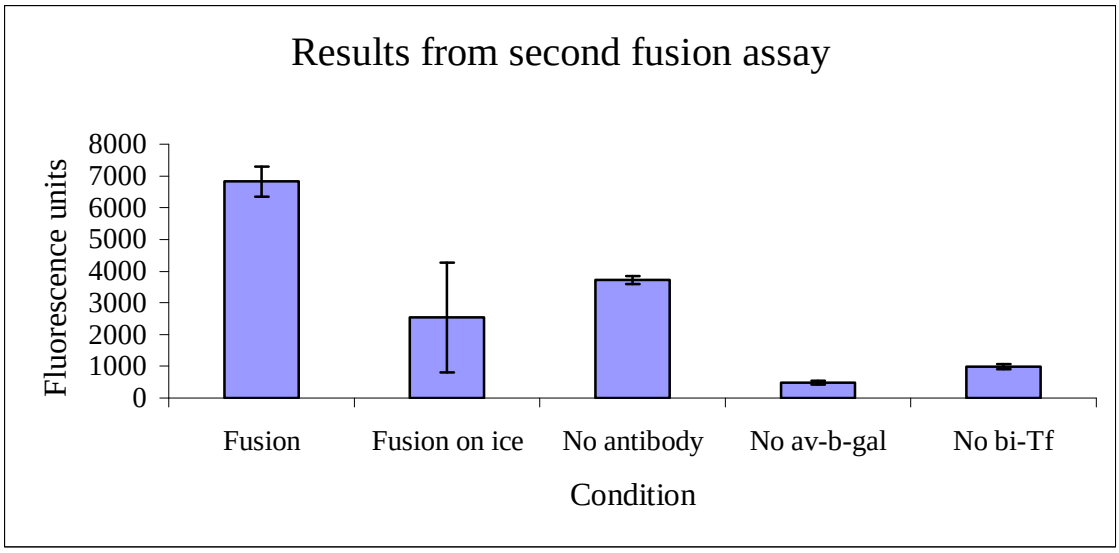

Figure 13. Fusion assay performed in the cold room. The difference between 37°C fusion sample and a 4°C control sample was about 3000 fluorescence units for gain 2000. Conversely there was much higher background arising from the fluorescence values of the sample incubated in uncoated wells.

Modifications of fusion assay

In view of the fact that we could not reprodicibly observe temperature dependent fusion a number of different variations on this method were attempted. These included using wells from an alternative manufacturer, alterations in composition of CTR cocktail, different method of PNS preparation or addition of extra cytosol to the fusion mixture. However we were unable to reproducibly obtain temperature / energy dependent fusion.

One mole of avidin binds to 4 moles of biotin, 1 mole of transferrin binds 4 moles of biotin and 1 mole of β-gal binds 1 mole of avidin. The probes combine at a ratio 1:1 and this is why equal protein concentrations of both types of PNS were used. But 1 mole of β-gal can bind up to 4 moles of avidin and therefore different combinations of mixed PNS were attempted. But again we did not observe any temperature dependent fusion.

The composition of reagents in the CTR cocktail was also assessed. The ATP preparation used contained magnesium and a possibility exists that the Mg^{2+} concentration was in excess. Fusion assays were therefore performed without the addition of $MgCl_2$ (Fig. 14). However the results show that this did not improve the assay.

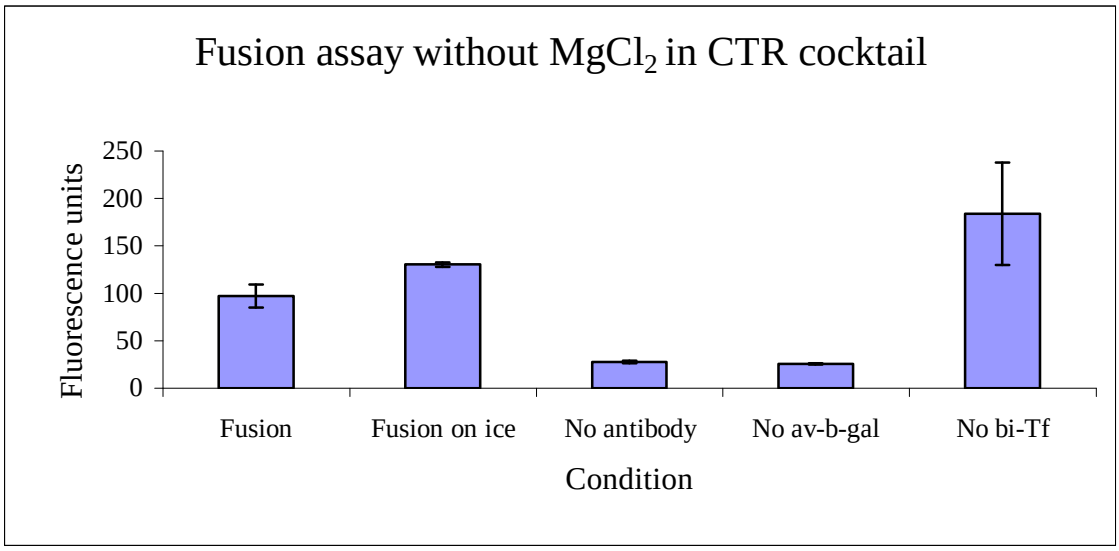

Figure 14. The assay was performed with CTR cocktail lacking extra added Mg^{2+}, which was assumed to inhibit fusion. Protein concentration of samples was 2.2 mg/ml.

The PNS used in these experiments was prepared with the stainless steel ball homogeniser (cell cracker). There may be a possibility that this method could be too aggressive and organelles like endosomes and lysosomes could be broken thus releasing the internalized probes. Released lysosomal enzymes could degrade the markers and inhibit fusion. There is an alternative method for PNS preparation. A freez-thaw method with violent mixing was then attempted (Fig. 15). The cells with endocytosed marker were resuspended in two pellet volumes of BB and snap frozen in liquid nitrogen. The samples were gently thawed and then vortexed vigorously. The process was repeated three times. PNS was divided from intact cells and nuclei by centrifugation. However the PNS from these experiments did not support fusion. Moreover the integrity of lysosomes, unlike endosomes, is not maintained during freezing and thawing (Wessling-Resnick M. *et al.*, January 1990). Another possible method for PNS

preparation is passing the cells through a narrow gauge needle, but this method was not investigated.

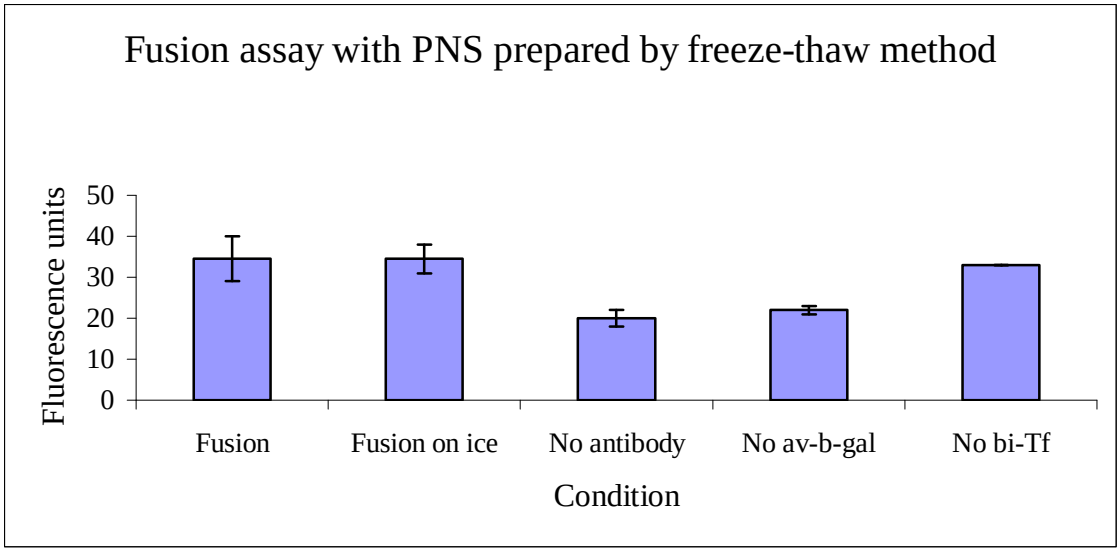

Figure 15. Fusion assay performed with PNS prepared by freezing-thawing with violent mixing. The signal of sample incubated at 37°C did not differ from the sample incubated on ice.

Finally experiments were performed to investigate whether supplementing fusion assays with prepared cytosol could enhance the low fusion signal. Various amounts of cytosol giving concentration of 0.5; 1.0 and 2.0 mg/ml were investigated and the results are shown in Fig. 16. The signal at 37°C was not enhanced in any of these cytosol supplemented assays. It is likely that cytosol did not have a positive effect on fusion, because there might have been enough factors important for fusion of EE. It seems like the fusion was detected, because samples incubated on ice gave lower values of fluorescence than samples incubated at 37°C. Once again the data shown in Figure 16 was not consistently observed and in subsequent assays the 4°C control values were higher than those in tubes incubated at 37°C.

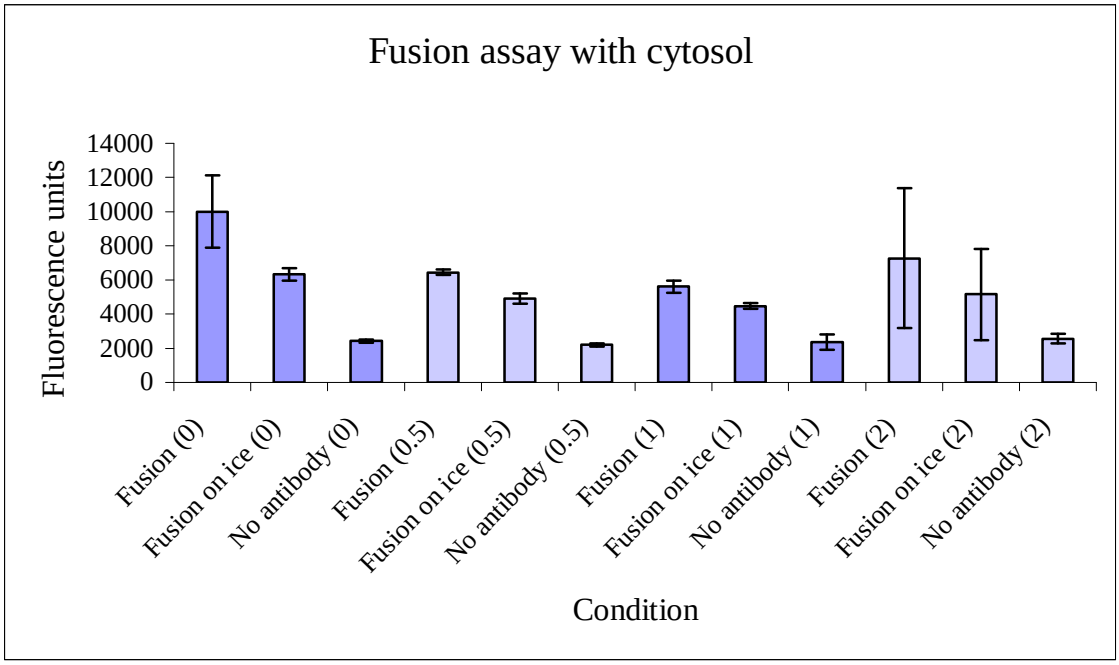

Figure 16. The effect of supplementing the fusion assay with cytosol was assessed. The numbers in brackets represent protein concentration of cytosol in the incubation solution. Four sets of samples were prepared: 0 mg/ml, 0.5 mg/ml, 1 mg/ml and 2 mg/ml additional protein concentration. First three colums serve as a standard where no cytosol was added. The total protein concentration of samples was 2.9; 3.4; 3.9 and 4.9. Gain 2000.

There was a concern that the probes were outside the endosomes where they could even in the presence of insulin-biotin combine and lead to false results. A simple modification was attempted whereby the endosomes containing internalized probes were isolated from the PNS via centrifugation at 25000 rcf for 10 min at 4°C. The resulting pellet was washed with BB, centrifuged again and resuspended in BB. This 'purified' PNS and extra cytosol were added into incubation buffer and fusion assay was performed. There was no improvement in fusion compared with previous assays.

Despite the fact that the MBG assays was developed and specific binding of the β-gal-av-bi-Tf to the transferrin antibody in the wells was shown, we were unable to reprodicibly observe fusion at 37°C. Thus we were unable to determine the effects of CPP on early endosome fusion.

5.6. Assay for measuring lysosomal degradation of avidin-β-galactosidase

One possibility for the the low efficiency of fusion was degradation of the av-β-gal in the lysosomes of the cells. We therefore determined whether we could measure the degradation of this probe under similar conditions to those used for the fusion assays.

The degradation assay is based on a published method (Nieland J. F. T. *et al*, 2004). Confluent K562 cells were collected and washed 3 × with PBS. Cells were resuspended in twice the pellet volume of ice-cold uptake buffer containing 0.5 mg/ml av-β-gal and incubated with intermitent mixing for 1 hour at 19.5°C. A sample without enzyme was prepared for a control. The samples were washed 5 × with ice cold PBS to remove any non-internalized marker and were resuspended in uptake buffer without BSA and av-β-gal. Aliquots for chasing at 37°C and control on ice were then prepared. During this 37°C incubation, the marker was expected to follow the endocytic pathway from EE through endosomal carrier vesicles to LE and lysosomes. LE and mainly lysosomes contain degradation enzymes which will cleave and degrade av-β-gal and therefore the fluorescence which is measured afterwards is expected to decrease with the time of incubation. After the chase, the cells were resuspended in twice the pellet volume of ice-cold BB and were broken. Freezing-thawing method previously described for cell breakage was used. The samples were centrifuged for 5 min at 1000 rcf at 4°C and PNS was collected. The protein concentration of all the samples was measured and MBG assay was performed. PNS was mixed with lysis buffer (10 µl) to release av-β-gal from organelles and BB was added up to 50 µl. The protein concentration of this solution was adjusted to 1 mg/ml. MBG buffer (300 µl) was added and the samples were incubated for 1 hour at 37°C. The reaction was stopped with developing buffer (1 ml) and the fluorescence was measured (Fig. 17).

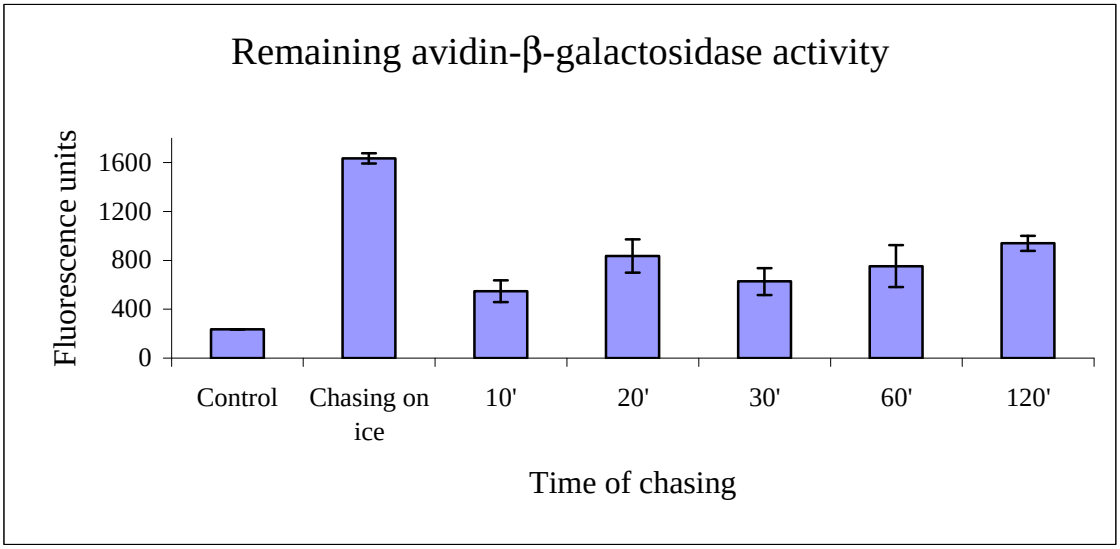

Figure 17. In control sample no enzyme was endocytosed. Chasing on ice did not allow the probe to move from EE and no degradation was detected.

As expected the maximal fluorescence was observed in samples incubated on ice after the initial pulse. However we were surprised to observe that even 10 min of chase resulted in a significant reduction in cell associated av-β-gal activity. This rapid decrease was not observed in reports utilising the same method (Nieland J. F. T. *et al*., 2004)

6. CONCLUSIONS

This diploma thesis was carried out under the sponsorship of Erasmus program. The aim of this study was to set up an *in vitro* assay of early endosome fusion at the Welsh School of Pharmacy. The method was originally described by Braell and Wessling-Resnick using internalized probes in K562 cells (Wessling-Resnick M. *et al*., January 1990). Two complementary probes (avidin-β-galactosidase and biotin-transferrin) were separately introduced into early endosomes. Once the PNS was prepared conditions suitable for fusion were applied and the resulting complex was determined using fluorimetry. Initially it was necessary to develop a β-gal activity assay in the laboratory, and determine the specificity of complex binding to the transferrin antibody on the wells. These aims were successfully completed and this then allowed progress to development of the full fusion assay. Unfortunately despite numerous attempts utilising a number of different conditions, fusion could not be reproducibly measured. Time-constraints hindered further experiments that may have yielded more promising data.

Once this assay will be set up it can be used to answer many questions under the remit of drug delivery on the endocytic pathway. One of these as previously noted involves the effects of cell penetrating peptides on endosome integrity and fusion.

The experiment for measuring activity of endocytic probe during passage through endocytic pathway *in vitro* was performed and temperature-dependent degradation was observed. The signal of the probe decreased with the time of incubation at 37°C suggesting the probe was degraded by lysosomal enzymes. The results obtained show that there is a significant and rapid reduction in β-gal activity with chasing at 37°C. This needs to be further investigated for example by performing the same experiments at lower temperatures to assess whether any degradation may be occuring under these conditions.

7. SHRNUTÍ

Tato diplomová práce byla zpracována pod záštitou programu SOCRATES/ ERASMUS na Welsh School of Pharmacy v Cardiffu.

Cílem mé práce bylo modifikovat již vyvinutou metodu (Wessling-Resnick M. *et al*., January 1990) a přizpůsobit ji podmínkám tamní laboratoře. Předmětem studia byla subpopulace tzv. časných endosomů, které vznikají těsně při povrchu plasmatické membrány po rozpadu klatrinového pláště. Jednou z jejich vlastností je schopnost spojovat se, fúzovat, čímž vzniká nadbytečná membrána potřebná pro další osud těchto organel.

Ve svých experimentech jsem pracovala se dvěma komplementárními markery. Jedním z nich byl avidin navázaný na enzym β-galaktosidasu, která štěpí kromě laktosy také syntetické substráty za vzniku příslušných produktů. V této studii byl produktem specifické hydrolýzy methylumbeliferylgalaktosidu intenzivně fluoreskující methylumbeliferon. Druhým markerem byl biotinylovaný transferin. Podstatou komplementarity markerů je vysoká afinita avidinu k biotinu. Dvě skupiny buněk byly inkubovány se zmíněnými markery a po jejich akumulaci v endosomech následovala mechanická destrukce buněk za uvolnění organel. Oba typy endosomů byly smíchány v prostředí podporujícím jejich fúzi. Spojením dvou endosomů nesoucích různé markery došlo k jejich rychlé kombinaci za vzniku komplexu β-galaktosidasa – avidin – biotin – transferin. Přidáním detergentu se rozpadla membrána spojené organely a uvolnil se její obsah. Ke kvantifikaci komplexu byl použit ELISA test. Celý komplex byl prostřednictvím transferinu zachycen na povrchu jamky s navázanou protilátkou proti transferinu a po přidání substrátu pro β-galaktosidasu byla změřena fluorescence vzorku.

Ačkoli přípravné experimenty poskytovaly optimistické výsledky, samotná fúze neprobíhala reprodukovatelně jako proces závislý na teplotě a energii. Z časových důvodů se mi nepodařilo přizpůsobit podmínky pro optimální průběh metody. V současné době na ní pracuje zbytek týmu. Po úspěšném zavedení metody fúze časných endosomů *in vitro* budou vytvořeny podmínky pro testování nejrůznějších léčiv, i těch potenciálních, a jejich vlivu na tento krok endocytosy. Cílem je najít selektivní inhibitory fúze s potenciálem rozštěpit endosomální membránu. Tím by byla

otevřena cesta široké škále léčiv, která jsou za normálních okolností zachycena v endosomálním kompartmentu a následně hydrolysována v lysosomech.

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