Analysis of Two Members of the EXT Family, EXT1 and EXT2 Bearing ER-, Golgi-, and Secretory Vesicle-Sorting Motifs

by

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ABSTRACT

EXT1 and EXT2 are type II transmembrane glycosyltransferases involved in the biosynthesis of heparan sulfate (HS) polysaccharide chains (McCormick *et al.*, 2000). These two glycoproteins are found predominantly in the ER when overexpressed individually but form a Golgi-localized heterocomplex when co-transfected together (Kobayashi *et al.*, 2000; McCormick *et al.*, 2000). To examine the roles of the N-terminal domains of EXT1 and EXT2, a series of chimeric cDNAs were constructed with ER-, Golgi- and secretory vesicle-sorting motifs. These constructs were transiently expressed in mammalian cells and tested for *in vivo* HS biosynthetic activity, heterocomplex formation, and subcellular localization. All EXT1 chimeras were able to synthesize HS *in vivo* as determined by their ability to restore herpes simplex virus infectivity to normally resistant HS-deficient Sog9 cells, though with varying efficiencies. Sec-EXT1 and Sec-EXT2 were able to form a heterocomplex together despite the loss of their wild-type (wt) cytoplasmic N-termini, putative transmembrane domains and 10 aa of their stem regions, suggesting that the N-termini of EXT1 and EXT2 are not essential for heterocomplex formation. The steady state subcellular distribution of the chimeras was determined by indirect immunofluorescence microscopy. As specified by their sorting motifs, ER-EXT1 and ER-EXT2 distributed in staining patterns distinctive of the ER, and Sec-EXT1 and Sec-EXT2 displayed staining patterns indicative of secretory vesicles. Though Golgi-EXT1 and Golgi-EXT2 did result in perinuclear distribution, a majority of transfected cells exhibited staining patterns typically observed for ERGIC- or early Golgi-localization. Interestingly, when each of the EXT1 chimeric constructs was co-transfected with wt EXT2, both proteins co-localized together and exhibited a staining pattern characteristic of the Golgi

regardless of their respective sorting signals. This suggests that when a wt anchoring partner is present, chimeric EXT1s are able to form a heterocomplex with wt EXT2 and localize to the Golgi. Taken together, the results presented in this thesis suggest that the major Golgi-localization mechanism used by EXT1 and EXT2 is hetero-oligomer formation as described in the kin recognition model (Nilsson *et al.*, 1993) which hypothesizes that Golgi-resident proteins form complexes too large to enter transport vesicles and are thus retained in the Golgi.

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List of Abbreviations

aa	-	amino acid
BSA	-	bovine serum albumin
COPI	-	coat protein I
COPII	-	coat protein II
ER	-	endoplasmic reticulum
ERGIC	-	ER-Golgi intermediate compartment
GAG	-	glycoaminoglycan
GlcA	-	D-glucuronic acid
GlcA-T	-	D-glucuronic acid transferase
GlcNAc	-	N-acetylglucosamine
GlcNAc-T	-	N-acetylglucosamine transferase
HME	-	hereditary multiple exostoses
HPLC	-	high performance liquid chromatography
HS	-	heparan sulfate
HSPG	-	heparan sulfate proteoglycan
HSV-1	-	herpes simplex virus type 1
HSV-2	-	herpes simplex virus type 2
Ii	-	invariant chain
Iip31	-	invariant chain protein isoform 31
Iip33	-	invariant chain protein isoform 33
LOH	-	loss of heterozygosity
M.O.I.	-	multiplicity of infection
NDST	-	N-deacetylase/N-sulfotransferase
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PenStrep	-	penicillin-streptomycin
pfu	-	plaque forming unit
PM	-	plasma membrane
TBS	-	Tris buffered saline
TGN	-	*trans*-Golgi network
TMB	-	3,3',5,5'-tetramethylbenzidine
wt	-	wild-type

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1. Introduction

1.1 Hereditary Multiple Exostoses (HME)

Hereditary multiple exostoses (HME) is one of the most common hereditary skeletal dysplasias in humans with an estimated prevalence of 1:50,000 (Schmale *et al.*, 1994) though this increases to 1:1,000 among the Chamorros of Guam (Krooth *et al.*, 1961). It is characterized by the formation of multiple benign cartilage-capped tumours (exostoses) at the growth plate of the epiphyses of long bones (Solomon, 1963). These exostoses are present or develop shortly after birth and continue to grow into puberty. They can cause a variety of orthopedic deformities such as limb length discrepancies and short stature. Most exostoses are non-problematic but surgical removal of exostoses may be required in instances where the exostoses are pressing on nerves or surrounding soft tissues (Paik *et al.*, 2000). In severe presentation of the disorder, limb length equalization procedures may also be necessary (Dahl, 1993). Another serious complication of HME occurs in 0.5-2% of HME patients in which the benign tumours undergo malignant transformation into chondrosarcomas (Hennekam, 1991).

Linkage studies show that mutations in either *EXT1* on chromosome 8q24.1 (Cook *et al.*, 1993) or *EXT2* on chromosome 11p11-p12 (Wu *et al.*, 1994) are responsible for the majority of HME cases though *EXT3* on chromosome 19p (Le Merrer *et al.*, 1994) has also been implicated in the disease as well.

1.2 EXT1 and EXT2

The human and mouse *EXT1* and *EXT2* genes have been cloned and sequenced (Ahn *et al.*, 1995; Lin and Wells, 1997; Stickens *et al.*, 1996; Stickens and Evans, 1997) as have the *Drosophila melanogaster* homologues of *EXT1* and *EXT2* (Bellaiche *et al.*, 1998; The *et al.*, 1999), and the *Caenorhabditis elegans* (*C. elegans*) homologue of *EXT2* (Clines *et al.*, 1997). Amino acid (aa) comparisons between the human, mouse and *Drosophila* EXT1 homologues show the greatest similarity in the C-terminal half of the protein (Cheung *et al.*, 2001). Human, mouse and *C. elegans* homologues of EXT2 also show the C-terminal end of the aa sequence as being the most conserved (Clines *et al.*, 1997). The finding that the C-terminal domains of EXT1 and EXT2 is the most conserved part of both proteins implies that these two domains are important, and in agreement with this, all etiological missense mutations identified to date are localized in the C-termini of EXT1 and EXT2 (Philippe *et al.*, 1997; Wuyts and Van Hul, 2000).

EXT1 and *EXT2* are expressed ubiquitously (Ahn *et al.*, 1995; Stickens *et al.*, 1996) and encode the exostosin-1 (EXT1) and exostosin-2 (EXT2) proteins, respectively (Fig. 1A). Further analysis of these two proteins revealed that they have a type II transmembrane configuration (Fig. 1B), with a cytoplasmic N-terminus, a single putative transmembrane domain, a stem region with little/no predicted secondary structure followed by a large lumenal C-terminus (McCormick *et al.*, 2000). They are 31% identical (Wuyts *et al.*, 1996) with *EXT1* predicted to encode a 746 aa protein, and *EXT2*, a 718 aa protein.

A

EXT1 amino acid sequence

1 8 27

MQAKKRYFILLSAGSCLALLFYFGGLQFRASRSHSRREEHSGRNGLHHP
SPDHFWPRFPEPLRPFVPWDQLENEDSSVHISPRQKRDANSSIYKGKKC
100
RMESCFDFTLCKKNGFKVYVYPQQKGEKIAESYQNILAAIEGSRFYTSDPS
QACLFVLSLDTLDRDQLSPQYVHNLRSKVQSLHLWNNGRNHLIFNLYSGT
WPDYTEDVGFDIGQAMLAKASISTENFRPNFDVSIPLFSKDHPRTGGERG
FLKFNTIPPLRKYMLVFKGKRYLTGIGSDTRNALYHVHNGEDVVLLTTCKH
GKDWQKHKDSRCDRDNTEYEKYDYREMLHNATFCLVPRGRRLGSFRFL
EALQAACVPVMLSNGWELPFSEVINWNQAAVIGDERLLLQIPSTIRSIHQD
KILALRQQTQFLWEAYFSSVEKIVLTTLEIIQDRIFKHISRNSLIWNKHPGGL
FVLPQYSSYLGDFPYYYANLGLKPPSKFTAVIHAVTPLVSQSQPVLKLLVA
AAKSQYCAQIIVLWNCDKPLPAKHRWPATAVPVVVIEGESKVMSSRFLPY
DNIITDAVLSLDEDTVLSTTEVDFAFTVWQSFPERIVGYPARSHFWDNSKE
RWGYTSKWTNDYSMVLTGAAIYHKYYHYLYSHYLPASLKNMVDQLANCE
DILMNFLVSAVTKLPPIKVTQKKQYKETMMGQTSRASRWADPDHFAQRQ
SCMNTFASWFGYMPLIHSQMRLDPVLFKDQVSILRKKYRDIERL

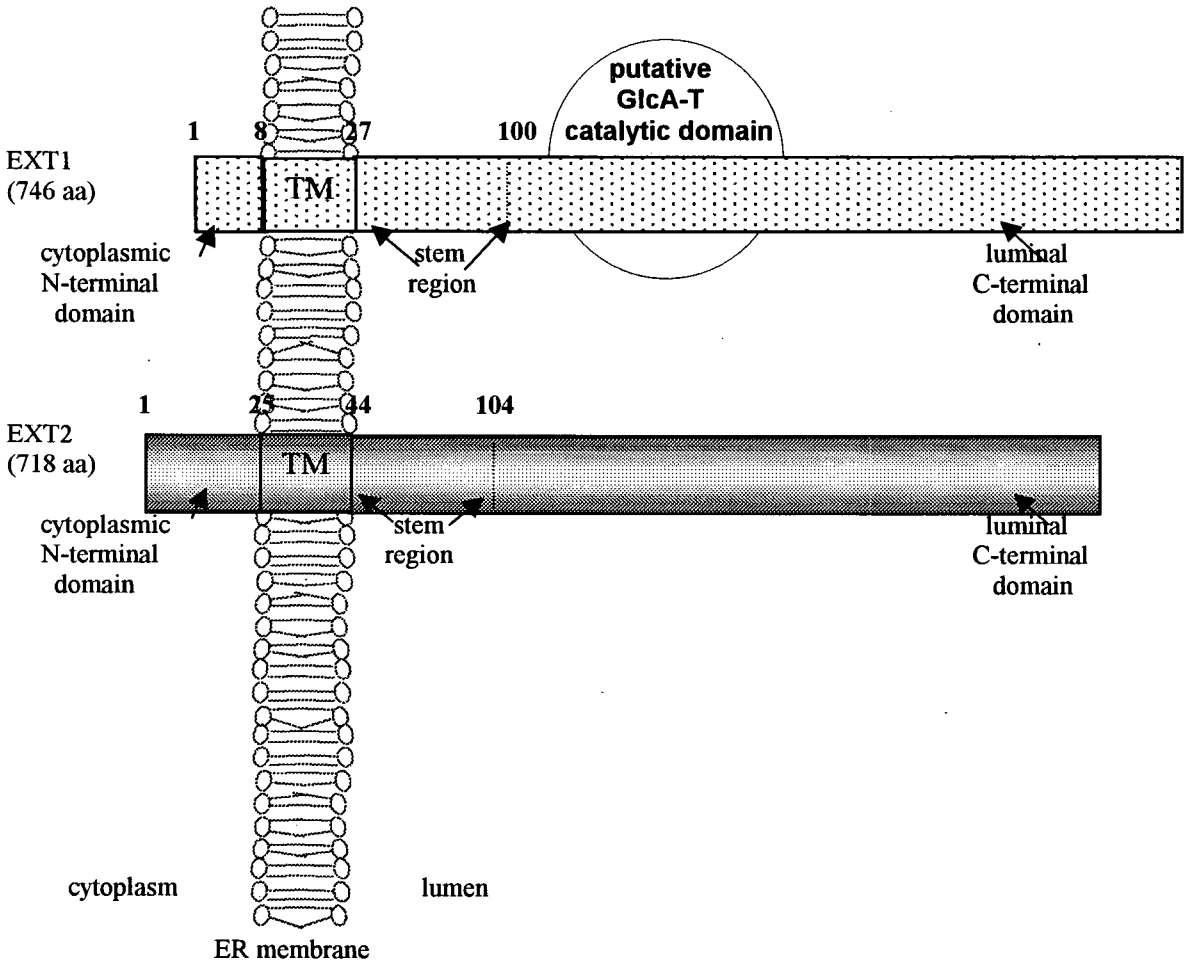
EXT2 amino acid sequence

1 25 44

MCASVKSNIRGPALIPRMKTKHRIYYVTLFSIVLLGLIATGMFQFWPHSIES
SSDGGVEKRTIREVPVVRLPTDSPIPERGDLSCRMHTCFDVYRCGFNPK
104
NKIKVYIYPLKKYVDDAGVPVSSAISREYNELLTAISDSDYYTDDINRACLFV
PSIDVVNQNPLRIKETAQALAQLSRWDRGTNHLLFNMLPGAPPDYNTALD
VPRDRALLAGGGFSTWTYRQGYDVSIPVFSPLSAEMALPEKAPGPRRYFL
LSSQMAIHPEYREELEALQAKHQESVLVLDKCTNLSEGVLSVRKRCHQHQ
VFDYPQVLQEATFCTVLRRARLGQAVLSDVLQAGCVPVVIADSYILPFSEIL
DWKKASVVVPEEKMSDVYSILQNIPQRQIEEMHRQARWFWEAYFQSIKAI
ALATLQIINDRIYPYAAISYEEWNDPPAVKWASVSNPLFLPLIPPQSQGFTAI
VLTYDRVESLFRVITEVSKVPSLSKLLVVWNNQNKNPPEESLWPKIRVPLK
VVRTAENKLSNRFFPYDEIETEAVLAIDDDIIMLTSDELQFGYEVWREFPDR
LVGYPGRLHLWDHEMNKWKYESEWTNEVSMVLTGAAFYHKYFNYLYTY
KMPGDIKNWVDAHMNCEDIAMNFLVANVTGKAVIKVTPRKKFKCPECTAI
DGLSLDQTHMVERSECINKFASVFGTMPLKVVEHRADPVLYKDDFPEKLK
SFPNIGSL

letters in bold denote the cytoplasmic N-terminal domain
letters shaded in gray denote the putative transmembrane domain
letters underlined denote the predicted stem region
numbers denote amino acid residues

B



numbers in black denote amino acid residue

Figure 1 EXT1 and EXT2.

A) The amino acid (aa) sequence of EXT1 and EXT2 are presented. Important domains are indicated by different colored letters. Letters in bold indicate the cytoplasmic N-terminal domain while letters shaded in gray indicate the putative transmembrane domain. Finally, the letters underlined indicate the stem region (area of little/no secondary structure) as predicted by J-PRED (a secondary structure prediction server).

B) EXT1 and EXT2 are both type II transmembrane (TM) proteins with a short N-terminal cytoplasmic domain, a single hydrophobic TM-spanning domain followed by a large C-terminal domain containing the stem region and catalytic domain(s). Amino acid residue numbers indicate the start of the different putative domains.

Loss of heterozygosity (LOH) studies using polymorphic markers linked to *EXT1* and *EXT2* indicate that these loci are lost in the chondrosarcomas of HME patients, suggesting these two genes encode proteins with tumour suppressor functions (Hecht *et al.*, 1995; Raskind *et al.*, 1995). But their function was unknown until recently when EXT1 was demonstrated to be involved in *in vivo* heparan sulfate (HS) biosynthesis (McCormick *et al.*, 1998). Subsequently, both EXT1 and EXT2 were discovered to harbour N-acetylglucosamine transferase (GlcNAc-T) and D-glucuronic acid transferase (GlcA-T) activities *in vitro* (Lind *et al.*, 1998). Remarkably, EXT1 and EXT2 were shown to form a hetero-oligomeric complex *in vivo* and that this complex possesses substantially higher glycosyltransferase activity *in vitro* than either EXT1 or EXT2 alone (McCormick *et al.*, 2000). This finding has since been confirmed by studies in yeast, which do not exhibit endogenous HS polymerase activity (Senay *et al.*, 2000). Interestingly, both EXT1 and EXT2 are found predominantly in the ER when overexpressed alone but re-localize to the Golgi when co-expressed in the same cell (Kobayashi *et al.*, 2000; McCormick *et al.*, 2000).

EXT1 and *EXT2* are members of the EXT family of putative tumor suppressors which also currently includes three EXT-like genes, *EXTL1* (Wise *et al.*, 1997), *EXTL2* (Wuyts *et al.*, 1997), and *EXTL3* (Van Hul *et al.*, 1998). These three EXTL genes encode proteins shown to possess GlcNAc-T *in vitro* activity as well (Kim *et al.*, 2001; Kitagawa *et al.*, 1999), suggesting that the members of the EXT family may all be involved in polysaccharide chain elongation.

Most HME cases have been attributed to missense or frameshift mutations in either *EXT1* or *EXT2* (Philippe *et al.*, 1997; Wuyts and Van Hul, 2000), and until recently, it was

believed that HME was due to deficient HS polymerase activity. However, recent data have shown that some etiological EXT1 mutants still possess HS enzymatic activity as determined by the HSV assay and high performance liquid chromatography (HPLC) analysis of cell surface glycosaminoglycans (GAGs) (Cheung *et al.*, 2001). This implies that other proteins may be involved in the disorder or that EXT1 and EXT2 encode another function yet undescribed. It is also possible that the etiological mutants produce abnormal levels/types of HS which are undetectable in our lab assays, and that adversely affect the controlled signalling required for proper proliferation, differentiation and apoptosis of chondrocytes.

1.3 Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans (HSPGs) are found ubiquitously on cell surfaces and in the extracellular matrix. These complex molecules of which there are three major families, the syndecans, the glypicans and the perlecans consist of a protein core covalently attached via a tetrasaccharide linker to HS chains. Perlecans are components of cartilage and basement membranes, and they, along with the transmembrane syndecans and the glycosylphosphoinositide-linked glypicans, mediate a variety of biological processes through the binding of their HS chains to different cellular ligands.

HS is a member of the GAG family of unbranched polysaccharide chains composed of repeating disaccharide units. Depending on the disaccharide composition, GAGs can be grouped into three different groups: chondroitin/dermatan sulfate, heparin/heparan sulfate and keratan sulfate. The biosynthesis of HS chains occurs in the Golgi apparatus with the initial attachment of a tetrasaccharide linker region (xylose, galactose, galactose and glucuronic acid) to a serine residue of a core protein (Fig. 2). The EXTL2 protein is then believed to add the first N-acetylglucosamine (GlcNAc) to the linker to start the HS chain (Kitagawa *et al.*, 1999). Repeating disaccharide units of D-glucuronic acid (GlcA) and GlcNAc are then added on by the copolymerase EXT1 and EXT2 (Lind *et al.*, 1998; McCormick *et al.*, 2000; McCormick *et al.*, 1998). Furthermore, recent data indicate that the other two members of the EXTL proteins, EXTL1 and EXTL3 harbour *in vitro* GlcNAc-T activity likely involved in the synthesis of HS chains (Kim *et al.*, 2001). The HS polysaccharide chains are modified by N-deacetylase-N-sulfotransferases (NDSTs), 6-O-sulfotransferases, 3-O-sulfotransferases, 2-O-sulfotransferases and epimerases to create highly variable and complex sulfated structures.

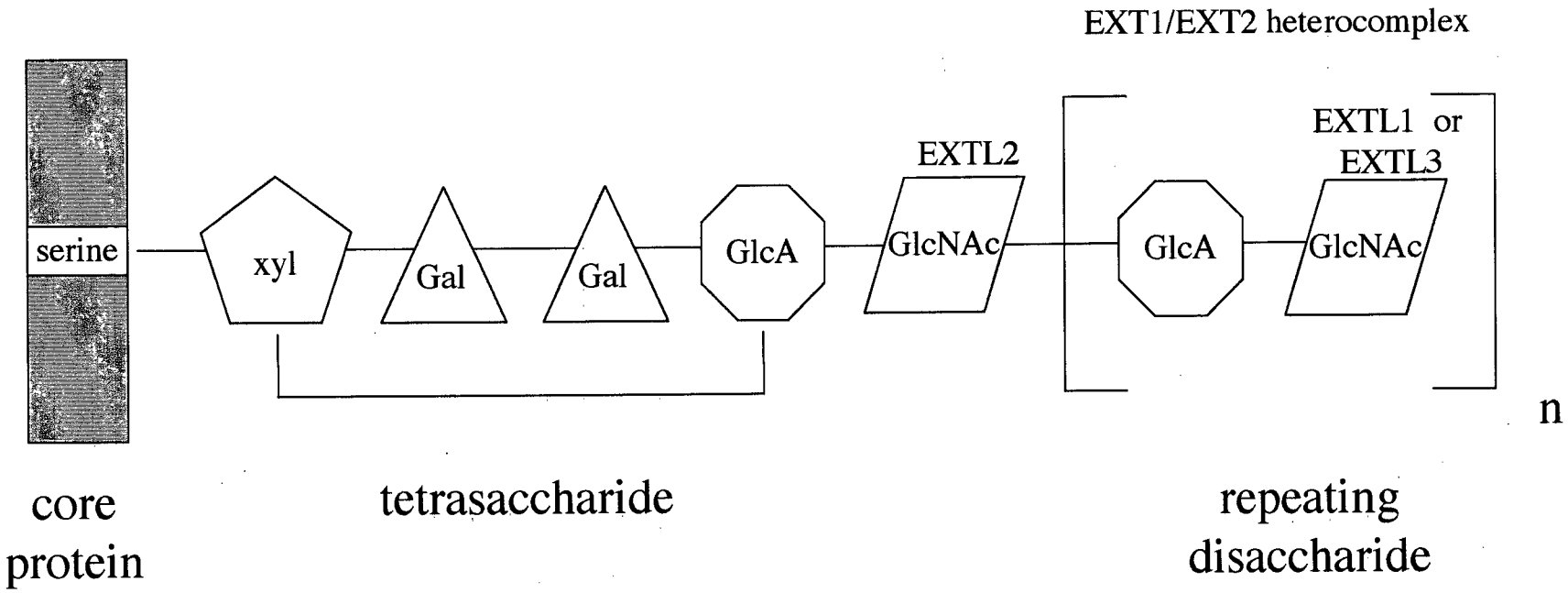


Figure 2 Heparan sulfate polysaccharide chain.
Glycoproteins have heparan sulfate (HS) chains attached via a tetrasaccharide linker composed of xylose (xyl), galactose (gal), gal, and glucuronic acid (GlcA) to a serine residue on the protein. The EXTL2 enzyme then adds the first N-acetylglucosamine (GlcNAc) to start the repeating disaccharide chain of GlcA and GlcNAc which are polymerized by the EXT1/EXT2 heterocomplex. EXTL1 and EXTL3 may also help catalyze the addition of GlcNAc onto the HS chain.

These remarkably diverse HS molecules interact with a variety of different proteins to regulate a diverse range of cellular functions. They have been shown to mediate cell-cell adhesion, serve as co-receptors for soluble factors, mediate protein internalization, and effect conformational change thus enhancing enzymatic activity (Table I). Furthermore, cell surface HSPGs are also exploited by microbial pathogens from bacteria to viruses as ligands for attachment to host cells (Table II). HSPGs are also shed from the cell surface thus they can further regulate cell surface HSPG-ligand interactions by competing for available ligands in the extracellular matrix.

Table I Examples of heparan sulfate-ligand interactions.

Protein ligand	Effect of binding HS
Fibroblast growth factor-2	Mediate interaction with fibroblast growth factor-2 receptor (Yayon *et al.*, 1991, Rapraeger, 1991 #23)
Lipoprotein lipase	Internalization (Fuki *et al.*, 1997; Saxena *et al.*, 1990)
Laminin	Adhesion (Utani *et al.*, 2001)
Antithrombin III	Induce conformational change thus increasing its activity (Olson *et al.*, 1992)

Table II Microbial pathogens that bind to heparan sulfate.

Microbial pathogen	Protein ligand
Herpes simplex virus	glycoprotein C (Herold *et al.*, 1991)
Listeria monocytogenes	ActA (Alvarez-Dominguez *et al.*, 1997)
Bordetella pertussis	major subunit of fimbriae (Geuijen *et al.*, 1996)
Pseudorabies virus	glycoprotein C (Karger and Mettenleiter, 1996)

With all these regulatory functions it is not surprising that mutating or knocking out HS biosynthetic enzymes in *Drosophila* or mice would produce dramatic *in vivo* effects. Mutations in *tout velu* (*ttv*) which encodes the *Drosophila* homologue of *EXT1* result in defects in wing anterior-posterior patterning (Bellaiche *et al.*, 1998) while homozygous *ttv* mutants die during the pupal stage (The *et al.*, 1999). *Sugarless* (*sgl*), also a *Drosophila* gene, encodes a protein homologous to mammalian UDP-glucose dehydrogenase which generates UDP-GlcA for HS chain polymerization. Mutations in *sgl* result in abnormal segmental patterning of the embryonic epidermis as it affects the signalling of Wingless, a secreted heparin-binding morphogen involved in patterning during embryogenesis (Binari *et al.*, 1997; Hacker *et al.*, 1997; Haerry *et al.*, 1997). Further evidence of HS's regulatory role in development can also be found in mammals. *EXT1* homozygous mutants in mice die during the embryonic stage due to defects in mesoderm and extraembryonic tissue formation (Lin *et al.*, 2000). Furthermore, mice homozygous for a gene trap mutation that disrupts the HS 2-O-sulfotransferase gene exhibit kidney agenesis, abnormal eye and skeletal growth, and die in the neonatal stage (Bullock *et al.*, 1998). All these *in vivo* data clearly show that HS plays an important role in controlling embryogenesis and is essential for proper development in invertebrates and vertebrates.

1.4 Herpes Simplex Virus Type 1 & Type 2

Herpesviruses are eukaryotic viruses responsible for several important human and animal diseases. All herpesviruses have a linear double-stranded DNA genome, icosahedral capsid, tegument (amorphous proteinaceous layer surrounding the capsid), and a lipid envelope containing numerous viral glycoproteins involved in attachment and entry.

The family *Herpesviridae* is divided into three subfamilies of *alpha-*, *beta-*, and *gammaherpesvirinae* based on biological characteristics and genomic analysis. Though herpesviruses have been isolated from a wide range of vertebrates there is a high degree of host specificity with eight human herpesviruses identified to date. They are the herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella-zoster virus of the *alphaherpesvirinae*, human cytomegalovirus, human herpesvirus-6 and human herpesvirus-7 of the beta subfamily, and Epstein-Barr virus and human herpesvirus-8 of the *gammaherpesvirinae*.

The complete sequence of the 152.3 kb genome of HSV-1 was published in 1988 (McGeoch *et al.*, 1988) while HSV-2's 154.7 kb genome sequence was only published recently (Dolan *et al.*, 1998). The two genomes are closely related to each other with an 83% nucleotide sequence homology (Dolan *et al.*, 1998).

The entry of HSV into host cells is a multistep process beginning with the initial attachment of HSV to cell surface GAGs followed by a stable attachment between HSV and secondary receptors. The final step in the process is the fusion of the viral envelope with the PM of the host cell. The initial attachment of both HSV-1 and HSV-2 to the host cell surface is primarily mediated by the binding of viral envelope glycoproteins (gB & gC) to HS which serves as the host-cell primary receptor (Herold *et al.*, 1994; Herold *et al.*, 1991; Williams and Straus, 1997). The absence of cell surface HS GAGs due to either heparanase digestion or defects in HS-biosynthesis (i.e. mutant cell lines) results in dramatically reduced infectivity by HSV of these host cells (Banfield *et al.*, 1995; WuDunn and Spear, 1989). Stable attachment of HSV to host cells is mediated by the binding of gD to one of several cell surface receptors. Herpes virus entry mediator (Hve) A, a member of

the tumour necrosis receptor family, and HveC, a member of the immunoglobulin (Ig) family can mediate stable attachment between the gD of both HSV-1 and HSV-2 to the host cell (Geraghty *et al.*, 1998; Montgomery *et al.*, 1996). In constrast, HveB, also a member of the Ig family can only mediate adhesion of HSV-2 and some laboratory strains of HSV-1 (Warner *et al.*, 1998). Finally, viral penetration of the host cell involves the viral glycoproteins gB, gH-gL required for the fusion of the viral envelope with the cell PM (Cai *et al.*, 1988; Fuller *et al.*, 1989; Roop *et al.*, 1993).

Although both HSV-1 and HSV-2 use HS as a receptor for initial attachment, and HveA and HveC for stable attachment, the two serotypes differ epidemiologically with HSV-1 mainly causing oral lesions and HSV-2 primarily associated with genital lesions. However, the results of a recent study suggests that HSV-1 is increasingly responsible for genital infections (Nilsen and Myrmel, 2000). Differences in the ability of HSV-2 to bind HveB which most strains of HSV-1 are unable to use may help explain some of the epidemiological differences seen between the two serotypes. Additionally, HS GAGs are very heterogeneous with varying degrees of N-acetylation, N-sulfation, O-sulfation and epimerization of glucuronic acid to iduronic acid. Thus another possibility is that differences in the interaction with these chain modifications between HSV-1 and HSV-2 may further account for the differences seen between the two serotypes. Indeed, when the addition of O-desulfated heparins on HSV-1 and HSV-2 infection was examined, it was found that HSV-2 infection was inhibited while there was little or no inhibitory effects on HSV-1 infection (Herold *et al.*, 1996). Furthermore, Herold *et al.* found that this phenotype was due to glycoprotein C of HSV-2 (gC-2) and that when gC-2 was transferred to an HSV-1 background this led to the mutant HSV-1 being inhibited by O-desulfated heparins.

Thus, the expression level of HveB or the degree of O-sulfation in various cell types may partly account for the different cell trophism seen between these two serotypes.

1.5 Secretory Pathway

EXT1 and EXT2 must traverse the secretory pathway to reach the Golgi where they polymerize the polysaccharide chains of HS (Fernandez and Warren, 1998). Eventually these glycosyltransferases are secreted out of the cell where their truncated forms can be isolated from serum (Lind *et al.*, 1998).

Intracellular trafficking and eventual secretion of proteins is one of the most complex biological phenomena in the field of biology. The transport pathway of proteins destined for secretion or the plasma membrane (PM) originate in the lumen of the rough ER and then pass through the ER-Golgi intermediate compartment (ERGIC) to the Golgi apparatus before being packaged into secretory vesicles (Fig. 3). Movement of proteins from the ER to the cell surface is mediated by vesicles which bud from the donor membrane then dock and fuse with the acceptor membrane thus delivering their cargo to the targetted location (Nickel *et al.*, 1998). Transport vesicles examined to date have been shown to contain cytosolic coat proteins such as clathrin, coat protein I (COPI) and coat protein II (COPII). Clathrin-coated vesicles contain clathrin attached to the donor membrane via adaptor proteins and are involved in transporting proteins between the PM and the *trans*-Golgi network (TGN) (Pearse and Robinson, 1990). COPII-coated vesicles are non-clathrin-coated vesicles which bud from the ER and are involved in the forward movement (anterograde transport) of proteins to the ERGIC (Aridor *et al.*, 1995; Barlowe *et al.*, 1994). COPI-coated vesicles are also non-clathrin-coated but their role in the

transport pathway is less clear. They have been shown to be involved in anterograde transport from the ER-Golgi intermediate compartment (ERGIC) to the Golgi as well as in intracisternal protein transport through the Golgi (Aridor *et al.*, 1995; Orci *et al.*, 1986). Furthermore, COPI vesicles have also been implicated in the retrieval of escaped ER resident proteins back from the Golgi as COPI protein has been demonstrated to bind the C-terminal Lys-Lys-Xaa-Xaa motif which serves as an ER-retrieval signal in the cytoplasmic tail of type I transmembrane proteins (Cosson and Letourneur, 1994; Teasdale and Jackson, 1996).

Sorting of proteins within the secretory pathway is accomplished by the selective packaging of cargo molecules into coated vesicles (Gu *et al.*, 2001). These transport vesicles then bud and fuse with the target membranes. Targetting proteins used by transport vesicles include members of the SNAp-REceptor (SNARE) family which are also required for membrane fusion between the vesicle and the target organelle (Sollner *et al.*, 1993).

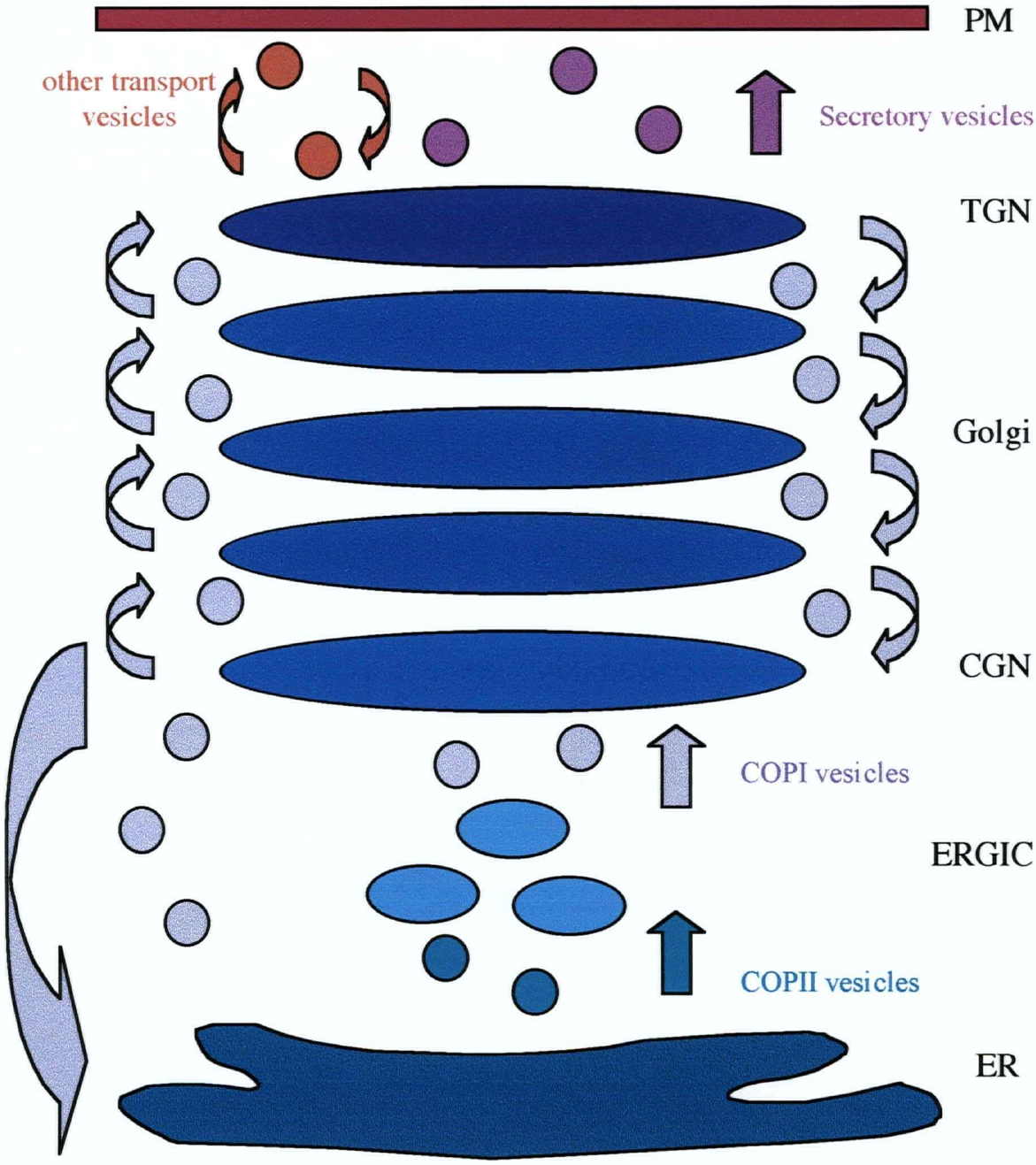


Figure 3 Secretory pathway.
Proteins destined for secretion or the plasma membrane (PM) travel through the transport pathway to reach the cell surface. Proteins translated in the endoplasmic reticulum (ER) are packaged into COPII vesicles which carry them to the ER-Golgi intermediate (ERGIC). From there, they travel via COPI vesicles to the cis-Golgi network (CGN) and through the Golgi stacks to the trans-Golgi network (TGN). Once in the TGN, they are packaged into secretory/exocytic vesicles destined for the cell surface. Other transport vesicles can also be found cycling between the TGN and the PM. Escaped proteins may be retrieved back to their compartments by COPI vesicles.

1.6 Subcellular Localization

A typical eukaryotic cell has a diverse number of organelles and compartments each containing its own unique set of proteins. Thus the sorting of proteins to the appropriate location within the cell is vital to its function. This is usually accomplished by signal sequences encoded in the gene.

1.6.1 ER-localization

The ER is a network of membranes extending throughout the cytoplasm of a cell (Solomon *et al.*, 1993). The ER is composed of two morphologically distinct regions called the rough ER (RER) and the smooth ER (SER). The RER is a series of parallel flattened sacs characterized by the presence of ribosomes on the outer surface (cytosolic side) of its membrane. The SER on the other hand does not have ribosomes attached to its membranes and resembles a branched tubular network rather than flattened sacs. Many ER resident proteins or those destined for compartments along the secretory pathway are synthesized on the ribosomes of the RER and translocated into the lumen of the ER. ER resident proteins contain ER localization motifs that allow them to be maintained in the ER either by retention or retrieval via a retrograde transport flow from the Golgi back to the ER by COPI vesicles (Teasdale and Jackson, 1996).

Human invariant chain (Ii) is a type II transmembrane protein involved in the assembly of the major histocompatibility complex class II in the ER (Janeway Jr. and Travers, 1996). Due to alternative initiator methionines in the Ii mRNA, 2 different protein products are produced; Iip31 and Iip33 (Strubin *et al.*, 1986). Iip31 is rapidly transported out of the ER while Iip33 is maintained in the ER (Lotteau *et al.*, 1990). The transplantation of the cytoplasmic N-terminus of the ER-localized isoform onto N-

acetylglucosaminyltransferase (GlcNAc-T I) relocalized it from the medial Golgi to the ER (Nilsson *et al.*, 1994) indicating that the N-terminus of Iip33 contained an ER-localization signal. In agreement with this, a double arginine motif in close proximity to the N-terminus of Iip33 was identified as an ER-localization signal sufficient to localize a chimeric transferrin receptor (a type II cell surface membrane protein) to the ER (Schutze *et al.*, 1994).

1.6.2 Golgi Localization

The Golgi apparatus consists of a stack of flattened membraneous sacs and was first described by Camillo Golgi in 1898 (Solomon *et al.*, 1993). The Golgi serves as a site of post-translational modifications as well as a sorting point for proteins and lipids to different organelles (Gu *et al.*, 2001). These two functions are performed by various Golgi resident proteins, some of which are the glycosyltransferases responsible for the addition of oligosaccharide chains onto nascent proteins to produce glycoproteins.

All Golgi glycosyltransferases cloned to date are type II transmembrane proteins with a short cytoplasmic amino-terminus, a single transmembrane domain, a stem region of little secondary structure followed by a large carboxy-terminus in the lumen of the Golgi apparatus (Colley, 1997). There is very little sequence homology among the different glycosyltransferases which suggest that the Golgi localization signal used by these enzymes must involve structural/conformational features instead of a specific amino acid sequence. There are currently two main models to explain how Golgi residents are retained within the Golgi: the bilayer thickness model (Bretscher and Munro, 1993) and the kin-recognition model (Nilsson *et al.*, 1993). Though both models are supported by experimental evidences, neither model alone can account for how all Golgi-resident glycosyltransferases

are retained within the Golgi.

1.6.2.1 Bilayer thickness model

Differences in the length of the transmembrane domain of Golgi proteins (shorter) and plasma membrane proteins (longer), and the finding that increasing the amount of cholesterol resulted in increased membrane thickness (Nezil and Bloom, 1992) led to the proposal of the bilayer thickness model by Bretscher and Munro in 1993. This model (Fig. 4) postulates that the shorter length of the transmembrane domain of Golgi-resident proteins excludes the Golgi proteins from entering cholestrol-rich transport vesicles destined for the PM (Bretscher and Munro, 1993). The membrane spanning domain of Golgi residents have been shown to be sufficient in localizing reporter molecules to the Golgi (Munro, 1991; Nilsson *et al.*, 1991). In addition, Munro demonstrated that the amino acids of the transmembrane domain of Golgi residents could be altered without changing the Golgi localization, but that extending the transmembrane domain length resulted in PM localization (Munro, 1995).

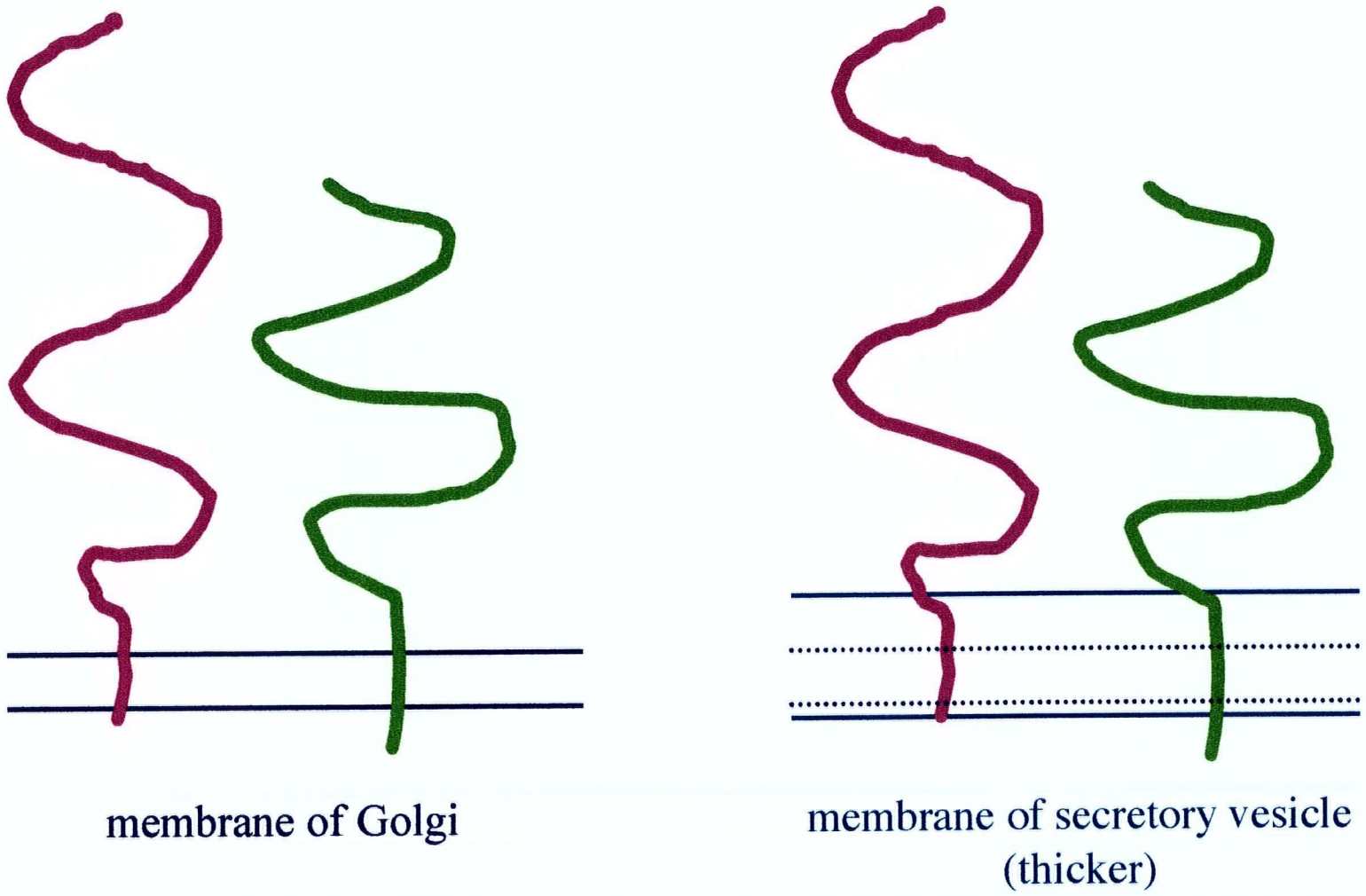


Figure 4 Bilayer thickness model.
The bilayer thickness model hypothesizes that Golgi-resident proteins are retained in the Golgi due to the shorter length of their transmembrane domain versus those of plasma membrane proteins (Bretscher and Munro, 1993). The shorter length of their transmembrane domain prevents the Golgi-residents from entering secretory vesicles and thus retains them in the Golgi.

1.6.2.2 Kin recognition model

Initial work on the Golgi localization of coronavirus M glycoprotein E1 demonstrated that Golgi retention of this protein was associated with the formation of insoluble homo-oligomers (Weisz *et al.*, 1993). This led to the kin-recognition model (Fig. 5), which proposes that resident proteins of the Golgi oligomerize to form large homo- and hetero-oligomers whose large structure prevents their entry into transport vesicles destined for the cell surface, and are thus retained in the Golgi (Nilsson *et al.*, 1993). In support of this model, the substitution of an ER localization signal for the cytoplasmic N-terminus of GlcNAc-T I not only resulted in its relocalizaion to the ER but also caused another medial-Golgi protein to relocalize there as well (Nilsson *et al.*, 1994). Furthermore, recent data on β-galactoside α2,6-sialyltransferase have demonstrated that the major mechanism of Golgi localization used for this glycosyltransferase is formation of Triton-insoluble mono-oligomers (Chen *et al.*, 2000).

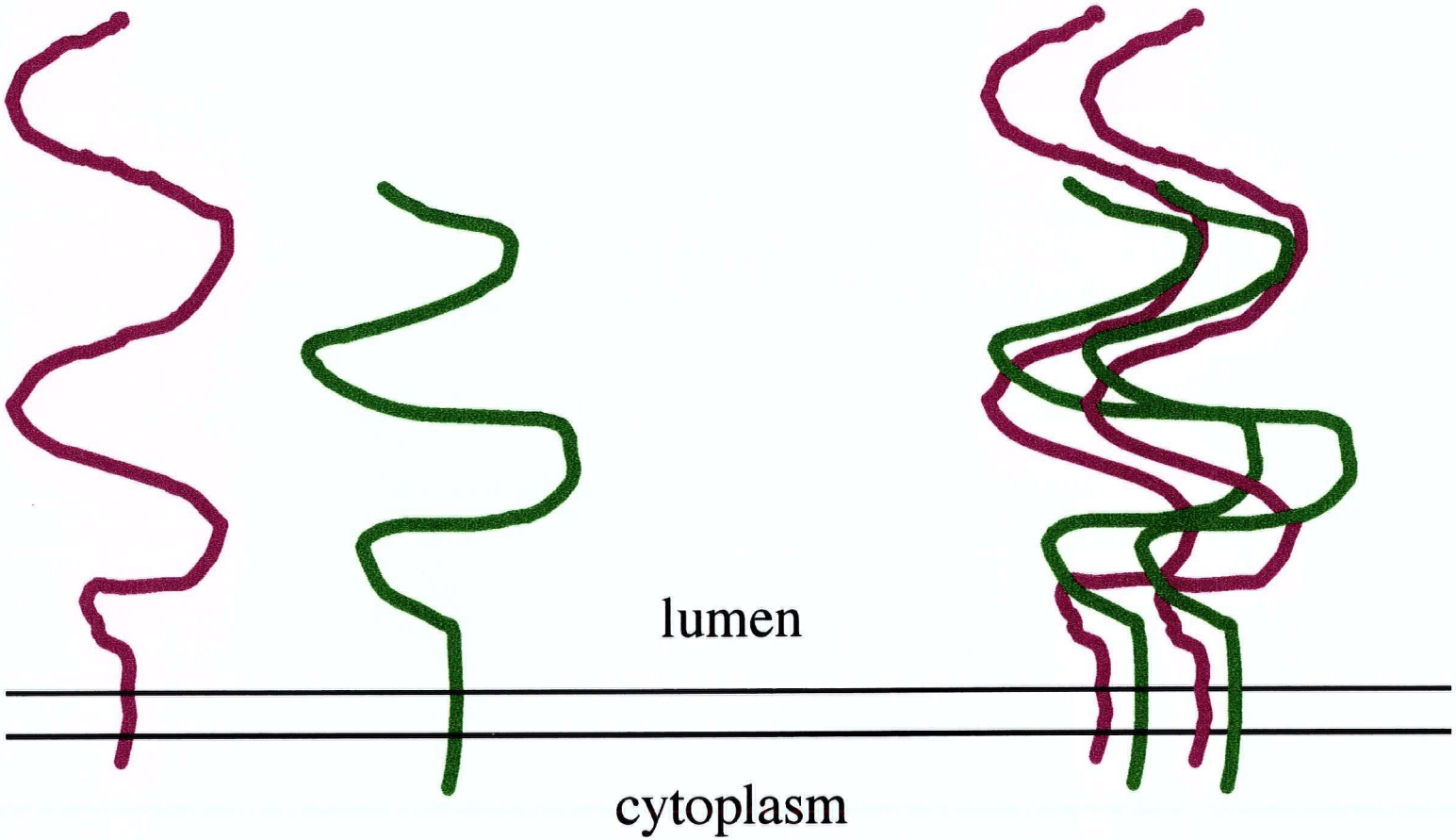


Figure 5 Kin recognition model.
The kin recognition model hypothesizes that Golgi-residents oligomerize to form large homo- and hetero-oligomers (Nilsson, T *et al.*, 1993). The large complexes formed by these Golgi-residents immobilize the proteins in the Golgi membrane and prevent their entry into vesicles destined for the cell surface.

1.6.3 Secretory Vesicles

Once processed by the Golgi apparatus, secretory proteins and other substances destined for transport out of the cell are packaged into secretory vesicles. These vesicles move from the Golgi to the PM where they fuse with the PM and release their contents into the extracellular space. Several cytosolic coat proteins have been described for vesicles exiting from the TGN (Gu *et al.*, 2001; Jones *et al.*, 1993; Ladinsky *et al.*, 1994) though other studies have not found evidence of a distinct class of coat complex (Nickel *et al.*, 1994). Thus at present, the biogenesis of secretory vesicles remains unclear.

1.7 Research Objectives

The glycosyltransferases EXT1 and EXT2 are type II transmembrane proteins involved in the biosynthesis of heparan sulfate (HS) polysaccharide chains (McCormick *et al.*, 2000). They are found predominantly in the ER when overexpressed individually but form a Golgi-localized heterocomplex when co-expressed in the same cell (Kobayashi *et al.*, 2000; McCormick *et al.*, 2000). All published EXT1 and EXT2 missense mutations identified in HME patients are located in their lumenal C-termini (Philippe *et al.*, 1997; Wuyts and Van Hul, 2000), and studies on these EXT1 mutants show that some of these mutations are capable of abrogating *in vivo* HS biosynthetic activity (Cheung *et al.*, 2001). Furthermore, the GlcA-T catalytic domain has been localized to the central region of the EXT1 protein, indicating that the lumenal domain is important for enzymatic activity (McCormick *et al.*, 2000; Wei *et al.*, 2000). By contrast, little is known regarding the role of the N-terminal domains of EXT1 and EXT2. Thus I decided to investigate the importance of the N-terminal domains of these two proteins in i) *in vivo* HS biosynthesis,

ii) heterocomplex formation, and iii) subcellular localization. Chimeric constructs of EXT1 and EXT2 were made in which different subcellular localization signals were grafted onto their cytoplasmic N-termini. HS biosynthesis is thought to occur in the Golgi (Fernandez and Warren, 1998) and as the lumenal C-terminus is considered to contain the catalytic domain, the N-terminal region should not be involved in the enzymatic activity of the EXT proteins. I therefore hypothesized that only subcellular localization signals directing the EXT1 proteins to compartments other than the Golgi would abrogate *in vivo* function as determined by our HSV assay. To test this hypothesis, EXT1 and EXT2 chimeras containing ER-, and Golgi-localization signals as well as secretory forms of the two EXT proteins were created and analyzed to determine the roles of their respective N-terminal domains.

2. Materials and Methods

2.1 Cell Culture

BHK and Sog9 [HS-deficient murine L-cell line (Banfield *et al.*, 1995)] cells were maintained as monolayers in a 37°C, 5% CO_2 humidified incubator. All cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum, 50 U/mL penicillin and 50 μg/mL streptomycin. All cell culture reagents were from Life Technologies, Burlington, Ont., Canada.

2.2 EXT Constructs

All primers used in the creation of chimeric EXTmyc or EXT1gfp constructs were obtained from Life Technologies, Burlington, Ont., Canada.

2.2.1 EXTmyc constructs

The chimeric EXT1 constructs were created by PCR of the human EXT1 gene from pEXT1myc (McCormick *et al.*, 1998) while the chimeric EXT2 constructs were created by PCR of the murine EXT2 gene from pmEXT2myc (McCormick *et al.*, 2000). The constructs were created with a myc tag downstream of the gene to enable easy detection of the protein products.

2.2.1.1 Sec-EXT1myc

Sec-EXT1myc was constructed by PCR of the EXT1 coding region starting 10 aa downstream of the putative transmembrane domain by using the forward primer BamHI-EXT1stem (5'-CCG GAT CCC GAG AGA AGA ACA CAG CGG TAG G-3') and the reverse primer EcoRI-EXT1rev (5'-GGA ATT CCA AGT CGC TCA ATG TCT CG-3').

The forward primer contains a *Bam*HI restriction enzyme site directly upstream of the EXT1 sequence, and the reverse primer contains an *Eco*RI site excluding the EXT1 stop codon to allow for in-frame tagging. After digestion with *Bam*HI (Life Technologies, Burlington, Ont., Canada) and *Eco*RI (Life Technologies, Burlington, Ont., Canada), the Sec-EXT1 PCR product was then ligated into the *Bam*HI and *Eco*RI site of pSecTag2 "C" (Invitrogen, Burlington, Ont., Canada) such that the EXT1 coding region was in frame with pSecTag2's secretion signal (immunoglobulin kappa chain leader sequence).

The PCR reaction was performed as follows: 2 min initial denaturation at 94°C, 30 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 2 min 30 sec with an additional 10 min elongation step at 72°C at the end.

2.2.1.2 Sec-EXT2myc

Sec-EXT2myc was constructed in a similar fashion as described for Sec-EXT1myc using the primers BamHI-mEXT2stem (5'-CGG GAT CCC GAG TGA TGG GGG TGT GGA GAA ACG-3') containing a *Bam*HI site, and EcoRI-mEXT2rev (5'-GGA ATT CCT AAG CTG CCA ATG TTG GGG AAG CTC TTC-3') containing an *Eco*RI site. The Sec-EXT2 PCR product was also ligated in the pSecTag2 vector.

2.2.1.3 ER-EXT1myc

ER-EXT1myc was constructed by PCR of the EXT1 coding region by using the primers hEXT1-ERfor (5'-GCG GAT CCT CCC ACC ATG CAC AGG AGG AGA AGC TAT TTC ATC CTG TCA GCT GGC-3') containing a *Bam*HI site in the 5'-end, and NotTagclone8 (5'-TTT TTC TTT TGC GGC CGC TTT TTT CCT TAA GTC GCT CAA TGT CTC GGT A-3') containing a *Not*I site in the 3'-end. After digestion with *Bam*HI (Life Technologies, Burlington, Ont., Canada) and *Not*I (Life Technologies, Burlington,

Ont., Canada), the ER-EXT1 PCR product was then ligated into the *Bam*HI and *Not*I site of pcDNA3.1mycHis "B" (Invitrogen, Burlington, Ont., Canada).

2.2.1.4 ER-EXT2myc

ER-EXT2myc was constructed by ligating the amplified chimeric EXT2 PCR product into the *Bam*HI and *Sst*II (Life Technologies, Burlington, Ont., Canada) site of pcDNA3.1mycHis "B". The primers used were mEXT-ERfor (5'-GCG GAT CCG CCC ACC ATG CAC AGG AGG AGA AGC ATC TAC TAC GTC ACC CTG TTC TCC-3') and SstIImEXT2rev (5'-TCC CCG CGG GGA TAA GCT GCC AAT GTT GGG GAA-3'). The forward primer, mEXT-ERfor contains a *Bam*HI restriction enzyme site while the reverse primer, SstIImEXT2rev contains a *Sst*II site.

2.2.1.5 Golgi-EXT1myc

Golgi-EXT1myc is a chimeric construct containing the N-terminus, transmembrane domain and part of the stem region of murine N-deacetylase/N-sulfotransferase 2 (NDST2) (Kusche-Gullberg *et al.*, 1998) fused to the stem region and catalytic domain of EXT1. It was constructed in 2 fragments, the first PCR fragment containing the mNDST N-terminus, transmembrane domain and 129 nucleotides of the stem region along with 24 nucleotides of the EXT1 gene at the 3'-end [constructed using the primers mHSNST-Bgl-fwd (5'-GAA GAT CTT CCC ACC ATG CTC CAG CTG TGG AAG GT-3') containing the *Bgl*II site, and mNDST2/hEXT1-Golgi(rev) (5'-GCT GTG TTC TTC TCT CCG GCT GTG GCT CCT AGT TGT TTC TGG AGG CCT TTG GGG-3'), a hybrid primer containing 24 nucleotides of the EXT1 gene]. The second PCR fragment contained the C-terminus of EXT1 starting in the stem region along with 24 nucleotides of the NDST2 gene at the 5'-end. It was created using mNDST2/hEXT1-Golgi(for) (5'-CCT CGA CCC CAA AGG

CCT CCA GAA ACA ACT AGG AGC CAC AGC CGG AGA GAA GAA-3'), a hybrid primer with containing 24 nucleotides of the NDST2 gene, and EXT1-L360rev (5'-ATA GTT TAG CGG CCG CAT TCT TAT TCA GAG CAT CAC AGG GAC GCA GGC-3') containing the *Ppu*MI site. The two PCR products were mixed together, denatured for 5 min at 92°C, and allowed to slowly reanneal at room temperature. The 3'-ends of the reaction products were extended using Taq DNA polymerase (72°C for 20 min) to form double stranded "polished" ends. This was then used as the template for a PCR reaction using the primers mHSNST-Bgl-fwd and EXT1-L360rev. The PCR reaction was performed as follows: a 2 min initial denaturation step at 94°C followed by 5 cycles of 94°C for 30 sec, 30°C for 30 sec, 72°C for 2 min 30 sec then 25 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 2 min 30 sec with an additional 10 min elongation step at 72°C at the end. The PCR product was then digested with *Bgl*II (Life Technologies, Burlington, Ont., Canada) and *Ppu*MI (New England Biolabs, Mississauga, Ont., Canada) and ligated into a *Bam*HI and *Ppu*MI digested pEXT1myc (McCormick *et al.*, 1998).

2.2.1.6 Golgi-EXT2myc

Golgi-EXT2myc was constructed in the same manner as described above. The primers used were mHSNST-Bgl-fwd (sequence as listed above) and mNDST2/mEXT2-Golgi(rev) (5'-CAC ACC CCC ATC ACT GGA GGA CTC AAT GGA AGT TGT TTC TGG AGG CCT TTG GGG-3'), containing 24 nucleotides of the EXT2 gene in the 3'-end, for the first fragment of the chimeric protein. The NDST2/EXT2 hybrid primer, mNDST2/mEXT2-Golgi(for) (5'-CCT CTA CCC CAA AGG CCT CCA GAA ACA ACT TCC ATT GAG TCC TCC AGT GAT GGG-3'), and EXT2-E367rev (5'-CCG CCG CTC GAG CGG TCA CTC TGG AAC GAC CAC AGA TGC-3') containing the *Blp*I site were

used for the second fragment. After the final PCR with the primers mHSNST-Bgl-fwd and EXT2-E367rev, the PCR fragments were digested with *Bgl*II and *Blp*I (New England Biolabs, Mississauga, Ont., Canada) and ligated into *Bam*HI and *Blp*I digested pEXT2myc (McCormick *et al.*, 1998). PCR was performed as described for Golgi-EXT1myc.

2.2.2 EXT1gfp constructs

The myc-tagged chimeric EXT1 constructs were re-created with a gfp tag downstream of the gene to enable differentiation of cotransfected chimeric proteins.

2.2.2.1 Sec-EXT1gfp

Sec-EXT1gfp was constructed by amplifying the EXT1 gene containing the N-terminal immunoglobulin kappa chain leader sequence from Sec-EXTmyc using the primers Sec1&2gfp(for) (5'-GAA GAT CTT CTA GCC ACC ATG GAG ACA GAC ACA CTC-3') and EcoRI-EXT1rev (sequence as listed above). The PCR fragment was then digested with *Bgl*II and *Eco*RI, and subcloned into the *Bgl*II and *Eco*RI restriction enzyme sites of the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA).

2.2.2.2 ER-EXT1gfp

ER-EXT1gfp was also created in a similar fashion, the gene was amplified with the forward primer hEXT1-ERfor (sequence listed above) and the same reverse primer as the one used in the construction of Sec-EXT1gfp. The PCR fragment was digested with *Bam*HI and *Eco*RI and subcloned into the *Bgl*II- and *Eco*RI-digested pEGFP-N1.

2.2.2.3 Golgi-EXT1gfp

The chimeric NDST2/EXT1 gene was amplified from Golgi-EXT1myc with mHSNST-Bgl-fwd and EcoRI-EXT1rev (both sequences listed above). Golgi-EXT1gfp

was created by digesting with *Bgl*II and *Eco*RI followed by ligation of the PCR fragment into pEGFP-N1.

2.3 Transient Transfections

Cells were transfected using LipofectAmine Plus (Life Technologies, Burlington, Ont., Canada) according to manufacturer's instructions. Briefly, for the transfection of cells in one well of a 6-well dish, 1 μg of DNA was complexed with 6 μl of PLUS Reagent in 100 μl of DMEM. 15 min later, the addition of 4 μl of LipofectAMINE in 100 μl of DMEM was combined with the PLUS-complexed DNA. After another 15 min, this mixture was added to the well containing 0.8 ml of DMEM and incubated for 3 hrs at 37°C in a 5% CO_2 humidified incubator. The transfection media was then replaced with DMEM containing 10% FBS and 1% PenStrep.

2.4 Immunofluorescence staining

All α-myc and α-gfp antibodies used in this and other experiments involving these two antibodies were from Invitrogen, Burlington, Ont., Canada. The antibodies were provided at a concentration of approximately 1mg/mL. The α-myc antibodies were mouse monoclonal antibodies (9E10.2), and the α-gfp antibodies were rabbit polyclonal antibodies.

Approximately 175,000 BHK cells were seeded on acid-etched coverslips (Fisher, Nepean, Ont., Canada) and transiently transfected (as described above) the following day. The cells were fixed with 4% paraformaldehyde, EM-grade (Canemco Inc., St. Laurent, Que., Canada) 24-28 hrs following transfection and blocked overnight with 1% BSA/PBS.

Cells were then incubated with α-myc antibodies diluted at 1:200 in 0.25% saponin/1% BSA/PBS for 1 hr at room temperature in a humidified chamber. Bound primary antibodies were visualized using goat α-mouse antibodies conjugated to Texas Red (Cedar Lane, Hornby, Ont., Canada), Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or FITC (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) at 1:100 in 1% BSA/PBS. Cells were visualized and images acquired digitally using either the Zeiss DVC fluorescent microscope or the Bio-Radiance Plus Confocal microscope.

2.5 Immunoblotting

Subconfluent BHK cells were transiently transfected and lysed 40 hrs later in 100 μL of Triton X-100 lysis buffer (2% Triton X-100/20 mM Tris•HCl pH 7.4/150 mM NaCl containing complete protease inhibitors [Boehringer Mannheim, Indianaplis, IN, USA]) for 5 min at 4°C. The cell lysates were then centrifuged at 15,000 x g for 15 min and the supernatant separated by 6% SDS-PAGE. Following separation, the proteins were transferred onto Immobilon-P membrane (Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus (Pharmacia Biotech, Baie d'Urfe, Que., Canada) according to manufacturer's instructions. The membrane was blocked with 5% skim milk/TBS at 4°C O/N then incubated with α-myc and/or α-gfp antibodies diluted 1:2500 in 3% skim milk/TBS for 3 hrs. The membrane was then washed 3X (5 min each time) in 0.05% Tween-20/TBS followed by the addition of horseradish peroxidase-conjugated (HRP) α-mouse (Kirkegoard and Perry Laboratories, Gaithersburg, MD, USA) or HRP-conjugated α-rabbit (Kirkegoard and Perry Laboratories, Gaithersburg, MD, USA) antibodies at 1:2000 in 3% skim milk/TBS. Following another 3 washes, the proteins were detected

using the LumiGLO Chemiluminescent Substrate Kit (Kirkegoard and Perry Laboratories, Gaithersburg, MD, USA) followed by exposure to BioMAX Light Film (Kodak, Toronto, Ont., Canada).

2.6 Immunoprecipitation

At 16-18 hrs post-transfection, the BHK cells were radiolabelled with 3.7 MBq/mL L-[^{35}S]methionine (trans ^{35}S-label, ICN, Montreal, Que., Canada) in methionine-free DMEM (ICN, Montreal, Que., Canada) supplemented with 4% dialyzed fetal bovine serum and 2 mM L-glutamine (Life Technologies, Burlington, Ont., Canada). Cells were then lysed in 1 mL of Triton X-100 lysis buffer and centrifuged at 15,000 x g for 15 min at 4^{o}C. The supernatant was pre-cleared with Protein G-sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Baie d'Urfe, Que., Canada) for 1 hr at 4^{o}C on a rotator before being centrifuged for 15 sec at 15,000 x g. One μg of either α-gfp or α-myc was added to the pre-cleared supernatant and allowed to bind overnight while rotating at 4^{o}C. The following day, protein G-sepharose was added and allowed to rotate for an additional 2 hrs at 4^{o}C followed by centrifugation. The supernatant from this was then separated by 10% SDS-PAGE (Hoeffer apparatus) and transferred onto Immobilon-P membrane (Millipore, Bedford, MA, USA) using the semi-dry transfer apparatus followed by exposure to BioMAX MR Film (Kodak, Toronto, Ont., Canada).

2.7 HSV Assay

Approximately 1 million Sog9 cells were seeded and transfected with various EXT constructs the following day. At 40 hrs post-transfection, the transfected Sog9 cells were

washed in PBS and infected with β-galactosidase expressing herpes simplex viruses (either HSV type 1 [G207] or HSV type 2 [LIBR1]) at an M.O.I. of 0.1 pfu/cell for 1 hr in serum-free media. Cells were then washed 3X in PBS and incubated in 0.1% IgG/10% FBS/DMEM for 10 hrs (for HSV-1) or 6 hrs (for HSV-2). The cells were then fixed in 4% paraformaldehyde, blocked with 1% BSA/PBS, and stained with X-gal.

2.8 Sandwich Enzyme Linked Immunosorbent Assay (ELISA)

The bottom of 96-well plastic plates were coated with α-myc antibodies at 1:100 in TBS for 5 hrs at room temperature then blocked overnight with 3% skim milk/TBS at 4^0C. Approximately 500,000 BHK cells were transiently transfected and lysed in 150 μL of Triton X-100 lysis buffer, centrifuged at 15,000 x g for 15 min at 4^0C and the supernatant added to the α-myc antibody-coated wells for 2 hrs at room temperature. Wells were washed 3X each time with 0.05% Tween 20/TBS before the addition of the next substance. α-gfp antibodies at a dilution of 1:100 in TBS were added to the wells next for 2 hrs followed by the addition of HRP-conjugated secondary α-rabbit antibodies at 1:100 in TBS for 1 hr. Homo-/hetero-complex formation was detected by the addition of 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma, Oakville, Ont., Canada). The reaction was stopped with the addition of 100 μL of 0.5M H_2SO_4 and the relative amount of chromophore generated was determined by spectrophotometry (λ_{abs}: 450 nm) using the Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Baie d'Urfe, Que., Canada).

3. Results

3.1 EXT constructs

To determine the importance of the N-terminal regions of EXT1 and EXT2 for *in vivo* HS biosynthetic activity as well as to study its role in EXT1/EXT2 heterocomplex formation, several chimeric EXT constructs were made (Fig. 6). Specifically, the N-terminal sequences of EXT1 and EXT2 were replaced with other known subcellular-localization signal sequences, which have been shown to direct chimeric proteins to the ER, Golgi or for secretion. All EXT1 and EXT2 chimeric constructs were C-terminally tagged with either myc or gfp for easy detection of the chimeras.

To construct the ER-EXT1 and ER-EXT2 constructs (Fig. 6A), the cytoplasmic N-termini of EXT1 and EXT2 were deleted up to 1 aa before the putative transmembrane domain and substituted with MHRRRS, an ER-localization motif from human invariant chain protein 33. These 6 aa residues were sufficient to retain the human transferrin receptor, a type II membrane protein, to the ER and thus abrogate normal cell-surface expression (Schutze *et al.*, 1994).

The transmembrane domain and adjacent sequences of α2,6-sialyltransferase and GlcNAc-TI, both Golgi-localized glycosyltransferases, have been shown to be sufficient to localize non-Golgi proteins to the Golgi (Burke *et al.*, 1994; Munro, 1991). Thus to localize EXT1 and EXT2 to the Golgi, the N-terminus, putative transmembrane domain and part of the stem region of both EXT1 and EXT2 were replaced with the N-terminus, transmembrane domain and part of the stem region of murine N-deacetylase/N-

sulfotransferase 2, an isozyme of human NDST1 which localizes to the trans-Golgi network (Humphries *et al.*, 1997; Kusche-Gullberg *et al.*, 1998) (Fig. 6B).

For the secretory constructs of EXT1 and EXT2, the cytoplasmic N-terminus, putative transmembrane domain along with 10 amino acids of the stem region were excised and substituted with the immunoglobulin kappa chain secretory signal tag supplied in the pSecTag2 vector (Invitrogen) thus targetting the chimeric protein for secretion (Fig. 6C).

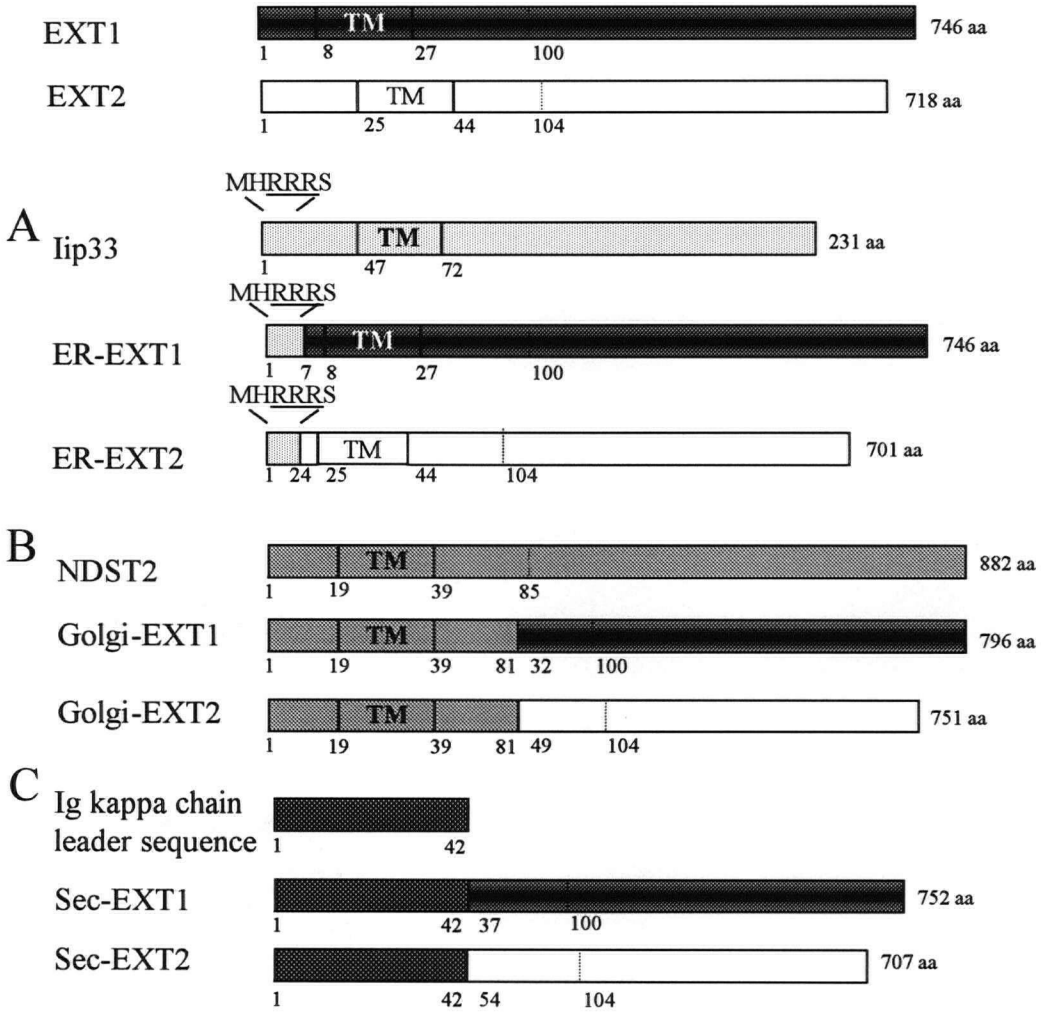


Figure 6 Chimeric EXT constructs.

All chimeric constructs were C-terminally tagged with either myc or gfp for easy detection.

A) The N-termini of EXT1 and EXT2 were replaced with the ER-localization signal MHRRRS (Schutze, M. *et al.*, 1994) to generate the ER-EXT1 and ER-EXT2 chimeric constructs.

B) Munro, S., 1991 proposed that the cytoplasmic N-terminus, transmembrane domain and part of the stem region of type II Golgi-residents were sufficient to localize type II membrane proteins to the Golgi. Thus to generate the Golgi-EXT1 and Golgi-EXT2 chimeric constructs, the N-termini

of EXT1 and EXT2 were substituted with the NDST cytoplasmic N-terminus, the transmembrane domain and part of the stem region.

C) EXT1 and EXT2 minus the cytoplasmic N-terminus, transmembrane domain and 10 aa of the stem region were subcloned into the secretory vector, pSecTag2 (InVitrogen) which contains the immunoglobulin kappa chain leader sequence for efficient secretion of expressed proteins (Coloma, MJ *et al.*, 1992).

3.2 Transient expression of EXT constructs in mammalian cells

All EXT constructs were verified by DNA sequencing and tested for expression in mammalian BHK cells. Expression of chimeric EXT proteins was detected via their myc or gfp C-terminal tags. ER-EXT1gfp and Sec-EXT1gfp were predicted to have a molecular weight of 114 and 116 kDa, respectively, while Golgi-EXT1gfp was expected to be slightly larger at 120 kDa. Analysis of the chimeric EXT1gfp expression showed protein bands with molecular masses that were larger than predicted (Fig. 7A). As there are two putative N-glycosylation sites on EXT1, it is possible that they were glycosylated thus causing the increase in molecular weight observed. The myc-tagged EXT2 chimeras were expected to be 83, 85 and 88 kDa for ER-EXT2, Golgi-EXT2 and Sec-EXT2 respectively. Bands migrating higher than expected were observed for the chimeric EXT2myc proteins (Fig. 7B). Like EXT1, there are also two putative N-glycosylation sites on EXT2 which may become N-glycosylated thereby resulting in an increase in molecular weight. Thus within the resolution of the gel system used, all chimeric EXT constructs were expressed in mammalian cells, and were larger than expected likely due to glycosylation of the proteins.

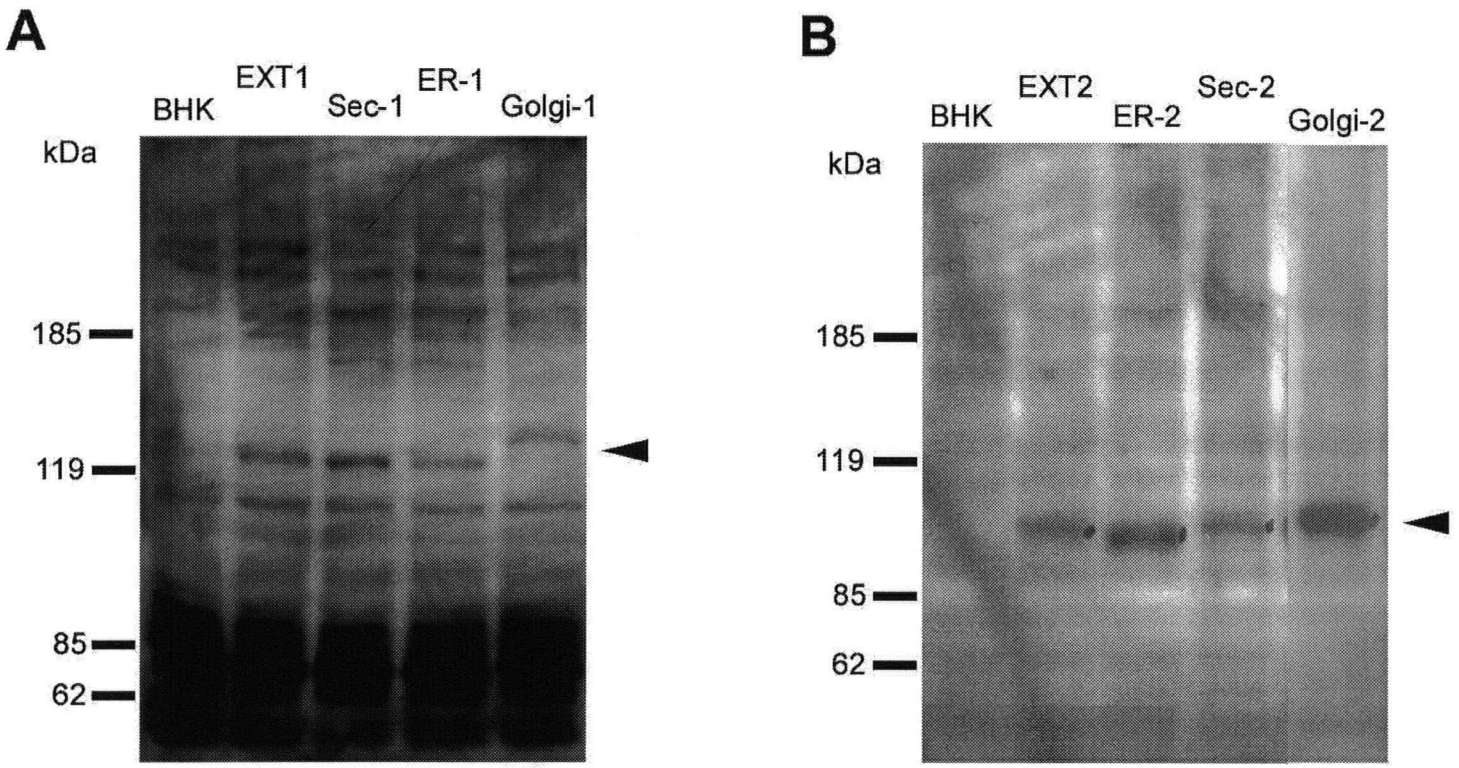


Figure 7 Western blot analysis of EXT constructs.

EXT constructs were transiently transfected into BHK cells and lysed 40 hrs later. The cell lysates were then separated electrophorectically and transferred onto Immobilon-P membrane followed by detection with antibodies.

(A) Chimeric EXT1gfp constructs were transiently expressed, the cell lysates were separated and the tagged proteins detected by α-gfp antibodies.

(B) Chimeric EXT2myc proteins were probed with α-myc antibodies following transfer onto membrane.

3.3 Golgi-EXT1, ER-EXT1 and Sec-EXT1 were all able to restore HSV susceptibility in Sog9 cells

To determine whether the substitution of different subcellular localization signals would perturb the *in vivo* enzymatic function of EXT1, all EXT1 constructs were transiently expressed in Sog9 cells. Sog9 cells are a murine L-cell derived line (Banfield *et al.*, 1995) that contains a specific defect in the EXT1 gene, predicting a truncated EXT1 protein (McCormick *et al.*, 2000) and are thus deficient in heparan sulfate biosynthesis. Due to the lack of cell surface heparan sulfate expression, and therefore of the initial attachment receptor, Sog9 cells are 99.5% resistant to HSV infection (Banfield *et al.*, 1995). At 40 hrs following transfection, Sog9 cells expressing the various EXT constructs were infected with HSV strains expressing β-galactosidase, and 6-10 hrs following infection, the cells were fixed and stained with the β-galactosidase substrate, X-gal.

EXT2 is unable to compensate for the defect in HS biosynthesis present in Sog9 cells (McCormick *et al.*, 2000) and in agreement with this, none of the chimeric EXT2myc constructs were capable of restoring Sog9 susceptibility to HSV infection (Fig. 8). By contrast, all the EXT1gfp constructs tested were able to synthesize HS *in vivo* as determined by the HSV assay though at different efficiencies (Fig. 9). As GlcA-T and GlcNAc-T activity is believed to occur in the Golgi (Fernandez and Warren, 1998), the transfection of Golgi-EXT1 into Sog9 cells was expected to allow HSV to infect Sog9 cells. Not only was this observed, but the Golgi construct allowed 15-20% more Sog9 cells to be infected by HSV than wt EXT1 (Fig. 9D). Interestingly, ER-EXT1 also restored HS biosynthesis in Sog9 cells despite an ER localization signal (Fig. 9C). The HSV infectivity of ER-EXT1-transfected Sog9 cells was slightly lower at 90-95% of wt EXT1 level. The

ability of ER-EXT1 to allow HSV to infect transfected Sog9 cells may be an artifact of overexpression as the ER may be overwhelmed with the amount of proteins present leading to leakage of ER-EXT1 to the Golgi. Remarkably, the secretory construct of EXT1 (Sec-EXT1) was also able to synthesize HS, and thus enabled HSV to infect Sog9 cells although at a greatly reduced level (Fig. 9E). The infectivity of the Sec-EXT1-transfected Sog9 cells was only 20-30% of wt EXT1 level but this still suggests that the Sec-EXT1 protein was functional and likely being retained within the cell long enough for the polymerization of heparan sulfate chains to occur on proteoglycans.

Since HSV-1 and HSV-2 differ slightly in their interactions with HS (Herold *et al.*, 1996), all the EXT1 constructs were tested with both HSV serotypes. Similar results were obtained with HSV-1 as with the HSV-2 assay (data not shown).

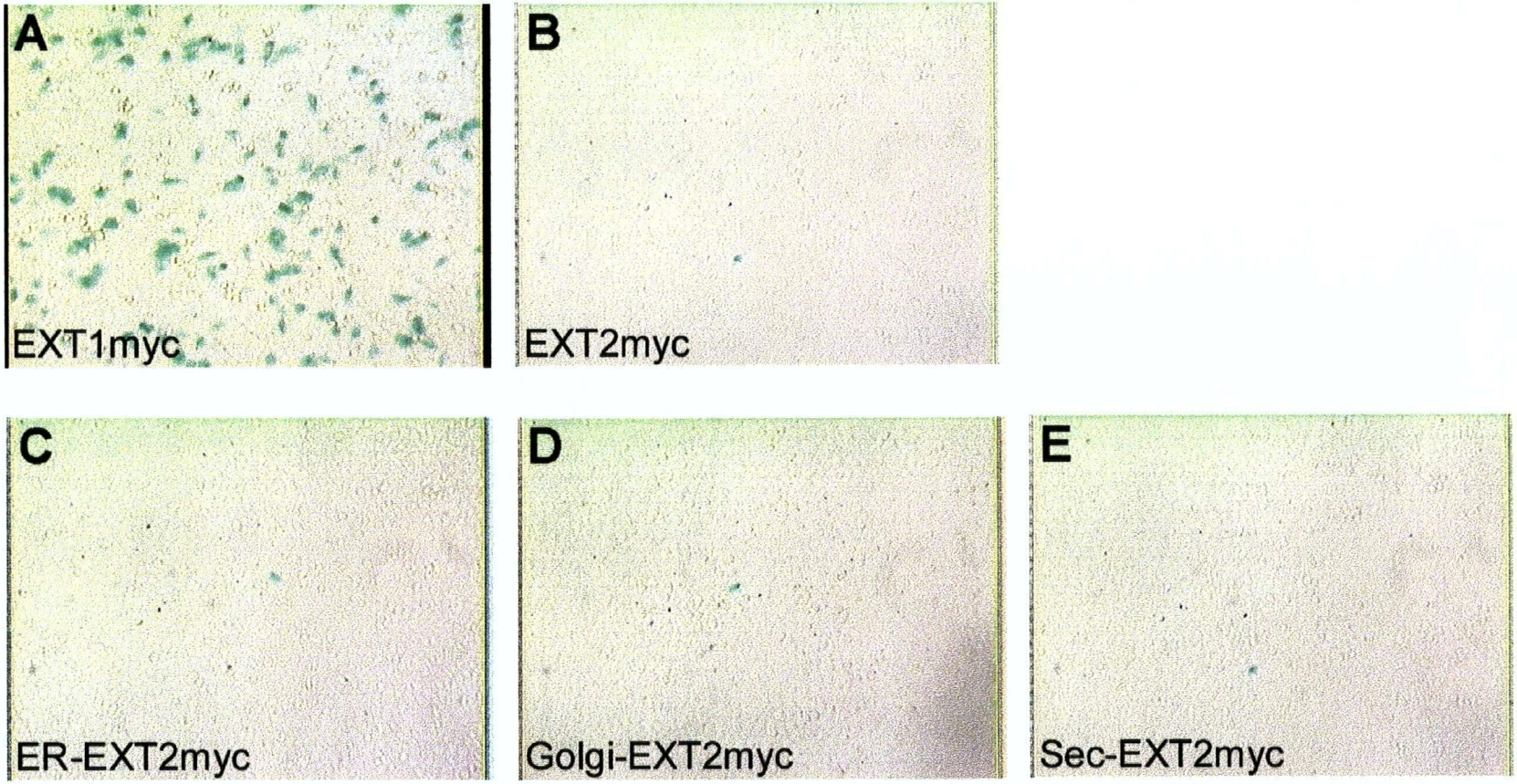


Figure 8 HSV assay of EXT2myc constructs in Sog9 cells.
Sog9 cells which are deficient in HS biosynthesis and are thus highly resistant to HSV infection were transfected with **(A)** EXT1myc (positive control), **(B)** EXT2myc (negative control), **(C)** ER-EXT2myc, **(D)** Golgi-EXT2myc or **(E)** Sec-EXT2myc. At 40 hrs post-transfection, the cells were infected with the β-galactosidase expressing HSV-2 strain, LIBR1. The cells were then fixed with paraformaldehyde 6 hrs later, and stained with X-gal for the visualization of HSV-infected cells.

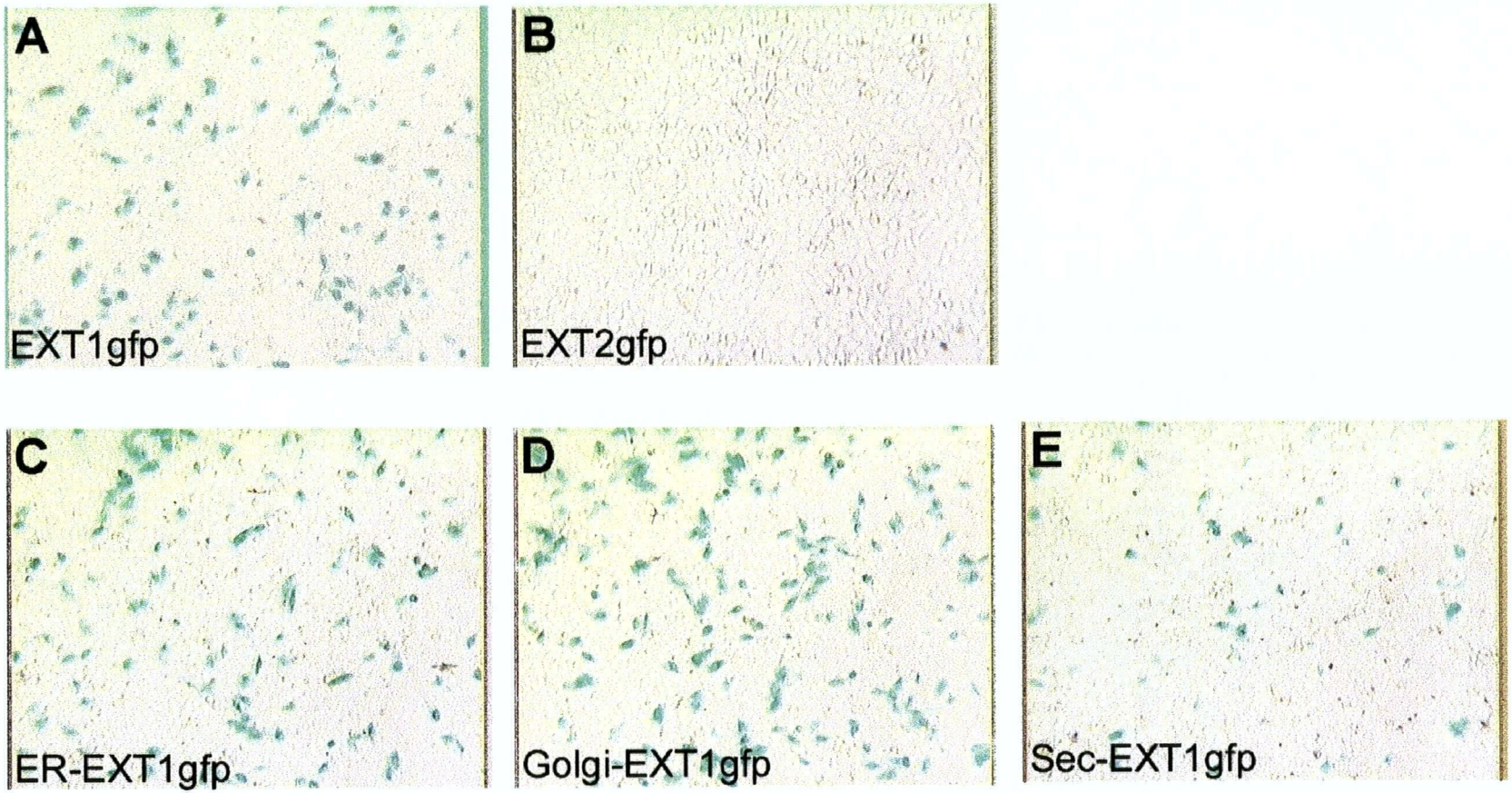


Figure 9 HSV assay of EXT1gfp constructs in Sog9 cells.

Sog9 cells which are deficient in HS biosynthesis and are thus highly resistant to HSV infection were transfected with **(A)** EXT1gfp (positive control), **(B)** EXT2gfp (negative control), **(C)** ER-EXT1gfp, **(D)** Golgi-EXT1gfp or **(E)** Sec-EXT1gfp. At 40 hrs post-transfection, the cells were infected with the β-galactosidase expressing HSV-2 strain, LIBR1. The cells were then fixed with paraformaldehyde 6 hrs later, and stained with X-gal for the visualization of HSV-infected cells.

3.4 Subcellular localization patterns of mono-transfected EXT constructs

Immunofluorescence studies of all chimeric EXT constructs were done to determine the effects the sorting signals were having on the subcellular distribution of the chimeras. Each EXT construct was overexpressed in BHK cells and its subcellular localization pattern observed by immunofluorescence microscopy. Immunofluorescence images depicting the predominant staining patterns of the chimeric proteins are shown in Figure 10.

Mono-transfected EXT1 and EXT2 (Fig. 10 A, B) displayed a staining pattern indicative of the ER in agreement with previously published data that the overexpression of either EXT1 or EXT2 leads to ER-localization (McCormick *et al.*, 2000; McCormick *et al.*, 1998). Similarly, ER-EXT1 and ER-EXT2 (Fig. 10 C, D) also distributed in a staining pattern distinctive of ER proteins. Interestingly, Golgi-EXT1 and Golgi-EXT2 (Fig. 10 F, G) exhibited staining patterns characteristic of the early Golgi cisternae or ERGIC, though some chimeras did distribute to a perinuclear location. Yet NDST2 (Fig. 10 E), which supplied the Golgi-localization signal used in the Golgi-EXT chimeras, localized predominantly to the Golgi as indicated by the immunofluorescence pattern and not to the ERGIC or ER. Mono-transfected Sec-EXT1 and Sec-EXT2 (Fig. 10 I, J) showed a staining pattern characteristic of secretory vesicles and SecPSA (prostate specific antigen with the same immunoglobulin kappa chain leader sequence in its N-terminus), a secretory control provided by Invitrogen (Fig. 10 H).

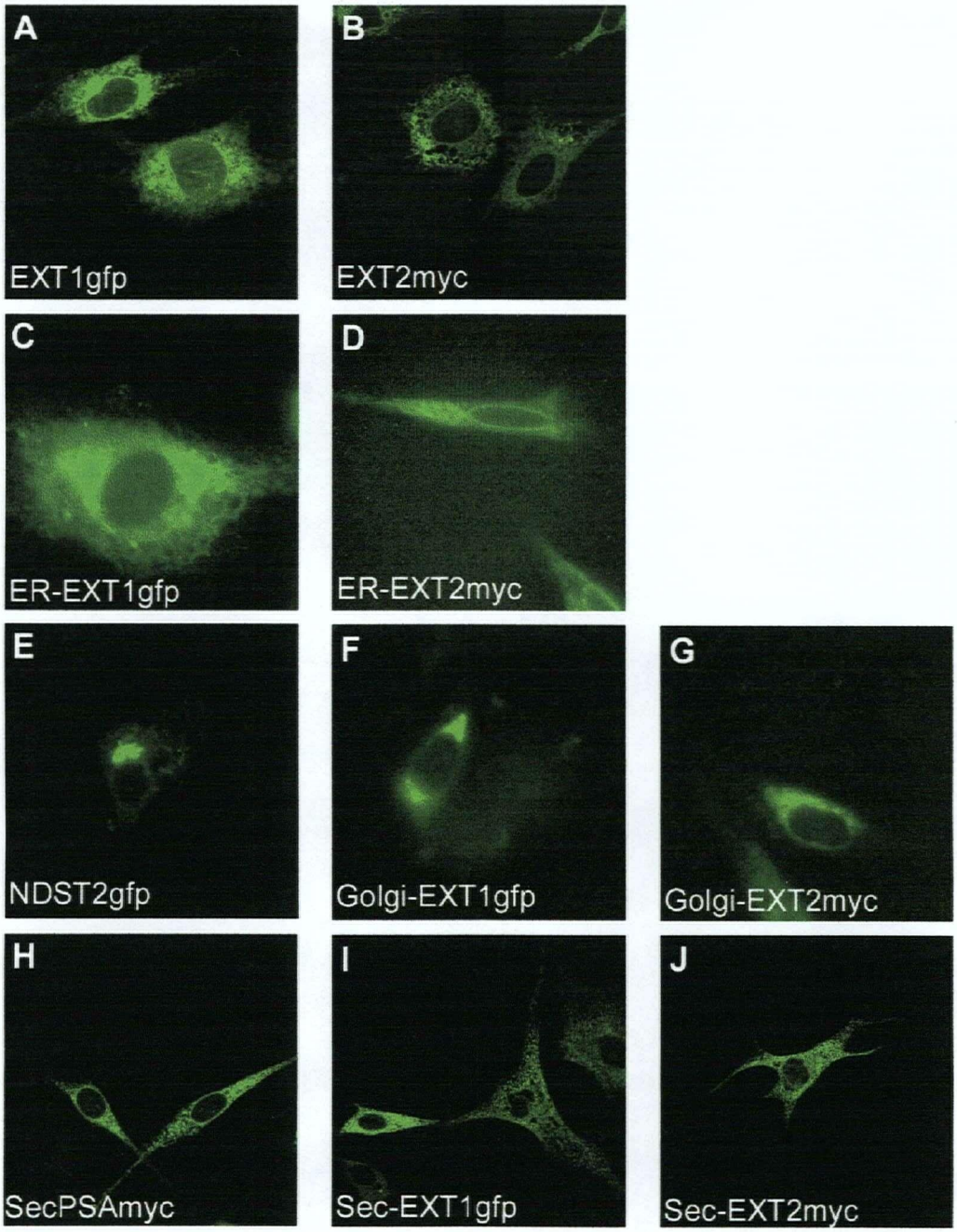


Figure 10 Subcellular localization patterns of mono-transfected chimeric EXT proteins.
Monolayers of BHK cells were mono-transfected with **A)** EXT1gfp, **B)** EXT2myc, **C)** ER-EXT1gfp, **D)** ER-EXT2myc, **E)** NDSTgfp, **F)** Golgi-EXT1gfp, **G)** Golgi-EXT2myc, **H)** SecPSAmyc, **I)** Sec-EXT1gfp and, **J)** Sec-EXT2myc.
Myc-tagged constructs were detected with α-myc antibodies followed by FITC-conjugated secondary antibodies. The subellular distribution of myc-tagged or gfp-tagged proteins were observed by immunofluorescence and images acquired using either the Zeiss DVC fluorescent microscope or the Bio-Radiance Plus confocal microscope.

3.5 Co-transfection of EXT1 constructs with wild-type (wt) EXT2 constructs

EXT1 and EXT2 localize to the ER when overexpressed individually but re-localize to the Golgi when transfected together (McCormick *et al.*, 2000). Furthermore, McCormick *et al.* have also shown that EXT1 and EXT2 are capable of forming a heterocomplex *in vivo*. Therefore, the subcellular localization patterns of chimeric EXT1 when co-expressed with wt EXT2 was investigated.

3.5.1 Immunofluorescence analysis of co-transfected chimeric EXT1 with wild-type EXT2

Data thus far showed that Sec-EXT1 was able to synthesize HS *in vivo* as demonstrated by HSV's ability to infect SecEXT1-transfected Sog9 cells, therefore it must be retained within the cell long enough for HS chain polymerization to occur. Hence the effects of co-transfecting gfp-tagged chimeric EXT1 with myc-tagged wt EXT2 was examined.

The distribution of cells with both co-transfected EXTs co-localizing in staining patterns characteristic of the ER, ERGIC, Golgi or secretory vesicles were assessed, and images depicting the immunofluorescence patterns of the majority of co-transfected cells are presented in Figure 11. The immunofluorescence images of chimeric EXT1gfp and wt EXT2myc were overlaid and shown in the third panels of each row. ER-EXT1gfp + EXT2myc (Fig. 11B) co-localized in a staining pattern characteristic of the Golgi despite the ER-localization motif in the cytoplasmic N-terminus of ER-EXT1gfp. The co-transfection of Golgi-EXT1gfp with wt EXT2myc (Fig. 11C) also resulted in both proteins localizing to what appears to be the Golgi. Interestingly though, Sec-EXT1gfp displayed a

staining pattern typical of Golgi-localization when co-transfected with wt EXT2myc (Fig. 11D). The data showed that all chimeric EXT1 constructs including the secretory construct of EXT1 re-localized predominantly to the Golgi when co-transfected with wt EXT2, suggesting that they are able to hetero-oligomerize.

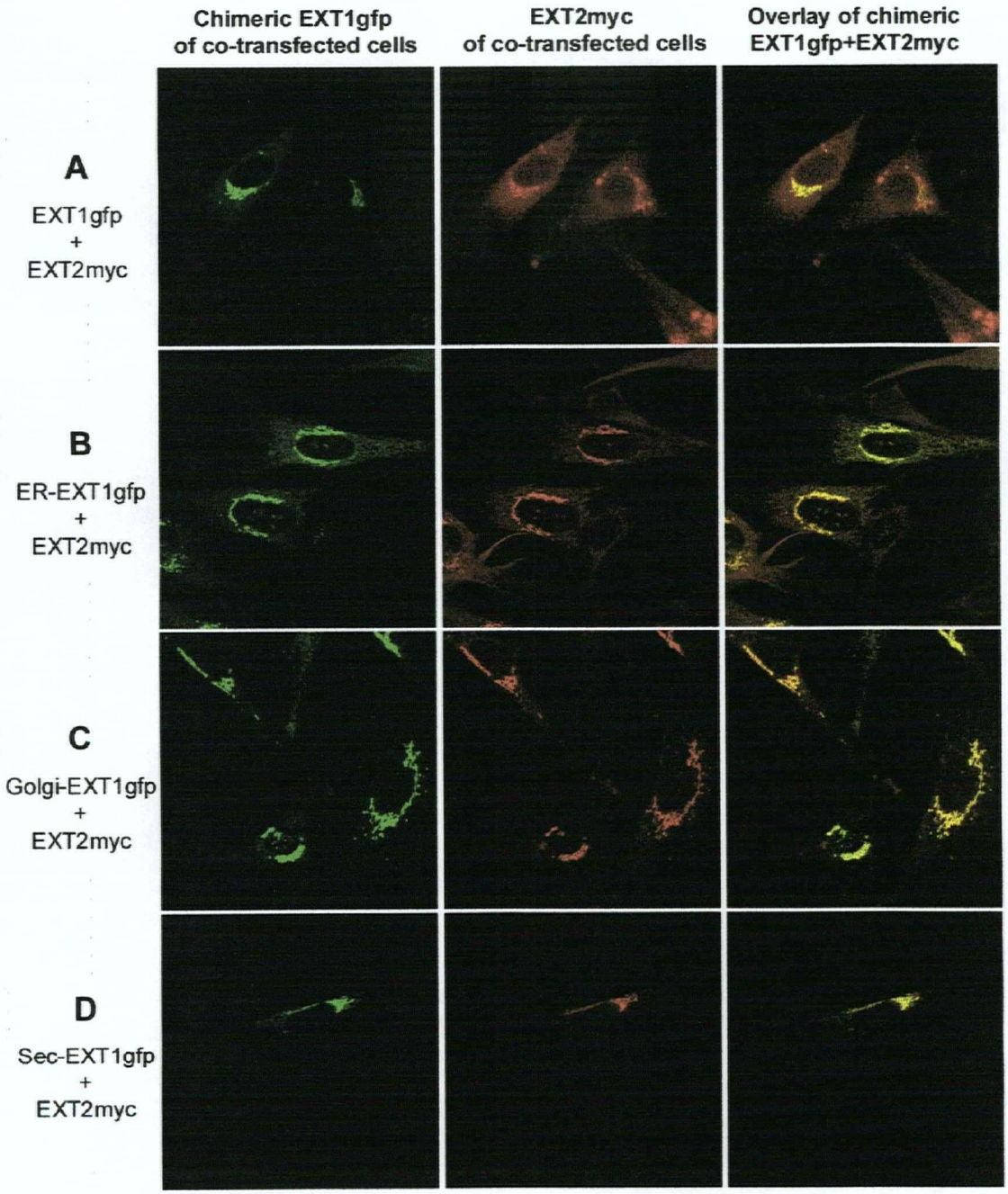


Figure 11 Subcellular localization patterns of co-transfected chimeric EXT1 with wild type EXT2.
BHK monolayers are co-transfected with **A)** EXT1gfp + EXT2myc, **B)** ER-EXT1gfp + EXT2myc, **C)** Golgi-EXT1gfp + EXT2myc and **D)** Sec-EXT1gfp + EXT2myc. Wild-type EXT2myc proteins were detected with a-myc antibodies followed by Cy5-conjugated secondary antibodies. The subcellular distribution of myc-tagged or gfp-tagged proteins were observed by immunofluorescence and images acquired using either the Zeiss DVC fluorescent microscope or the Bio-Radiance Plus confocal microscope.

3.5.2 All EXT1 constructs were able to form a heterocomplex with wild-type EXT2

To confirm that the co-localization of the EXT1 constructs with wild-type EXT2 was due to heterocomplex formation between the two proteins and not an artifact, sandwich enzyme-linked immunosorbent assay (ELISA) was performed. A 96-well plastic plate was coated with α-myc antibodies and cell lysates from either mono- or co-transfected BHK cells were added to the wells in triplicates. Rabbit α-gfp antibodies were then added followed by HRP-conjugated secondary α-rabbit antibodies. Heterocomplex formation between the EXT1 constructs and wild-type EXT2 was detected by the addition of the liquid substrate, TMB, and the soluble coloured product read at 450 nm. As can be seen in Figure 12, the substitution of the different N-terminal localization signals did not affect the oligomerization ability of chimeric EXT1 proteins with wt EXT2, suggesting that the N-terminal sequences was not necessary for hetero-oligomerization.

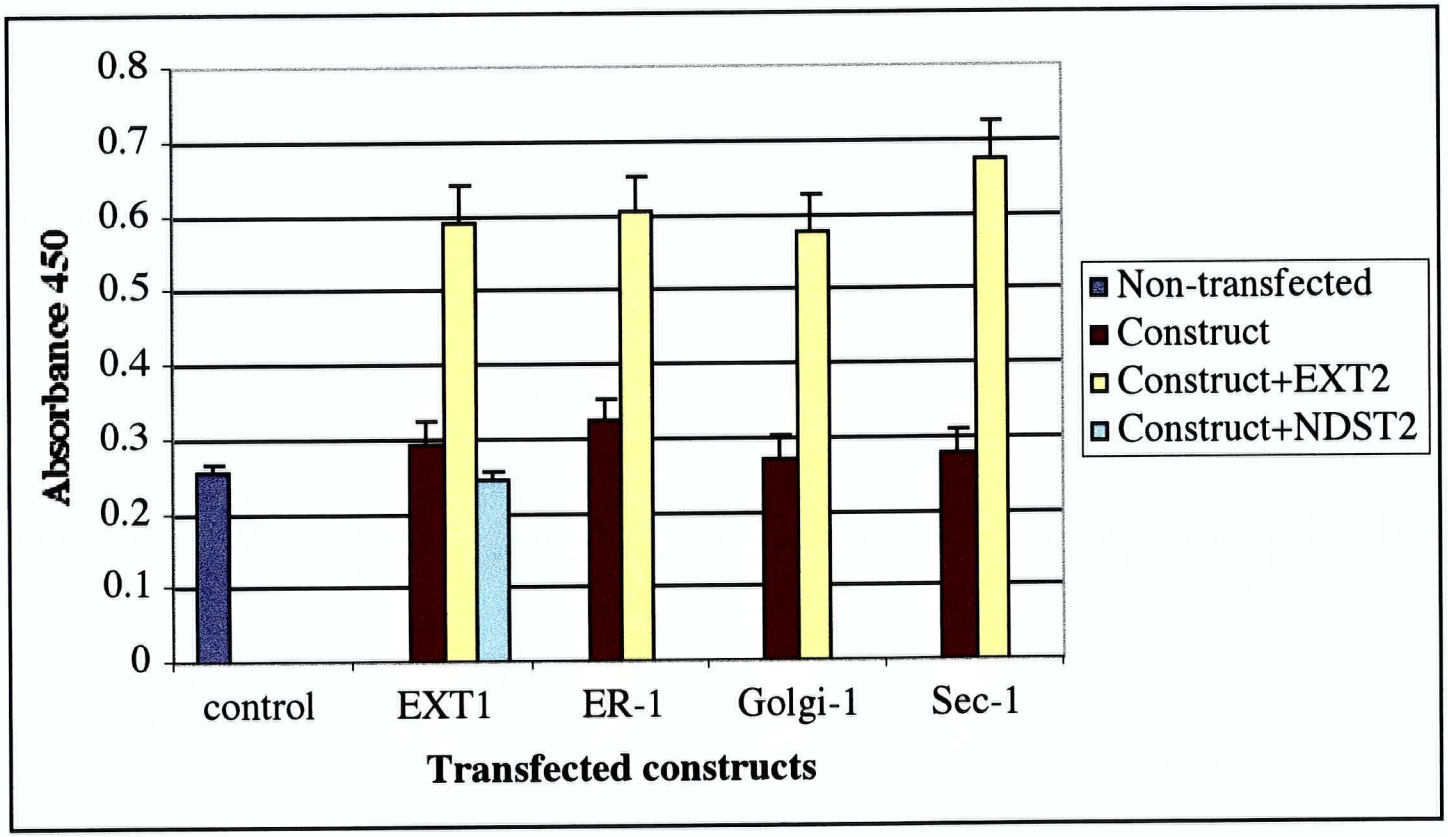


Figure 11 Analysis of heterocomplex formation of co-transfected chimeric EXT1 with wild-type EXT2.
The listed constructs were transfected into BHK cells and their cell lysates collected and analysed for chimeric EXT1/wild-type EXT2 hetero-oligomer formation using the enzyme linked immunosorbent assay. The chimeric EXT1myc protein was captured by α-myc antibodies coated on the bottom of wells, and hetero-oligomer formation with the co-transfected wild-type EXT2gfp protein or the NDST2gfp protein was detected using α-gfp antibodies and HRP-conjugated α-rabbit secondary antibodies. Complex formation was then revealed with the addition of the HRP substrate, TMB, producing a soluble substrate readable at 450 nm. The control was non-transfected cell lysate. A representative result from two experiments is presented.

3.6 Co-transfection of chimeric EXT1 with chimeric EXT2

As the above data indicated, chimeric EXT1 re-localized predominantly to the Golgi when co-transfected with wt EXT2 as suggested by the immunofluorescence patterns observed. Thus the ability of chimeric EXT1 to hetero-oligomerize with chimeric EXT2 and localize to the Golgi despite both proteins containing different subcellular localization signals was determined.

Immunofluorescence studies were done to observe the effects on subcellular localization of co-transfecting chimeric EXT1 and chimeric EXT2 together. The proportion of cells containing the two co-localized chimeric constructs displaying staining patterns characteristic of each subcellular compartment was ascertained, and Figure 13 shows the predominant immunofluorescence patterns of the chimeric proteins. When ER-EXT1 was co-transfected with either ER-EXT2 or Sec-EXT2, the staining patterns observed suggested the chimeras were co-localized among the ER, ERGIC and the Golgi with no obvious predominant localization. For the Golgi-chimeric constructs, the co-transfection of either Golgi-EXT1 or Golgi-EXT2 with another chimeric partner allowed both chimeras to immunofluoresce to a perinuclear localization indicative of the Golgi. Without an anchoring domain, the majority of co-transfected Sec-EXT1 and Sec-EXT2 exhibited staining patterns suggesting the chimeras were found in secretory vesicles when overexpressed. Thus the results of transfecting chimeric EXT1 with chimeric EXT2 were not as clear as the results of co-transfecting with wt EXT2.

To investigate whether the non-Golgi localization staining patterns observed for several of the chimeric EXT constructs were due to a lack of hetero-oligomerization, heterocomplex formation was tested using ELISA as described earlier. The absorbance

results obtained for the co-transfection of all combinations of chimeric EXT1 with chimeric EXT2 were similar to each other and to the absorbance reading observed for wt EXT1 and wt EXT2 (data not shown). This indicated that all the chimeric EXT1 constructs were able to form a heterocomplex with all the chimeric EXT2 constructs, suggesting that the inability of ER-EXT1 + ER-EXT2, ER-EXT1 + Sec-EXT2, Sec-EXT1 + ER-EXT2 and Sec-EXT1 + EXT2 to localize to the Golgi was not due to an inability to hetero-oligomerize.

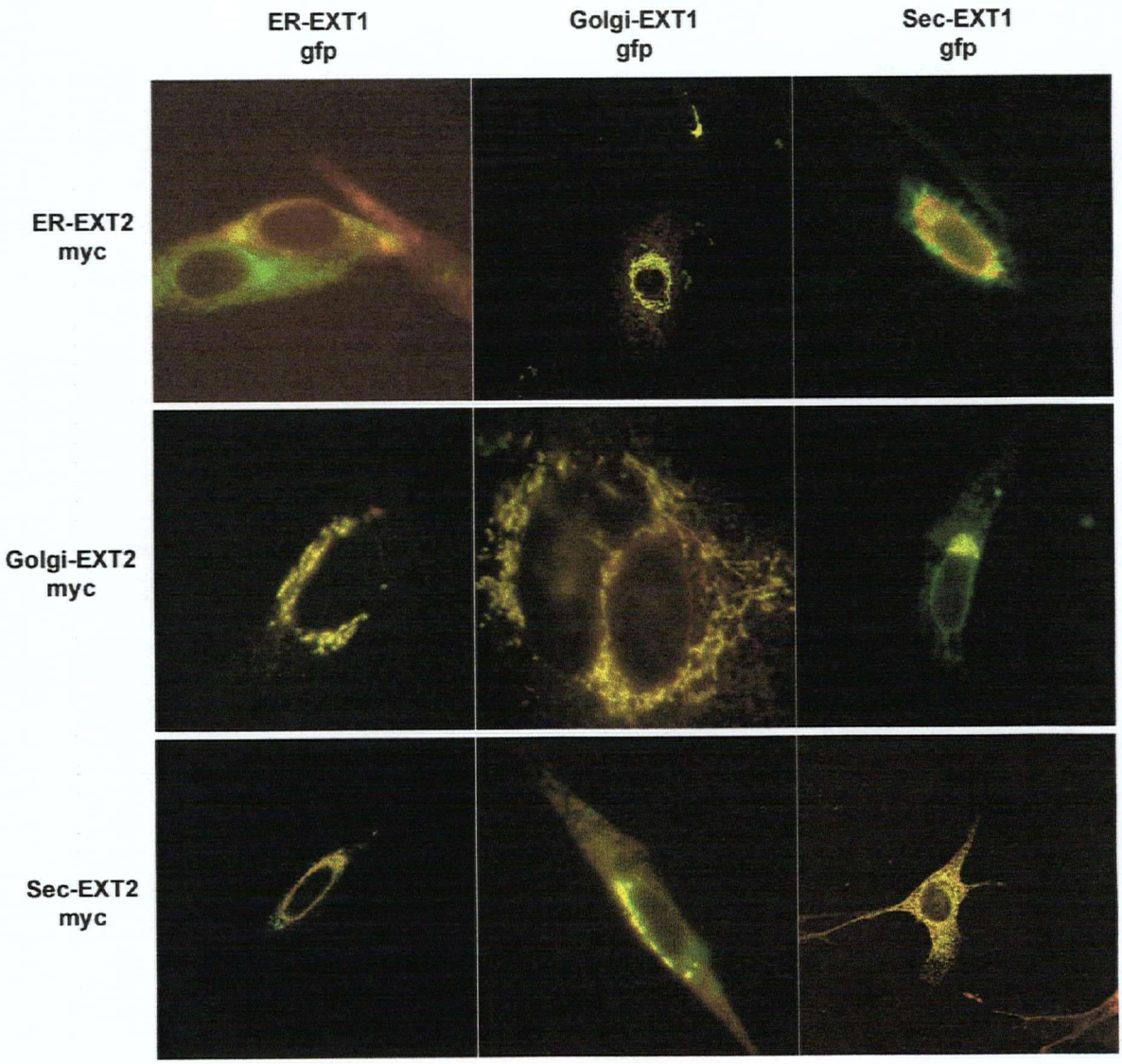


Figure 13 Subcellular localization patterns of co-transfected chimeric EXT1 with chimeric EXT2.

BHK monolayers were co-transfected with the listed chimeric EXT1gfp and chimeric EXT2myc constructs. Chimeric EXT2myc proteins were detected with α-myc antibodies followed by Cy5-conjugated secondary antibodies. The subellular distribution of myc-tagged or gfp-tagged proteins were observed by immunofluorescence and images acquired using either the Zeiss DVC fluorescent microscope or the Bio-Radiance Plus confocal microscope. Individual gfp- or myc-immunofluoresence images were overlaid together and presented here.

3.7 No other proteins involved in the EXT1/EXT2 heterocomplex were detected when either EXT1 or EXT2 were immunoprecipitated

To investigate whether there were other proteins involved in heterocomplex formation, a radiolabelled immunoprecipitation assay was performed. Approximately 18 hrs after the BHK cells were transfected with EXT1gfp and/or EXT2myc, they were radiolabelled overnight with [^{35}S]methionine. The cell lysates were collected and the tagged-proteins immunoprecipitated with either α-gfp or α-myc antibodies. The immunoprecipitated proteins were then separated by SDS-PAGE, transferred onto an Immobilon-P membrane and exposed to film. No other proteins were observed to be immunoprecipitated along with the EXT1/EXT2 heterocomplex within the detection sensitivity of the radiolabelled immunoprecipitation assay (Fig. 14). To further complicate the search for other proteins in the EXT1/EXT2 heterocomplex, a high degree of background bands were present despite several attempts to reduce non-specific binding.

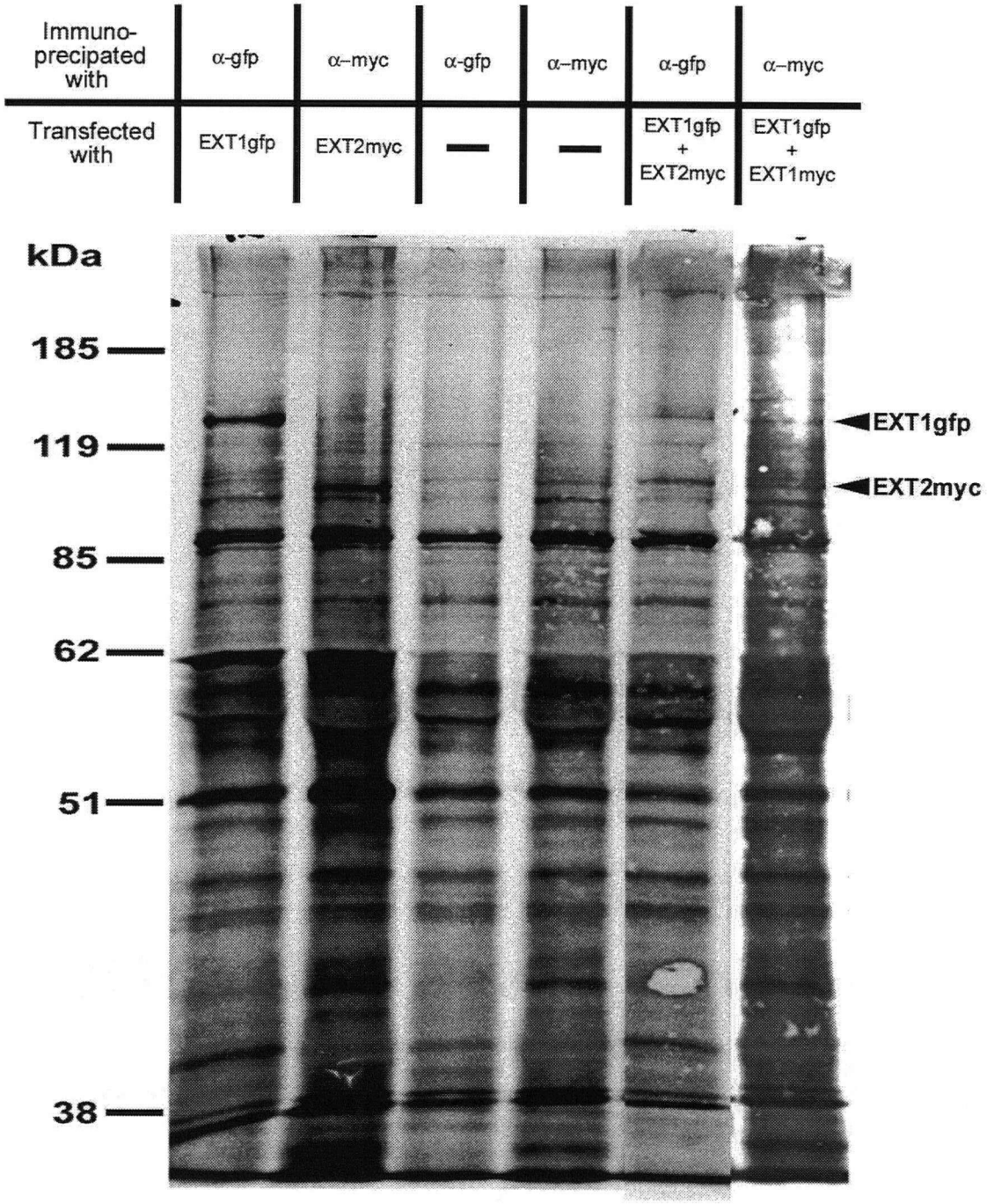


Figure 14 Radiolabelled immunoprecipitation analysis of EXT1-EXT2 heterocomplex formation.

BHK cells were transfected with the listed constructs and radiolabelled overnight with [^{35}S]methionine. Cells were then lysed and immunoprecipitated with the listed antibody followed by electrophoresis and exposure to film.

3.8 Co-transfection of chimeric EXT1 with chimeric EXT1

The kin recognition model hypothesizes that Golgi residents form homo- and/or hetero-oligomers in the Golgi preventing them from being packaged into vesicles destined for the PM (Nilsson *et al.*, 1993). Thus the ability of two different chimeric EXT1s to homo-oligomerize and the effect of this homo-oligomerization on subcellular localization were investigated.

BHK cells were co-transfected with different combinations of the EXT1 constructs, their subcellular localization patterns were analyzed using immunofluorescence microscopy, and the results presented in Figure 15. The staining patterns of co-transfected EXT1 + EXT1 (Fig. 15B), EXT1 + ER-EXT1 (Fig. 15C), EXT1 + Sec-EXT1 (Fig. 15E), ER-EXT1 + ER-EXT1 (Fig. 15F), ER-EXT1 + Sec-EXT1 (Fig. 15H) and Sec-EXT1 + Sec-EXT1 (Fig. 15K) were not characteristic of the Golgi, suggesting that they localized to subcellular compartments other than the Golgi. When Golgi-EXT1 was co-transfected with EXT1 (Fig. 15D), ER-EXT1 (Fig. 15G), Golgi-EXT1 (Fig. 15I) and Sec-EXT1 (Fig. 15J), it was able to partially co-localize the other chimeric EXT1 to the ERGIC or early-Golgi as suggested by the staining patterns observed. The immunofluorescence data indicated that homo-oligomer formation does not play a major role in Golgi localization in the case of EXT1 as the co-transfection of chimeric EXT1 constructs containing non-Golgi localization signals were not observed to re-localize in a staining pattern characteristic of the Golgi.

To confirm that chimeric EXT1s were capable of forming complexes with other chimeric EXT1s, homocomplex formation was studied by ELISA. The results indicated that the chimeric EXT1s were still able to form complexes with all the other chimeric EXT1 proteins (data not shown).

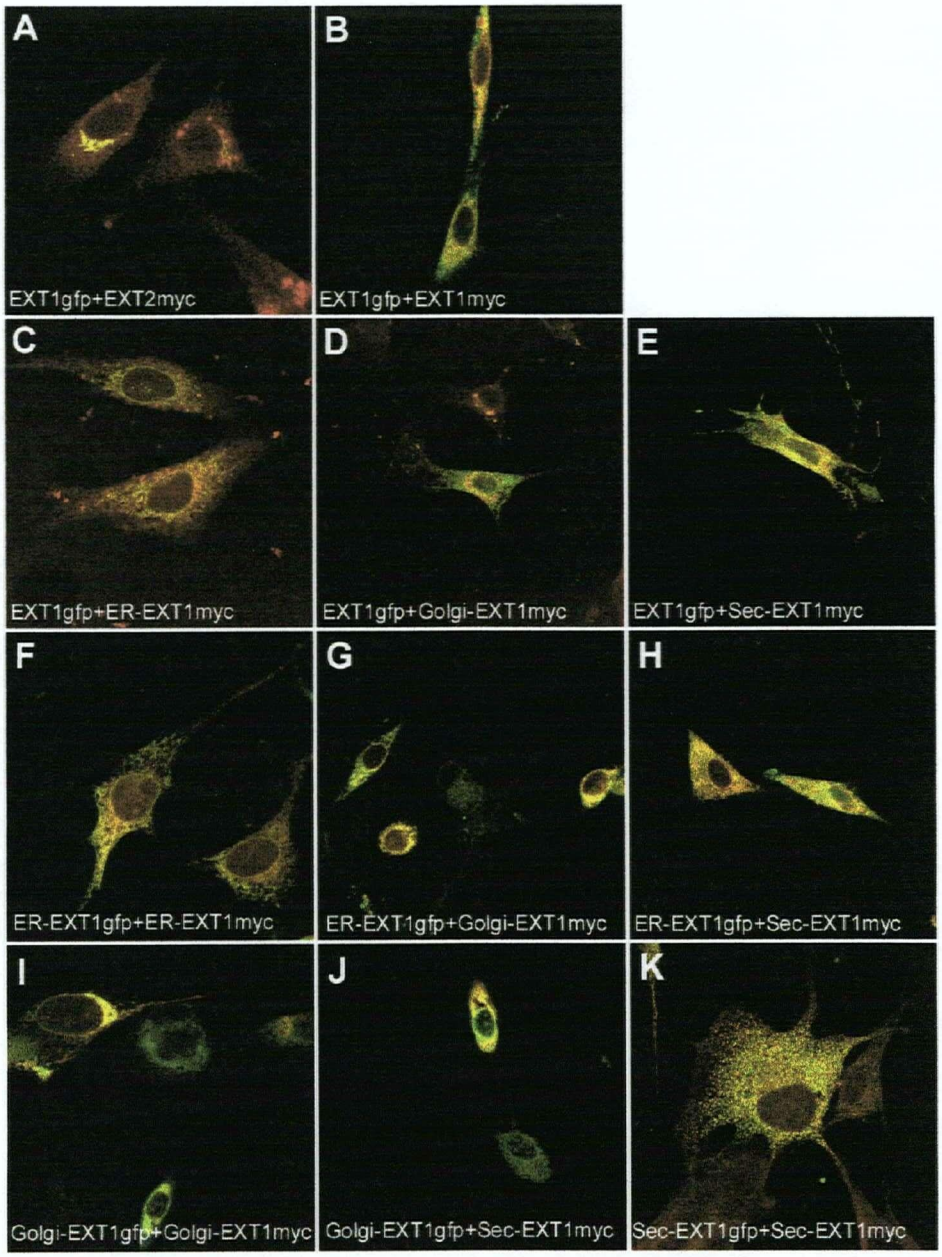


Figure 15 Subcellular localization patterns of co-transfected chimeric EXT1 with chimeric EXT1.

BHK monolayers were cotransfected with the following constructs: **A)** EXT1+EXT2, **B)** EXT1+EXT1, **C)** EXT1+ER-EXT1, **D)** EXT1+Golgi-EXT1, **E)** EXT1+Sec-EXT1, **F)** ER-EXT1+ER-EXT1, **G)** ER-EXT1+Golgi-EXT1, **H)** ER-EXT1+Sec-EXT1, **I)** Golgi-EXT1+Golgi-EXT1, **J)** Golgi-EXT1+Sec-EXT1, and **K)** Sec-EXT1+Sec-EXT1. One chimeric EXT1 partner was detected with α-myc antibodies followed by Cy5-conjugated secondary antibodies. The subellular distribution of myc-tagged or gfp-tagged proteins were observed by immunofluorescence and images acquired using either the Zeiss DVC fluorescent microscope or the Bio-Radiance Plus confocal microscope. Individual gfp- or myc-immunofluoresence images were overlaid together and presented here.

Discussion

An ER-, Golgi- and secretory form of EXT1 and EXT2 were created to determine the importance of the N-terminal region for *in vivo* HS polymerase activity, and its role in complex formation. The effects that the substituted localization signals had on Golgi-localization as indicated by immunofluorescence staining when chimeric EXT constructs were co-transfected together were also studied.

4.1 Subcellular localization of chimeric constructs

For type II transmembrane proteins, a double arginine motif in the cytoplasmic N-terminus of the ER-localized isoform of the human invariant chain has been identified as an ER-localization signal (Schutze *et al.*, 1994). This motif localized a type II transmembrane protein to the ER when transplanted onto the cell surface transferrin receptor. When this same motif was substituted into the cytoplasmic N-termini of EXT1 and EXT2, both chimeric constructs localized to the ER when overexpressed individually as suggested by the staining patterns observed (Fig. 10). But when co-transfected with their respective wt partners, they were unable to retain both themselves and their partner in the ER as might have been expected based on previously published results (Nilsson *et al.*, 1994). Nilsson *et al.* demonstrated that the substitution of an ER localization signal onto the medial Golgi protein, NAGTI caused another endogenous medial Golgi protein, MannII to relocalize to the ER. The results described in the present study suggest that the Golgi-localization mechanism used by EXT1 and EXT2 overrides the "RR" ER-localization motif.

The transmembrane domain and adjacent sequences of Golgi glycosyltransferases have been shown to be sufficient to localize type II transmembrane proteins to the Golgi (Burke *et al.*, 1994; Munro, 1991). Furthermore, the first 169 aa of the N-terminus of human NDST1 was able to localize a reporter protein to the Golgi (Humphries *et al.*, 1997). Thus the first 81 aa encompassing the transmembrane domain and adjacent sequences of murine NDST2, an isozyme of NDST1 were substituted into the N-termini of EXT1 and EXT2. From the staining patterns observed, this putative Golgi-localization signal was unable to localize the chimeric constructs of EXT1 and EXT2 strictly to the Golgi though it greatly improved the Golgi-localization of other chimeric constructs co-transfected with it (Fig. 11). This result suggests that some mechanism other than the TM domain and amino acids located immediately on either side is also involved in the Golgi-localization of EXT1 and EXT2. Variations in subcellular localization for the same Golgi localization signal have been seen in different cell types (Tang *et al.*, 1995). Thus it is possible that the NDST2-Golgi-localization signal is not sufficient to localize the chimeric constructs to the Golgi in BHK cells but would be adequate in other cell types though similar results were observed for the Golgi constructs in both BHK and Sog9 cells (data not shown).

The immunoglobulin kappa chain leader sequence in the SecTag2 vector appears to allow EXT1 and EXT2 to be efficiently packaged into secretory vesicles (Fig. 10). Interestingly, despite the secretory signal and the loss of its cytoplasmic N-terminus, TM domain and 10 aa of the stem region, it still localized to the Golgi as suggested by the perinuclear staining observed when co-transfected with its wt partner (Fig. 11). This suggests that it is forming a heterocomplex with its wt partner and its partner is anchoring both itself and the secretory EXT protein in the Golgi.

The subcellular localization as suggested by the different staining patterns observed when the chimeric constructs were mono-transfected or when various combinations of chimeric EXTs were co-transfected together should be confirmed in the future by co-localization with known subcellular markers. However, this study does provide information as to the effect the different sorting signals have on the chimeras as well as providing intriguing clues to Golgi localization due to heterocomplex formation.

4.2 *In vivo* heparan sulfate activity

Despite the substitutions of different subcellular signals on EXT1, all chimeric EXT1 constructs were still able to synthesize HS *in vivo* as determined by the HSV assay though at different efficiencies (Fig. 9).

The transient expression of Golgi-EXT1 (Fig. 9D) allowed better infection of Sog9 cells by HSV compared to wt EXT1 (Fig. 9A). This further supports the belief that HS polysaccharide chain polymerization occurs in the Golgi as suggested by the finding that GlcA-T and GlcNAc-T *in vitro* activity is coupled to the Golgi (Fernandez and Warren, 1998). In order for EXT1 to localize to the Golgi efficiently, heterocomplex formation with EXT2 appears to be required thus overexpressed wt EXT1 is constrained by the limiting amounts of endogenous EXT2 available. In contrast, Golgi-EXT1 is able to partially bypass the EXT2 requirement for Golgi-localization with its putative Golgi-localization signal thereby increasing the availability of the enzyme in the Golgi for HS biosynthesis.

Interestingly, ER-EXT1 also restored HS biosynthesis in Sog9 cells despite an ER localization signal (Fig. 9C). It is possible that the ability of ER-EXT1 to restore Sog9 susceptibility to HSV was an artifact of overexpression. The large amounts of proteins

expressed may have overwhelmed the ER leading to leakage of the chimera to the Golgi where it is then able to synthesize HS chains. Another possibility is that ER-EXT1 formed a heterocomplex with endogenous EXT2 thus localizing to the Golgi as suggested by the results of co-transfecting ER-EXT1 with wt EXT2 in which both proteins displayed immunofluorescence patterns characteristic of the Golgi when both partners are abundantly available.

In particular, the Sec-EXT1 construct was still able to synthesize HS though its putative transmembrane domain and adjacent sequences were replaced with a secretory signal. The GlcA-T activity has been localized to the middle of the lumenal C-terminus and the GlcNAc-T catalytic site is believed to be in the C-terminal region as well, thus Sec-EXT1's N-terminal substitution may not be expected to abrogate *in vitro* activity but surprisingly, it still did not abolish *in vivo* HS biosynthesis. Since it is able to synthesize HS *in vivo*, it further suggests that it is forming a heterocomplex with wt EXT2 in the Golgi and that this heterocomplex is stable enough for polysaccharide chain elongation to occur. The other possibility is that Sec-EXT1 is secreted out of the cell and polymerizing the HS polysaccharide chain onto nascent proteins in the extracellular space. This however, is highly unlikely as the substrates would have to be present in sufficient quantities for the enzyme to encounter. Furthermore, after the polysaccharide chains were synthesized, they would still have to undergo modification by other enzymes (i.e. NDST) to become sulfated.

The HSV assay is a useful assay for determining whether HS is being produced. As long as HS is present, HSV can use it to attach to the cell surface and infect the cells. However, the assay can't detect differences in the amount, length or in the type (i.e. degree

of sulfation) of HS being produced. Nevertheless, it is a useful assay for investigating the *in vivo* HS biosynthetic function of mutagenized EXT1.

4.3 Heterocomplex formation

From the ELISA results (Fig. 14, and data not shown), I conclude that the cytoplasmic N-terminus, TM domain and at least 10 aa of the stem region are not essential for heterocomplex formation. It appears that the lumenal C-termini of EXT1 and EXT2 alone are sufficient for heterocomplex formation as evidenced by the ability of Sec-EXT1 and Sec-EXT2 to oligomerize. The hetero-oligomerization between Sec-EXT1 and wt EXT2 is consistent with the results obtained in the HSV assay and the subcellular localization pattern analysis. Taken together, the data indicate that Sec-EXT1 is retained in the Golgi due to heterocomplex formation with endogenous wt EXT2 and is thus able to synthesize HS chains for HSV to bind and infect Sog9 cells. Further support that the lumenal portions of EXT1 and EXT2 are sufficient to generate the heterocomplex is provided by data showing an increase in the *in vitro* GlcNAc-T and GlcA-T activity of co-expressed EXT1 and EXT2 constructs lacking the TM domains, compared to individually expressed EXT1 or EXT2 (Senay *et al.*, 2000).

4.4 Involvement of other proteins

Yeast two-hybrid screens using a fragment containing a conserved portion of the EXT1 or EXT2 C-terminus with the human keratinocyte and lymphocyte cDNA libraries revealed 2 interacting proteins (Simmons *et al.*, 1999). EXT2 was found to interact with both GalNAc-T5 and TRAP1 (tumor necrosis factor type 1 associated protein) while EXT1

only interacted with TRAP1. Furthermore, they found that some etiological mutants failed to interact with TRAP1 and GalNAc-T5, suggesting a role for these two proteins in HME. GalNAc-T5 may be involved in HS biosynthesis while TRAP1, a heat shock protein with homology to the hsp90 family, has been demonstrated to interact with retinoblastoma protein (Rb) and type 1 receptor for tumor necrosis factor (Chen *et al.*, 1996; Song *et al.*, 1995). A recent study on TRAP1 suggests that in addition to playing a role in transforming growth factor-beta signalling, it acts as a chaperone for Smad2 as well (Wurthner *et al.*, 2001).

Thus I decided to see if I could detect any other proteins involved in the EXT1-EXT2 heterocomplex. Cells co-transfected with EXT1 and EXT2 were radiolabelled, the EXT proteins were immunoprecipitated, electrophoretically separated and analyzed. No other protein bands were observed compared to the negative controls (Fig. 14). EXT1 and EXT2 were able to form a heterocomplex resulting in Golgi localization despite being overexpressed in cells (McCormick *et al.*, 2000). This suggests that no other proteins are required for formation of the heterocomplex unless the other members of the complex were also being expressed in large quantities by the cell.

Unfortunately, there were a lot of background bands present which may interfere with the detection of other proteins involved in the heterocomplex. The background bands remained despite attempts to clear them up thus running a 2-D SDS-PAGE of the immunoprecipitated proteins may separate the different proteins enough to identify novel partners in the heterocomplex. Another approach to confirm that TRAP-1 and GalNAc-T5 are involved in the heterocomplex may be to immunoprecipitate EXT1 or EXT2 and probe specifically for TRAP-1 or GalNAc-T5. It is also possible that TRAP1, GalNAc-T5

or other interacting proteins are expressed at levels too low for detection by the radiolabelled immunoprecipitation assay or that the interactions between these proteins are only transient.

4.5 Model of Golgi retention

Golgi resident proteins must be maintained in the Golgi despite the large flow of proteins passing through heading for various destinations. Thus the mechanism of Golgi retention must be able to distinguish between Golgi residents and non-residents. To account for the maintenance of Golgi-residents but not transient proteins in the Golgi, two main models have been proposed: the bilayer thickness model and the kin-recognition model. The bilayer thickness model postulates that the shorter length of the transmembrane domain of Golgi-resident proteins versus those of the PM proteins prevents the protein from entering secretory vesicles and is thus retained within the Golgi (Bretscher and Munro, 1993). The kin-recognition model proposes that Golgi-residents oligomerize to form structures too large to enter transport vesicles, and are thus retained within the Golgi (Nilsson *et al.*, 1993). Although both models are supported by experimental evidence, neither model alone can account for how all Golgi-resident glycosyltransferases are retained. It is possible that different Golgi residents use different mechanisms to remain in the Golgi or that both models contribute to Golgi retention. The two models may work in concert, with the shorter length of the transmembrane of Golgi residents functioning to slow entry into transport vesicles thus allowing the proteins to interact with other Golgi proteins, thereby forming oligomers too large to exit the Golgi.

To further complicate the matter, the Golgi is composed of several discrete cisternae and individual Golgi residents are found only in certain cisternae and not others. In fact, many modification enzymes are found in the order in which they act on substrates. Thus further research needs to be done to account for the maintenance of distinct subpopulations of Golgi residents in specific compartments of the Golgi.

The co-localization patterns observed for the chimeric EXT1 and wt EXT2 proteins in the Golgi, especially with the chimeric Sec-EXT1 and wt EXT2, suggest that the major Golgi-localization mechanism used by EXT1 and EXT2 is hetero-oligomer formation as described by the kin recognition model (Nilsson *et al.*, 1993). Though the kin recognition model predicts that homo-oligomers also contribute to Golgi retention, they do not appear to contribute significantly to Golgi-localization in the case of EXT1 (data not shown). This is in agreement with the fact that overexpression of either EXT1 or EXT2 have been shown to lead to ER- and not Golgi-localization (McCormick *et al.*, 2000). Although EXT1 forms homo-oligomers, they may not be able to interact with other Golgi proteins (i.e. TRAP1) without the help of EXT2, or they may be unable to form a large enough complex to prevent entry into transport vesicles.

From all the data currently available, heterocomplex formation of EXT1 with EXT2 either allows a chaperone to bind and take the complex to the Golgi, or else EXT1 and EXT2 normally cycle between the ER and Golgi but upon heterocomplex formation, a conformational change occurs in one or both partners allowing the binding of more EXT1/EXT2 heterocomplexes or other Golgi-localized proteins in a multicomplex (Fig. 16). This multicomplex would then be retained in the Golgi and prevented from entering

secretory vesicles destined for the cell surface, or COPI vesicles for retrograde transport back to the ER.

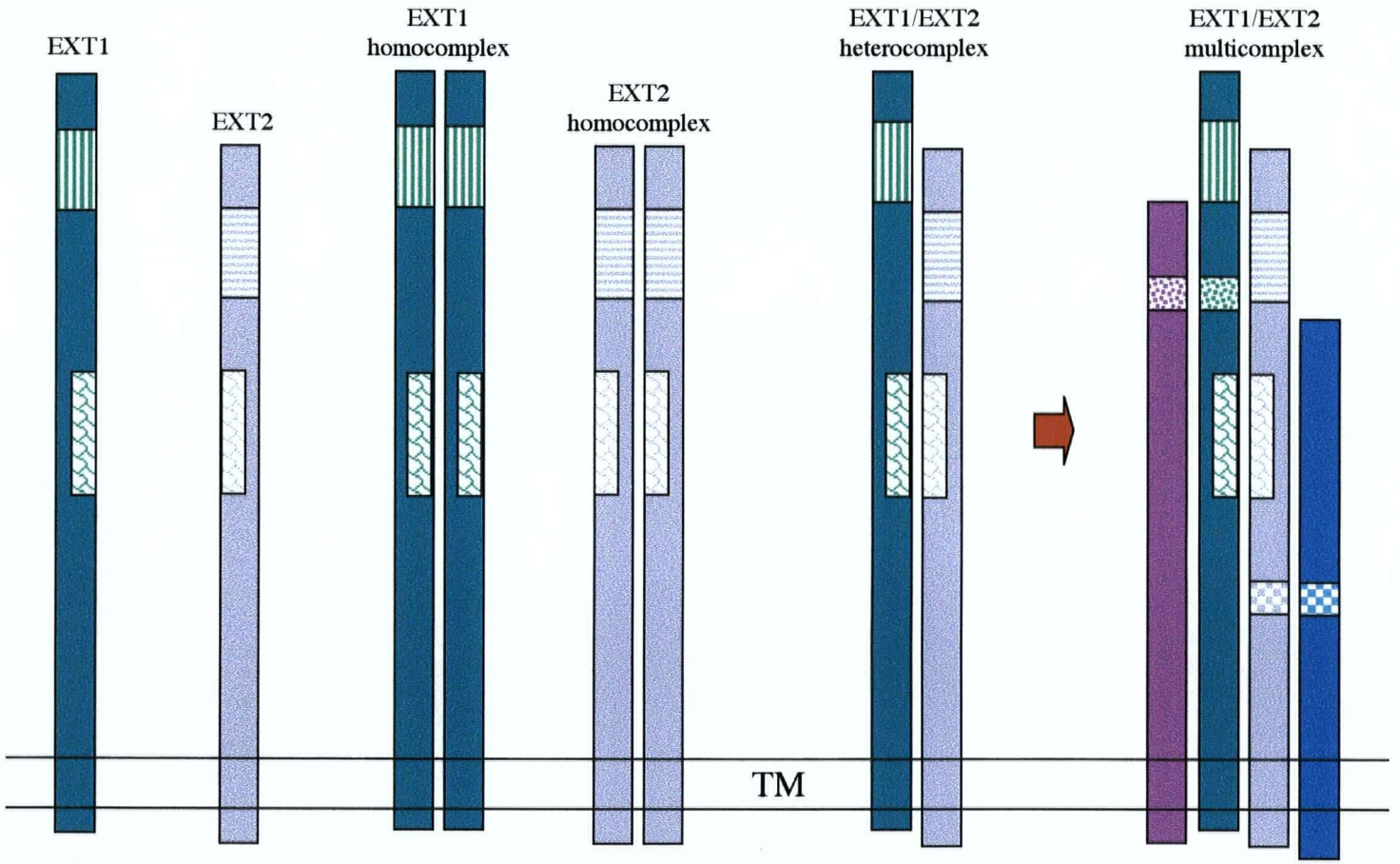


Figure 16 Model of EXT1 and EXT2 Golgi-localization mechanism.
EXT1 is able to complex with other EXT1 proteins, and EXT2 can bind to other EXT2s to form homo-oligomers *in vivo* (McCormick *et al.*, 2000). As well as forming homo-oligomers, EXT1 and EXT2 can complex with each other to form hetero-oligomers which result in efficient Golgi localization. As homo-oligomers are insufficient for Golgi localization, we propose that upon heterocomplex formation between EXT1 and EXT2, a conformational change occurs in one or both proteins. This would expose further binding sites for other Golgi-resident proteins to bind, and together with EXT1 and EXT2 form a multicomplex too large to enter transport vesicles budding from the Golgi.

4.6 Future directions

Sec-EXT1 and Sec-EXT2 were able to form a heterocomplex despite the loss of their cytoplasmic N-termini, putative transmembrane domains and 10 aa of their stem regions. Thus it would be very interesting to investigate which amino acids in the lumenal C-terimini of EXT1 and EXT2 are involved in heterocomplex formation. The heterocomplex appears to be relatively stable as it is able to withstand cell lysis and handling during the immunoprecipitation step, therefore disulfide bonds may be involved in the interaction between the two proteins. Destroying the ability of EXT1 and EXT2 to form intermolecular disulfide bonds by mutating cysteine residues in the lumenal domains of EXT1 and EXT2 may provide further information about heterocomplex formation. Three dimensional crystal structure analysis of the heterocomplex versus EXT1 or EXT2 alone should also provide clues to structure-function relationship as the heterocomplex contains higher glycosyltransferase activity *in vitro* than individual EXT1 or EXT2 alone (McCormick *et al.*, 2000). Furthermore, future experiments to determine the ability of etiological EXT1 mutants to hetero-oligomerize may help clarify the importance of heterocomplex formation in HME.

References

Ahn, J., Ludecke, H. J., Lindow, S., Horton, W. A., Lee, B., Wagner, M. J., Horsthemke, B., and Wells, D. E. (1995). Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). *Nat Genet* **11,** 137-143.

Alvarez-Dominguez, C., Vazquez-Boland, J. A., Carrasco-Marin, E., Lopez-Mato, P., and Leyva-Cobian, F. (1997). Host cell heparan sulfate proteoglycans mediate attachment and entry of Listeria monocytogenes, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition. *Infection and Immunity* **65,** 78-88.

Aridor, M., Bannykh, S. I., Rowe, T., and Balch, W. E. (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. *J Cell Biol* **131,** 875-893.

Banfield, B. W., Leduc, Y., Esford, L., Schubert, K., and Tufaro, F. (1995). Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. *J Virol* **69,** 3290-3298.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* **77,** 895-907.

Bellaiche, Y., The, I., and Perrimon, N. (1998). Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature* **394,** 85-88.

Binari, R. C., Staveley, B. E., Johnson, W. A., Godavarti, R., Sasisekharan, R., and Manoukian, A. S. (1997). Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. *Development* **124,** 2623-32.

Bretscher, M. S., and Munro, S. (1993). Cholesterol and the Golgi apparatus. *Science* **261,** 1280-1281.

Bullock, S. L., Fletcher, J. M., Beddington, R. S., and Wilson, V. A. (1998). Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev* **12,** 1894-1906.

Burke, J., Pettitt, J. M., Humphris, D., and Gleeson, P. A. (1994). Medial-Golgi retention of N-acetylglucosaminyltransferase I. Contribution from all domains of the enzyme. *J Biol Chem* **269,** 12049-12059.

Cai, W. H., Gu, B., and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J Virol* **62,** 2596-2604.

Chen, C., Ma, J., Lazic, A., Backovic, M., and Colley, K. J. (2000). Formation of insoluble oligomers correlates with ST6Gal I stable localization in the golgi. *J Biol Chem* **275,** 13819-13826.

Chen, C. F., Chen, Y., Dai, K., Chen, P. L., Riley, D. J., and Lee, W. H. (1996). A new member of the hsp90 family of molecular chaperones interacts with the retinoblastoma protein during mitosis and after heat shock. *Mol Cell Biol* **16,** 4691-4699.

Cheung, P. K., McCormick, C., Crawford, B. E., Esko, J. D., Tufaro, F., and Duncan, G. (2001). Etiological point mutations in the hereditary multiple exostoses gene EXT1: a functional analysis of heparan sulfate polymerase activity. *Am J Hum Genet* **69,** 55-66.

Clines, G. A., Ashley, J. A., Shah, S., and Lovett, M. (1997). The structure of the human multiple exostoses 2 gene and characterization of homologs in mouse and Caenorhabditis elegans. *Gen Res* **7,** 359-367.

Colley, K. J. (1997). Golgi localization of glycosyltransferases - more questions than answers. *Glycobiol* **7,** 1-13.

Cook, A., Raskind, W., Blanton, S. H., Pauli, R. M., Gregg, R. G., Francomano, C. A., Puffenberger, E., Conrad, E. U., Schmale, G., Schellenberg, G., Wijsman, E., Hecht, J. T., Wells, D., and Wagner, M. J. (1993). Genetic heterogeneity in families with hereditary multiple exostoses. *Am J Hum Genet* **53,** 71-79.

Cosson, P., and Letourneur, F. (1994). Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* **263,** 1629-1631.

Dahl, M. T. (1993). The gradual correction of forearm deformities in multiple hereditary exostoses. *Hand Clin* **9,** 707-718.

Dolan, A., Jamieson, F. E., Cunningham, C., Barnett, B. C., and McGeoch, D. J. (1998). The genome sequence of herpes simplex virus type 2. *Journal of Virology* **72,** 2010-2021.

Fernandez, C. J., and Warren, G. (1998). In vitro synthesis of sulfated glycosaminoglycans coupled to inter-compartmental Golgi transport. *J Biol Chem* **273,** 19030-19039.

Fuki, I. V., Kuhn, K. M., Lomazov, I. R., Rothman, V. L., Tuszynski, G. P., Iozzo, R. V., Swenson, T. L., Fisher, E. A., and Williams, K. J. (1997). The syndecan family of proteoglycans. Novel receptors mediating internalization of atherogenic lipoproteins *in vitro*. *J Clin Invest* **100,** 1611-1622.

Fuller, A. O., Santos, R. E., and Spear, P. G. (1989). Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J Virol* **63,** 3435-3443.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280,** 1618-1620.

Geuijen, C. A. W., Willems, R. J. L., and Mooi, F. R. (1996). The major fimbrial subunit of Bordetella pertussis binds to sulfated sugars. *Infection and Immunity* **64,** 2657-2665.

Gu, F., Crump, C. M., and Thomas, G. (2001). Trans-Golgi network sorting. *Cell. Mol. Life Sci.* **58,** 1067-1084.

Hacker, U., Lin, X., and Perrimon, N. (1997). The Drosophila sugarless gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124,** 3565-3573.

Haerry, T. E., Heslip, T. R., Marsh, J. L., and O'Connor, M. B. (1997). Defects in glucuronate biosynthesis disrupt Wingless signaling in Drosophila. *Development* **124,** 3055-3064.

Hecht, J. T., Hogue, D., Strong, L. C., Hansen, M. F., Blanton, S. H., and Wagner, M. (1995). Hereditary multiple exostosis and chondrosarcoma: linkage to chromosome 11 and loss of heterozygosity for EXT-linked markers on chromosomes 11 and 8. *Am J Hum Genet* **56,** 1125-1131.

Hennekam, R. C. (1991). Hereditary multiple exostoses. *J of Med Genet* **28,** 262-266.

Herold, B. C., Gerber, S. I., Belval, B. J., Siston, A. M., and Shulman, N. (1996). Differences in the susceptibility of herpes simplex virus types 1 and 2 to modified heparin compounds suggest serotype differences in viral entry. *Journal of Virology* **70,** 3461-3469.

Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R., and Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *J Gen Virol* **75,** 1211-1222.

Herold, B. C., WuDunn, D., Soltys, N., and Spear, P. G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J Virol* **65,** 1090-1098.

Humphries, D. E., Sullivan, B. M., Aleixo, M. D., and Stow, J. L. (1997). Localization of human heparan glucosaminyl N-deacetylase/N-sulphotransferase to the trans-Golgi network. *Biochem J* **325,** 351-357.

Janeway Jr., C. A., and Travers, P. (1996). "Immunobiology: the immune system in health and disease." Current Biology Ltd./Garland Publishing Inc.,

Jones, S. M., Crosby, J. R., Salamero, J., and Howell, K. E. (1993). A cytosolic complex of p62 and rab6 associates with TGN38/41 and is involved in budding of exocytic vesicles from the trans-Golgi network. *J Cell Biol* **122,** 775-788.

Karger, A., and Mettenleiter, T. C. (1996). Identification of cell surface molecules that interact with pseudorabies virus. *Journal of Virology* **70,** 2138-2145.

Kim, B. T., Kitagawa, H., Tamura, J., Saito, T., Kusche-Gullberg, M., Lindahl, U., and Sugahara, K. (2001). Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4- N-acetylglucosaminyltransferases that likely are involved in heparan sulfate/ heparin biosynthesis. *Proc Natl Acad Sci U S A* **98,** 7176-7181.

Kitagawa, H., Shimakawa, H., and Sugahara, K. (1999). The tumor suppressor EXT-like gene *EXTL2* encodes an α1, 4-*N*-acetylhexosaminyltransferase that transfers *N*-acetylgalactosamine and *N*-acetylglucosamine to the common glycosaminoglycan-protein linkage region. *J. Biol. Chem.* **274,** 13933-13937.

Kobayashi, S., Morimoto, K., Shimizu, T., Takahashi, M., Kurosawa, H., and Shirasawa, T. (2000). Association of EXT1 and EXT2, hereditary multiple exostoses gene products, in Golgi apparatus. *Biochem Biophys Res Commun* **268,** 860-867.

Krooth, R. S., Macklin, M. T., and Kilbish, T. F. (1961). Diaphyseal aclasis (multiple exostoses) on Guam. *J. Hum. Genet.* **13,** 340-347.

Kusche-Gullberg, M., Eriksson, I., Pikas, D. S., and Kjellen, L. (1998). Identification and expression in mouse of two heparan sulfate glucosaminyl N-deacetylase/N-sulfotransferase genes. *J Biol Chem* **273,** 11902-11907.

Ladinsky, M. S., Kremer, J. R., Furcinitti, P. S., McIntosh, J. R., and Howell, K. E. (1994). HVEM tomography of the trans-Golgi network: structural insights and identification of a lace-like vesicle coat. *J Cell Biol* **127,** 29-38.

Le Merrer, M., Legeai-Mallet, L., Jeannin, P. M., Horsthemke, B., Schinzel, A., Plauchu, H., Toutain, A., Achard, F., Munnich, A., and Maroteaux, P. (1994). A gene for hereditary multiple exostoses maps to chromosome 19p. *Hum Mol Genet* **3,** 717-722.

Lin, X., Wei, G., Shi, Z., Dryer, L., Esko, J. D., Wells, D. E., and Matzuk, M. M. (2000). Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice. *Dev Biol* **224,** 299-311.

Lin, X., and Wells, D. (1997). Isolation of the mouse cDNA homologous to the human EXT1 gene responsible for Hereditary Multiple Exostoses. *DNA Sequence* **7,** 199-202.

Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. (1998). The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. *Journal of Biological Chemistry* **273,** 26265-26268.

Lotteau, V., Teyton, L., Peleraux, A., Nilsson, T., Karlsson, L., Schmid, S. L., Quaranta, V., and Peterson, P. A. (1990). Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348,** 600-605.

McCormick, C., Duncan, G., Goutsos, K. T., and Tufaro, F. (2000). The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the Golgi apparatus and catalyzes the synthesis of heparan sulfate. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 668-673.

McCormick, C., Leduc, Y., Martindale, D., Mattison, K., Esford, L. E., Dyer, A. P., and Tufaro, F. (1998). The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. *Nature Genetics* **19,** 158-161.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., J., P. L., Scott, J. E., and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type I. *Journal of General Virology* **69,** 1531-1574.

Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87,** 427-436.

Munro, S. (1991). Sequences within and adjacent to the transmembrane segment of alpha-2,6-sialyltransferase specify Golgi retention. *Embo J* **10,** 3577-3588.

Munro, S. (1995). An investigation of the role of transmembrane domains in Golgi protein retention. *Embo J* **14,** 4695-4704.

Nezil, F. A., and Bloom, M. (1992). Combined influence of cholesterol and synthetic amphiphillic peptides upon bilayer thickness in model membranes. *Biophys J* **61,** 1176-1183.

Nickel, W., Brugger, B., and Wieland, F. T. (1998). Protein and lipid sorting between the endoplasmic reticulum and the Golgi complex. *Semin Cell Dev Biol* **9,** 493-501.

Nickel, W., Huber, L. A., Kahn, R. A., Kipper, N., Barthel, A., Fasshauer, D., and Soling, H. D. (1994). ADP ribosylation factor and a 14-kD polypeptide are associated with heparan sulfate-carrying post-trans-Golgi network secretory vesicles in rat hepatocytes. *J Cell Biol* **125,** 721-732.

Nilsen, A., and Myrmel, H. (2000). Changing trends in genital herpes simplex virus infection in Bergen, Norway. *Acta Obstet Gynecol Scand* **79,** 693-696.

Nilsson, T., Hoe, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hunte, F., Watzele, G., Berger, E. G., and Warren, G. (1994). Kin recognition between medial Golgi enzymes in HeLa cells. *EMBO J.* **13,** 562-574.

Nilsson, T., Lucocq, J. M., Mackay, D., and Warren, G. (1991). The membrane spanning domain of β-1,4-galactosyltransferase specifies trans Golgi localization. *EMBO Journal* **10,** 3567-3575.

Nilsson, T., Slusarewicz, P., Hoe, M. H., and Warren, G. (1993). Kin recognition: a model for the retention of Golgi enzymes. *FEBS Lett.* **330,** 1-4.

Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992). Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement. *Journal of Biological Chemistry* **267,** 12528-12538.

Orci, L., Glick, B. S., and Rothman, J. E. (1986). A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* **46,** 171-184.

Paik, N. J., Han, T. R., and Lim, S. J. (2000). Multiple peripheral nerve compressions related to malignantly transformed hereditary multiple exostoses. *Muscle Nerve* **23,** 1290-1294.

Pearse, B. M., and Robinson, M. S. (1990). Clathrin, adaptors, and sorting. *Annu Rev Cell Biol* **6,** 151-171.

Philippe, C., Porter, D. E., Emerton, M. E., Wells, D. E., Simpson, A. H., and Monaco, A. P. (1997). Mutation screening of the EXT1 and EXT2 genes in patients with hereditary multiple exostoses. *Am J Hum Genet* **61,** 520-528.

Raskind, W. H., Conrad, E. U., Chansky, H., and Matsushita, M. (1995). Loss of heterozygosity in chondrosarcomas for markers linked to hereditary multiple exostoses loci on chromosomes 8 and 11. *Am J Hum Genet* **56,** 1132-1139.

Roop, C., Hutchinson, L., and Johnson, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *J Virol* **67,** 2285-2297.

Saxena, U., Klein, M. G., and Goldberg, I. J. (1990). Metabolism of endothelial cell-bound lipoprotein lipase. Evidence for heparan sulfate proteoglycan-mediated internalization and recycling. *J Biol Chem* **265,** 12880-12886.

Schmale, G. A., Conrad, E. U., and Raskind, W. H. (1994). The natural history of hereditary multiple exostoses. *J Bone J Surg* **76,** 986-992.

Schutze, M. P., Peterson, P. A., and Jackson, M. R. (1994). An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic retiuculum. *EMBO J.* **13,** 1696-1705.

Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., Lidholt, K., Lindahl, U., and Kusche-Gullberg, M. (2000). The EXT1/EXT2 tumor suppressors: catalytic activities and role in heparan sulfate biosynthesis. *EMBO Reports* **1,** 282-286.

Simmons, A. D., Musy, M. M., Lopes, C. S., Hwang, L. Y., Yang, Y. P., and Lovett, M. (1999). A direct interaction between EXT proteins and glycosyltransferases is defective in hereditary multiple exostoses. *Hum Mol Genet* **8,** 2155-2164.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362,** 318-324.

Solomon, E. P., Berg, L. R., Martin, D. W., and Villee, C. (1993). "Biology." Saunders College Publishing,

Solomon, L. (1963). Hereditary multiple exostosis. *J Bone J Surg* **45,** 292-304.
Song, H. Y., Dunbar, J. D., Zhang, Y. X., Guo, D., and Donner, D. B. (1995). Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor. *J Biol Chem* **270,** 3574-3581.

Stickens, D., Clines, G., Burbee, D., Ramos, P., Thomas, S., Hogue, D., Hecht, J. T., Lovett, M., and Evans, G. A. (1996). The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. *Nat Genet* **14,** 25-32.

Stickens, D., and Evans, G. A. (1997). Isolation and characterization of the murine homolog of the human EXT2 multiple exostoses gene. *Biochem Mol Med* **61,** 16-21.

Strubin, M., Long, E. O., and Mach, B. (1986). Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. *Cell* **47,** 619-625.

Tang, B. L., Low, S. H., Wong, S. H., and Hong, W. (1995). Cell type differences in Golgi retention signals for transmembrane proteins. *Eur J Cell Biol* **66,** 365-374.

Teasdale, R. D., and Jackson, M. R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu Rev Cell Dev Biol* **12,** 27-54.

The, I., Bellaiche, Y., and Perrimon, N. (1999). Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan. *Mol Cell* **4,** 633-639.

Utani, A., Nomizu, M., Matsuura, H., Kato, K., Kobayashi, T., Takeda, U., Aota, S., Nielsen, P. K., and Shinkai, H. (2001). A unique sequence of the laminin alpha 3-G domain binds to heparin and promotes cell adhesion through syndecan-2 and -4. *J Biol Chem* **276,** 28779-28788.

Van Hul, W., Wuyts, W., Hendrickx, J., Speleman, F., Wauters, J., De Boulle, K., Van Roy, N., Bossuyt, P., and Willems, P. J. (1998). Identification of a third EXT-like gene (EXTL3) belonging to the EXT gene family. *Genomics* **47,** 230-237.

Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H., and Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* **246,** 179-189.

Wei, G., Bai, X., Gabb, M. M., Bame, K. J., Koshy, T. I., Spear, P. G., and Esko, J. D. (2000). Location of the glucuronosyltransferase domain in the heparan sulfate copolymerase EXT1 by analysis of Chinese hamster ovary cell mutants. *Journal of Biological Chemistry* **275,** 27733-27740.

Weisz, O. A., Swift, A. M., and Machamer, C. E. (1993). Oligomerization of a membrane protein correlates with its retention in the Golgi complex. *J Cell Biol* **122,** 1185-1196.

Williams, R. K., and Straus, S. E. (1997). Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans. *J Virol* **71,** 1375-1380.

Wise, C. A., Clines, G. A., Massa, H., Trask, B. J., and Lovett, M. (1997). Identification and localization of the gene for EXTL, a third member of the multiple exostoses gene family. *Gen Res* **7,** 10-16.

Wu, Y. Q., Heutink, P., de Vries, B. B., Sandkuijl, L. A., van den Ouweland, A. M., Niermeijer, M. F., Galjaard, H., Reyniers, E., Willems, P. J., and Halley, D. J. (1994). Assignment of a second locus for multiple exostoses to the pericentromeric region of chromosome 11. *Hum Mol Genet* **3,** 167-171.

WuDunn, D., and Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63,** 52-58.

Wurthner, J. U., Frank, D. B., Felici, A., Green, H. M., Cao, Z., Schneider, M. D., McNally, J. G., Lechleider, R. J., and Roberts, A. B. (2001). Transforming growth factor-beta receptor-associated protein 1 is a Smad4 chaperone. *J Biol Chem* **276,** 19495-19502.

Wuyts, W., and Van Hul, W. (2000). Molecular basis of multiple exostoses: mutations in the EXT1 and EXT2 genes. *Human Mutation* **15,** 220-227.

Wuyts, W., Van Hul, W., Hendrickx, J., Speleman, F., Wauters, J., De Boulle, K., Van Roy, N., Van Agtmael, T., Bossuyt, P., and Willems, P. J. (1997). Identification and characterization of a novel member of the EXT gene family, EXTL2. *Eur J Hum Genet* **5,** 382-389.

Wuyts, W., Van Hul, W., Wauters, J., Nemtsova, M., Reyniers, E., Van Hul, E. V., De Boulle, K., de Vries, B. B., Hendrickx, J., Herrygers, I., Bossuyt, P., Balemans, W., Fransen, E., Vits, L., Coucke, P., Nowak, N. J., Shows, T. B., Mallet, L., van den Ouweland, A. M., McGaughran, J., Halley, D. J., and Willems, P. J. (1996). Positional cloning of a gene involved in hereditary multiple exostoses. *Hum Mol Genet* **5,** 1547-1557.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64,** 841-848.