BIOLOGICAL RESEARCH REPORTS FROM THE UNIVERSITY OF JYVÄSKYLÄ

28

Ari Huovila

Assembly of Hepatitis B Surface Antigen

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 1992

BIOLOGICAL RESEARCH REPORTS FROM THE UNIVERSITY OF JYVÄSKYLÄ 28

Ari Huovila

Assembly of Hepatitis B Surface Antigen

Academic Dissertation

To be presented, with the permission of the Faculty of Mathematics and Natural Sciences of the University of Jyväskylä, for public criticism in Auditorium S212, on November 27, 1992, at 12 o'clock noon.

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella julkisesti tarkastettavaksi yliopiston vanhassa juhlasalissa (S212) marraskuun 27. päivänä 1992 kello 12.

UNIVERSITY OF JYVÄSKYLÄ, JYVÄSKYLÄ 1992

Assembly of Hepatitis B Surface Antigen

BIOLOGICAL RESEARCH REPORTS FROM THE UNIVERSITY OF JYVÄSKYLÄ 28

Ari Huovila

Assembly of Hepatitis B Surface Antigen

UNIVERSITY OF JYVÄSKYLÄ, JYVÄSKYLÄ 1992

URN:ISBN:978-951-39-9334-4
ISBN 978-951-39-9334-4 (PDF)
ISSN 0356-1062

Jyväskylän yliopisto, 2022

ISBN 951-680-902-2
ISSN 0356-1062

Copyright © 1992, by Ari Huovila
and University of Jyväskylä

Jyväskylän yliopiston monistuskeskus and
Sisäsuomi Oy, Jyväskylä 1992

Biol. Res. Rep. Univ. Jyväskylä 28: 1-73. 1992.

ASSEMBLY OF HEPATITIS B SURFACE ANTIGEN

Ari-Pekka Huovila

Huovila, A.-P. J. 1992: Assembly of hepatitis B surface antigen.
- Biol. Res. Rep. Univ. Jyväskylä 28: 1-73. ISSN 0356-6062.

Hepatitis B virus infected cells produce, in addition to virions, non-infectous particles composed of the major viral coat protein, hepatitis B surface antigen (HBsAg), and lipid. These particles are stabilized by extensive disulphide crosslinking. Cellular expression of HBsAg leads to its secretion as particles indistinguishable from those found in sera of infected patients. The assembly of these particles was classically believed to occur entirely in the endoplasmic reticulum (ER). This was paradoxical since the ER contains a high concentration of protein disulphide isomerase (PDI) which should resolve such extensive linkages.

HBsAg biosynthesis was studied using a stably transfected cell line. Kinetic studies showed that disulphide linked dimers begin to form during or immediately after the polypeptide synthesis. Their concentration increases rapidly until a peak at one hour after the initial synthesis, and then decreases monotonically as the dimers are crosslinked to higher oligomers. Several immunocytochemical techniques in combination with biochemical studies demonstrate that HBsAg is sorted rapidly after its dimerization in the ER to a morphologically and functionally distinct post-ER, pre-Golgi compartment in which the assembly is completed. This compartment excludes lumenal ER proteins and Golgi proteins. It was shown to be discontinuous with the ER. Two established intermediate compartment proteins, rab2 and the 72 kD KDEL-binding protein, colocalize to this compartment.

The exit from the ER was shown to be a requirement for the assembly, and the incubation of purified HBsAg particles with recombinant PDI led to the disassembly of highly crosslinked mature HBsAg multimers to dimers. This indicates that the PDI exclusion may be a condition for particle assembly.

Stereological analysis of electron microscopy data provided the first quantitation of an intermediate compartment, demonstrating that this compartment is a major and dynamic element of the secretory pathway.

Keywords: Endoplasmic reticulum, hepatitis B surface antigen, intermediate compartment, lipoprotein assembly; protein sorting, and secretory pathway.

A.-P. J. Huovila, European Molecular Biology Laboratory, Meyerhofstr. 1, D-6900 Heidelberg, Germany.

ABBREVIATIONS

β-COP, β-coatomer protein
BFA, brefeldin A
BiP, heavy chain binding protein, binding protein
CGN, *cis*-Golgi network
C-terminal, carboxy-terminal
DHBV, duck hepatitis B virus
ER, endoplasmic reticulum
GRP, glucose regulated protein
GSHV, ground squirrel hepatitis B virus
HA, hemagglutinin
HBcAg, hepatitis B core antigen
HBsAg, hepatitis B surface antigen
HBV, hepatitis B virus
HHBV, heron hepatitis B virus
HIV, human immunodeficiency virus
ICG, intracisternal granule
KDEL, lysine-aspartate-glutamate-leucine-COOH
NEM, N-ethyl maleimide
N-terminal, amino-terminal
ORF-S, open reading frame S (of HBV genome)
PCR, polymerase chain reaction
PDI, protein disulphide isomerase
PHC, primary hepatocellular carcinoma
RV, rubellavirus
SDS, sodium dodecyl sulphate
SDS-PAGE, SDS-polyacrylamide gel electrophoresis
TCR, T cell receptor
TGN, *trans*-Golgi network
TRAP, T cell receptor associated protein
vWF, von Willebrand factor
VSV, vesicular stomatitis virus
WGA, wheat germ agglutinin
WHV, woodchuck hepatitis virus

CONTENTS

Abstract 3
Abbreviations 4
Contents 5
List of original publications 7
1. Introduction 8
2. Review of the literature 11
2.1. HBsAg biosynthesis 11
2.2. Protein synthesis and assembly in the secretory pathway 18
2.2.1. Post-translational modifications in the endoplasmic reticulum 18
2.2.2. Disulphide bond formation in the secretory pathway 21
2.2.3. Molecular chaperones 25
2.2.4. Quality control in the endoplasmic reticulum 26
2.3. Could HBsAg mature in an ER-Golgi intermediate compartment? 29
2.4. Characterization of the intermediate compartment 34
2.5. Summary 34
3. Aims of the present study 36
4. Summary of methods 37
4.1. Cells 37
4.2. Antibodies used for immunocytochemistry and biochemical experiments 37
4.3. Immunocytochemistry 38
4.4. Conventional electron microscopy and morphometry 39
4.5. Pulse chase and immunoprecipitation experiments 39
4.6. Digital image analysis 40
5. Review of the results 41
5.1. Comparison of the alternative hypotheses for HBsAg assembly 41

5.1.1. HBsAg particles assemble after the exit from the ER 41
5.1.2. Time course of the interchain disulphide formation 42
5.2. Exit from the ER is required for the covalent oligomerization of HBsAg: Possible role of PDI exclusion 43
5.3 Characteristics of the HBsAg assembly compartment 44
5.3.1. HBsAg assembly occurs in a pre-Golgi compartment 44
5.3.2. Identity of the HBsAg maturation compartment 45
6. Discussion 46
6.1. Maturation pathway of HBsAg 47
6.2. Role of disulphide bonds in the assembly 49
6.3. Quality control and the HBsAg assembly compartment 51
6.4. The HBsAg assembly compartment and its relation to other exocytic compartments 53
7. Conclusions 55
Acknowledgements 57
Selostus 59
References 60

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which will be referred to by their Roman numerals:

I. Huovila, A.-P. J., A. M. Eder & S. D. Fuller. 1992. Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment.
- J. Cell Biol. 118:1305-1320.

II. Huovila, A.-P. J. & S. D. Fuller. 1992. An ER-Golgi intermediate compartment is not continuous with the endoplasmic reticulum. Submitted for publication in Molecular Biology of the Cell.

1. INTRODUCTION

Hepatitis B virus (HBV) is the major cause of chronic liver disease in man, infecting presently more than 250 million people (Sherker & Marion, 1991; Slagle et al., 1992). The most common course of primary HBV infection results in acute hepatitis B, an illness characterized by hepatocellular necrosis and and inflammatory responses, with severity varying from mild to fulminant. An estimated 5-10 % of HBV infected individuals develop a persistent, usually lifelong infection with complete virions or the viral surface antigen remaining in the circulation for decades (Beasley et al., 1981; Ganem & Varmus, 1987). Recent results obtained by polymerase chain reaction (PCR) methodology suggest that HBV infection may persist in a much greater percentage of people (Rogler, 1991). While persistent HBV infection is often associated with histologically and functionally normal or almost normal liver, sometimes it is accompanied by chronic persistent hepatitis B or chronic active hepatitis B. Chronic active hepatitis B is more severe and often progresses to cirrhosis, while chronic persistent hepatitis B is not progressive. In addition to chronic hepatitis B, persistent HBV infection is associated with development of one of the most common cancers, primary hepatocellular carcinoma (PHC; Beasley & Hwang, 1984). In comparison to age-matched noncarriers, chronic HBV carriers are at a 200-fold greater risk of developing PHC (Beasley et al., 1981). HBV is, in fact, second in importance only to tobacco as a known human carcinogen (Tiollais & Buendia, 1991). An estimated 1-2 million deaths due to severe hepatitis, cirrhosis, or PHC are attributed to HBV infection yearly (Sherker & Marion, 1991).

HBV is the type virus of the family *hepadnaviridae* (hepatotropic DNA viruses). In addition to HBV, the family comprises three other well characterized viruses, woodchuck hepatitis virus (WHV: Summers et al., 1978), ground squirrel hepatitis B virus (GSHV; Marion et al., 1980), and duck hepatitis B virus (DHBV; Mason et al., 1980). Other HBV-like viruses which infect herons (Sprengel et al., 1988), tree squirrels (Feitelson et al., 1986) and kangaroos (Cossart & Keirnan, 1984), have been reported. The four well studied hepadnaviruses show a relative hepatotropism (hence the family name) and a remarkable species specificity. For example, HBV infects only humans and other higher primates. No hepadnaviruses infecting conventional non-primate laboratory animals have been identified.

HBV is a small (42 nm) enveloped virus consisting of a nucleoprotein core containing a small (3200 bp), partially double stranded relaxed circular DNA genome (the smallest known animal virus DNA genome) (Dane et al., 1970; Tiollais et al., 1985). This is surrounded by a 27 nm T=3 icosahedral capsid composed of a core protein, hepatitis B core antigen (HBcAg) (C.-H. von Bonsdorff, M. Nassal, and S. Fuller, manuscript in preparation) The nucleocapsid is surrounded by an envelope in which the main protein component is hepatitis B surface antigen (HBsAg) (reviewed by Ganem, 1991).

One of the unique features of hepadnaviral infection is that in addition to the virions, the infected cells produce and secrete non-infectious lipoprotein particles composed only of HBsAg and lipid of host cell origin (Ganem, 1991; Ganem & Varmus, 1987). In all other viral infections the envelope proteins remain associated with the bilayer until interactions with other viral components trigger the budding step. This ensures that viral envelope proteins never exit the cell without enveloping the nucleocapsid. In contrast, noninfectious HBsAg particles are present in sera of infected patients at very high concentrations, exceeding that of the virus by up to a million fold. Production of these particles is conserved in the hepadnavirus family, suggesting an important role in viral pathogenesis. One proposed function of HBsAg particles is to provide immunological decoys which protect the infective viruses against the host immune

system, and so effect the horizontal spread of the infection in the liver (Ganem, 1991).

The main reasons for the wide interest in HBsAg particles are their strong antigenicity and the capability to induce a protective immune response. They form the basis of effective vaccines against hepatitis B itself (McAleer et al., 1984; Szmuness et al., 1980; Valenzuela et al., 1982; Wampler et al., 1985). The strong immunogenicity of these particles has raised the exciting possibility that they could serve as carriers for other antigens and hence for vaccination against other pathogens. In some cases this approach has produced encouraging results. Epitopes of human pathogens such as *Plasmodium falciparum* (a malaria parasite), human immunodeficiency virus (HIV) and poliovirus have been reported to produce antibody response when expressed on chimeric HBsAg particles (Delpeyroux et al., 1986; Michel et al., 1988; Michel et al., 1990; Rutgers et al., 1988). However, in other cases this approach has failed, perhaps because of inadequate understanding of HBsAg maturation and assembly.

To gain a better understanding of the events that lead to HBsAg lipoprotein particle assembly and of the environment of the cellular compartments in which they occur, the intracellular distribution of HBsAg was studied by immunofluorescence, confocal and immunoelectron microscopy, using different marker antibodies for exocytic compartments. Kinetics of disulphide bond formation were studied by pulse chase experiments to elucidate the role of disulphide crosslinking in the assembly process. The results show that HBsAg is transported to a compartment which is intermediate between the ER and the Golgi on the secretory pathway, and that disulphide linked oligomer formation precedes and overlaps the extrusion of the particles into the lumen of this intermediate compartment.

2. REVIEW OF LITERATURE

2.1. HBsAg biosynthesis

HBsAg is encoded by the open reading frame S (ORF-S) of the HBV genome (Fig. 1A). This frame contains three inframe translation initiation codons encoding, in addition to HBsAg, two larger proteins. Initiation at the first AUG of ORF-S mRNA generates a 39 kD pre-S_1 protein. The second AUG initiates the translation of 31 kD pre-S_2 protein, and the third initiation codon gives rise to HBsAg (pre-S_1, pre-S_2, and HBsAg are often called L, M, and S proteins, respectively).

Both pre-S proteins contain the HBsAg sequence at their carboxy terminus, and they differ by the length of the N-terminal extension. Each of the proteins occurs in two isoforms which differ by the presence or absence of N-glycosylation at the HBsAg domain (reviewed by Ganem & Varmus, 1987). Pre-S_1 is present in virions, but not significantly in HBsAg particles. It is essential for the virion formation (Bruss & Ganem, 1991), and it may function in binding to a host cell receptor. Pre-S_2 is present in both HBsAg particles and in virions, but is not needed for the assembly or secretion of either particle type.

All three proteins are translocated cotranslationally through the ER membrane. They are translated independently in the infected cells, and there is no precursor-product relationship between them; frame shift or deletion mutations within pre-S region do not affect HBsAg biosynthesis (Persing et al., 1985).

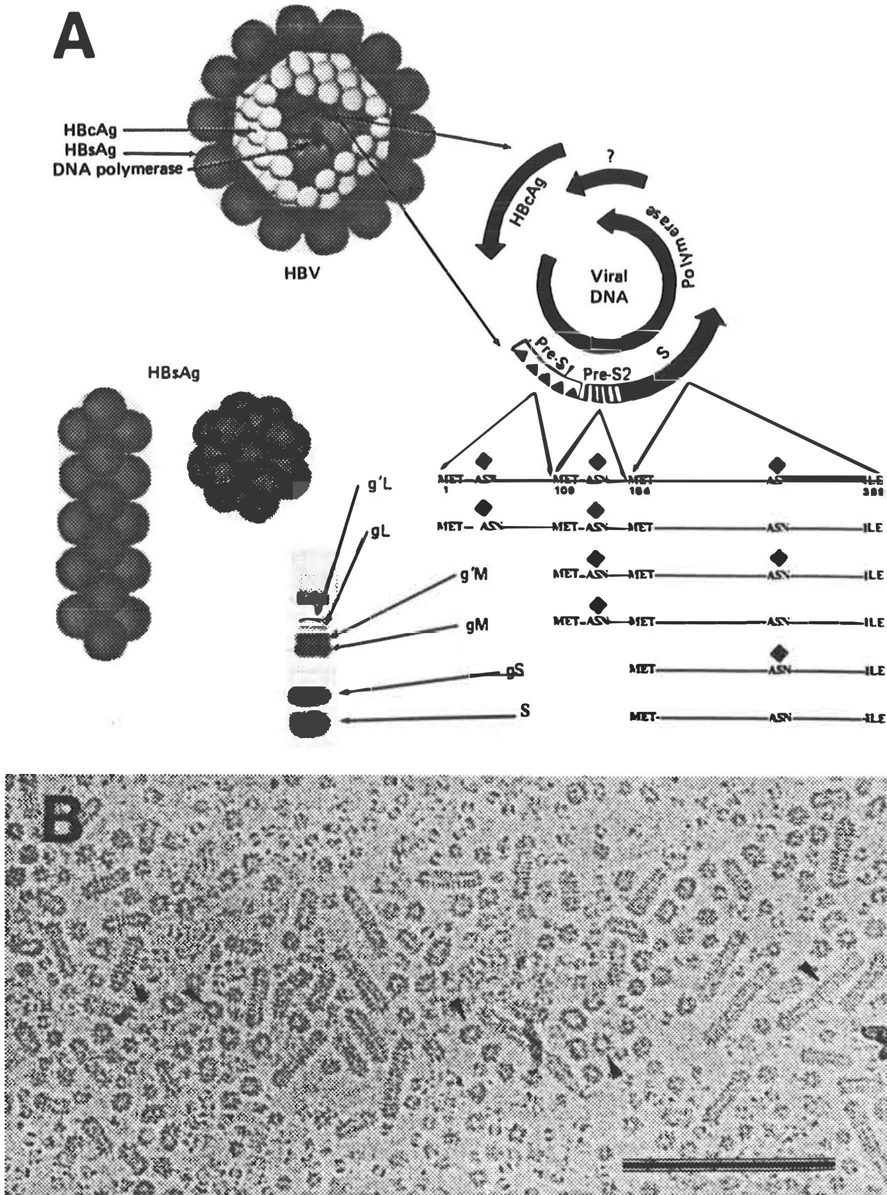

Figure 1. Hepatitis B virus and related structures. A) Upper left: hepatitis B virus. Lower left: the small and long forms of HBsAg particles. Upper right: the four open reading frames of the HBV genome, which code for the core antigen (HBcAg), an uncharacterized protein (here denoted with ?; also called ORF-X), a DNA polymerase, and the surface antigen proteins (pre-S_1, pre-S_2, and HBsAg). Lower right: the proteins encoded by ORF-S as seen on SDS-PAGE and as related to the pre-S and S-genes of ORF-S. The black squares indicate the N-glycosylation sites. (From Peterson, 1987.) B) A cryo-electron micrograph showing surface antigen particles from ground squirrel serum. Both the disk-like and tubular particles can be seen (indicated by arrowheads) (Stephen Fuller, unpublished.) Bar=200 nm.

```
HBV   M E N I T S G F L G P L L V L Q A G F F L L T R I L T I P Q  30
WHV   M - - S P S S L L G L L A G L Q V V Y F L W T K I L T I A Q
GSHV  M - - S P S G L L G L L A G L Q V V Y F L W T K I L T I A Q
DHBV  M S G T F G G I L A G L I G L L V S F F L L I K I L E I L R
HHBV  M G A T F G G I L A G L I G L L V G F F L L T K I L E I L R

HBV   S L D S W W T S L N F L G G S P V C L G Q N S Q S P T S N H  60
WHV   N L D W W W T S L S F P G G I P E C T G Q N S Q F Q T C K H
GSHV  S L D W W W T S L S F P G G I P E C T G Q N L Q F Q T C K H
DHBV  R L D W W W I S L S S P K G K M Q C A F Q D T G A Q I S P H
HHBV  K L D W W W I S L S S P K E K M L C A F Q N T G A Q T S P H

HBV   S P T S C P P T C P G Y R W M C L R R F I I F L F I L L L C  90
WHV   L P T S C P P T C N G F R W M Y L R R F I I Y L L V L L L C
GSHV  L P T S C P P T C N G F R W M Y L R R F I I Y L L I L L L F
DHBV  Y V G S C P W G C P G F L W T Y L R L F I I F L L L L L V A
HHBV  Y V G S C P W G C P G F L W T Y L R L F I I F L L L L L V A

HBV   L I F L L V L L D Y Q G M L P V C P L I P G S T T T S T G P  120
WHV   L I F L L V L L D W K G L I P V C P I Q P T T E T T V N - -
GSHV  L T F L L V L L D W K G L L P V C P M M P A T E T T V N - -
DHBV  A G L L Y L T A N G S T - - - - - - - - - - - - - - - - - -
HHBV  A G L L F L T E N K S T - - - - - - - - - - - - - - - - - -

HBV   C K T C T T P A Q G N S M F P S C C C T K P T D G N C T C I  150
WHV   C R G C T I S V Q D M Y T P P Y C C C L K P T A G N C T C W
GSHV  C R Q C T I S A Q D T F T T P Y C C C L K P T A G N C T C W
DHBV  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
HHBV  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

HBV   P I P S S W A F A K Y L W E W A S V R F S W L S L L V P F V  180
WHV   P I P S S W A L G N Y L W E W A L A R F S W L N L L V P L L
GSHV  P I P S S W A L G S Y L W E W A L A R F S W L S L L V P L L
DHBV  - - - - - - I L G K L Q W A S V S A L F S S I S S L L P S D
HHBV  - - - - - - I F E K L Q W E S V S A L S S S I Y S L L P S E

HBV   Q W F V G L S P T V W L S A I W M M W Y W G P S L Y S I V S  210
WHV   Q W L G G I S L I A W F L L I W M I W F W G P A L L S I L P
GSHV  Q W L G G I S L T V W L L L I W M I W F W G P V L M S I L P
DHBV  P K S L V A L - T F G L S L I W M T S S S A T Q T L V T L T
HHBV  P K S L V A L - T F G L F L I W T T S S S V T Q V L V T L T

HBV   P F I P L L P L F F C L W V Y I  226
WHV   P F I P I F V L F F L I W V Y I
GSHV  P F I P I F A L F F L I W A Y I
DHBV  Q L A T L S A L F Y K S - - - -
HHBV  Q L A T L S A L F F K N S G - -
```

Figure 2. Amino acid sequences predicted from the gene sequences coding for different hepadnaviral surface antigens. The residue numbering is according to human HBsAg. The cysteines are highlighted to emphasize their conservation in hepadnavirus family. HBV, hepatitis B virus, subtype adw (Valenzuela et al., 1979); WHV, woodchuck hepatitis virus (Galibert et al., 1982); GSHV, ground squirrel hepatitis B virus (Seeger et al., 1984); DHBV, duck hepatitis B virus (Mandart et al., 1984); HHBV, heron hepatitis B virus (Sprengel et al., 1988). The cysteines 48, 65 and 69 are essential for HBsAg secretion.

HBsAg is composed of 226 amino acids and exists in non-glycosylated 24 kD and in N-glycosylated 27 kD forms, which have identical amino acid sequences (Peterson, 1981); the reported

relative molecular weights vary from 23 kD to 25 kD for the nonglycosylated, and from 27 kD to 28 kD for the glycosylated HBsAg). The amino acid sequence predicts three hydrophobic regions, separated by two hydrophilic stretches. According to the current view, both N- and C-termini of the transmembrane form of HBsAg are on the lumenal side of the (ER) membrane (Eble et al., 1987; Peterson, 1987). Thus, the HBsAg polypeptide as well as the pre-S proteins must span the bilayer at least twice. This differs from most viral envelope proteins which span the membrane only once.

The *in vitro* evidence indicates that the translocations are mediated by the first and the second hydrophobic region (Eble et al., 1987). The first (N-terminal) hydrophobic segment functions as an uncleaved signal sequence (termed signal I) that initiates the chain translocation across the ER membrane and thereby delivers the N-terminus to the ER lumen (Eble et al., 1987). The second hydrophobic region (signal II, Eble et al., 1987) functions as an internal signal sequence that inserts into the bilayer in an inverted orientation (N-terminal end facing the cytosol, C-terminal end the ER lumen), translocating the C-terminal part of the protein to the ER lumen. As a result, most of the hydrophilic region between signals I and II is believed to reside in the cytosol. The C-terminal hydrophobic segment may be predominantly lumenal (Eble et al., 1987), or it may form an additional transmembrane helical hairpin (Eble et al., 1987; Peterson, 1987), but this has not been confirmed experimentally.

HBsAg particles occur in two distinct morphological forms: 22 nm diameter particles which appear spherical when negatively stained for electron microscopy, and filamentous particles of varying length and of the same diameter (Fig. 1.; Bayer et al., 1968; Dane et al., 1970; Robinson & Lutwick, 1976). Cryo-electron microscopy of unstained small particles in vitrified water reveals that they are disk-like (S. Fuller, unpublished data, Fig. 1B). The small particles are the major form of HBsAg in infected sera. They are the only form of HBsAg particles which has been extensively studied. In this thesis HBsAg particles refer to the small 22 nm particles.

HBsAg particles isolated from HBV infected sera are complex macromolecular assemblies having a molecular weight of 2-4 x 10^6 daltons (Dreesman et al., 1972; Kim & Tilles, 1973) and a buoyant density of about 1.20 (reported buoyant densities range from 1.18 to 1.21). They contain 75 % (w/w) protein and 25 % (w/w) host derived lipid, and carbohydrate (Gavilanes et al., 1982; Peterson et al.,1987). One particle contains approximately 100 HBsAg polypeptides (Aggerbeck & Peterson, 1985; Peterson, 1987). The major lipid classes in the particles are phospholipids (67 % by weight), free cholesterol (15 %), and cholesterol esters (14 %). The major phospholipid is phosphatidylcholine (Gavilanes et al., 1982). The carbohydrate is in the form of N-linked oligosaccharides on glycosylated HBsAg. Although the high cholesterol content is not consistent with particle maturation in the ER, it is possible that it reflects exchange of lipids after the particle formation.

The low lipid/protein ratio in HBsAg particles and the behavior of the particles at high pH suggest that the maturation pathway involves substantial reorganization of the host lipid, and that the proteins are not simply embedded in a conventional vesicle-like structure (Ganem & Varmus, 1987; Simon et al., 1988). An interesting feature of HBsAg particles which makes them unique among lipoproteins, is that their structure is stabilized by extensive disulphide crosslinking. This is essential for the antigenicity and immunogenicity of the particles (Imai et al., 1974; Peterson et al., 1987; Sukeno et al., 1972; Vyas et al., 1972). All of the 14 cysteines have been reported to be involved in disulphide bonds (Peterson et al., 1987). They are remarkably conserved in the primary structure of all the studied hepadnaviral surface antigens (11 out of 14 are conserved in the mammalian proteins, Fig. 2), suggesting an essential role for cysteines in the tertiary and quaternary structure of the surface antigen complexes.

The assembly process of HBsAg is now understood in some detail. The possibility of expressing HBV DNA in cell cultures and in cell-free assays has provided much information on the biogenesis and secretion of HBsAg particles. Cultured cells which are transfected with the coding region for HBsAg, assemble and secrete par-

ticles which are morphologically and biochemically indistinguishable from those in the sera of infected patients (Crowley et al., 1983; Dubois et al., 1980; Liu et al., 1982; Moriarty et al., 1981; Patzer et al., 1986; Patzer et al., 1984; Simon et al., 1988). This indicates that HBsAg assembles independently of other viral products. Thus, all of the information required for the cells to carry out the particle assembly and secretion resides within the HBsAg coding region of ORF-S.

Eble et al. (1986) demonstrated that in a cell-free translation system HBsAg is initially synthesized as a trans-membrane polypeptide that spans the membrane at least twice, and it was subsequently shown that this is the initial synthesis product in cultured cells (Simon et al., 1988). This transmembrane form was susceptible to protease digestion from the cytosolic side and resistant to alkaline carbonate extraction. Within an hour, the protein was converted to a protease resistant form. This protease resistance was interpreted to reflect a substantial conformational change and/or aggregation in the membrane. The protease resistant HBsAg was still integrally associated with the membrane as judged by the alkaline carbonate treatment. The protein became carbonate extractable with a half time which was very similar to that for the particle secretion. Carbonate extractability did not represent a conversion of HBsAg to a soluble form as such, but rather reflected the extrusion or budding of mature HBsAg particles into the lumen of the budding compartment. The authors concluded that the rate limiting step in the export process was the particle assembly in the membrane.

A fraction of the newly synthesized HBsAg is N-glycosylated cotranslationally in the ER lumen. All of the oligosaccharides on the secreted HBsAg particles in infected sera or in cell culture media are of the N-linked complex type, indicating transport through the Golgi complex before the secretion. Secretion was inhibited by monensin, also suggesting transport via the Golgi. However, all the detectable glycones on intracellular HBsAg were shown to be of the high mannose type (Patzer et al., 1984). Hence the rate limiting step in the particle formation must occur prior to medial Golgi. Electron

microscopy of HBsAg in infected or stably transfected cells revealed particles in the lumen of smooth intracellular membranes (Gerber et al., 1974; Gerber et al., 1972; Patzer et al., 1986; Shibayama et al., 1984), which have been interpreted as elements of the ER. These data are supported by the observation that no HBsAg colocalized with the lectin wheat germ agglutinin (WGA) within the HBsAg expressing cells (Patzer et al., 1986).

These studies have contributed to the classical view of the biogenesis of HBsAg particles. This model holds that the entire assembly process takes place in the ER, and that mature HBsAg particles are rapidly transported through the Golgi and secreted (reviewed by Ganem & Varmus, 1987 and Ganem, 1991). This model is largely based on morphological and immunocytochemical observations. Unfortunately, specific markers for the ER had not been used in immunocytochemical studies. The data on which the model was based indicate that HBsAg spends virtually all of its intracellular life time in compartment(s) upstream of the medial Golgi, probably proximal to the entire Golgi complex. The events occurring before or during the transport from the ER to the Golgi comprise the rate limiting step in the particle biosynthesis.

The classical view equates pre-Golgi and the ER, but several lines of recent biochemical and microscopy evidence support the existence of an intermediate compartment between the classical ER and the classical Golgi (Lippincott-Schwartz et al., 1990; Lodish et al., 1987; Saraste & Kuismanen, 1984; Saraste & Svensson, 1991; Schweitzer et al., 1988, 1990, 1991a). It has been suggested that oligomerization of some viral proteins may indeed occur in this compartment (Schweitzer et al., 1990; Tooze et al., 1988). The possibility that HBsAg might assemble in such a compartment has not been addressed. Thus, understanding the early steps in the secretory pathway is important for understanding HBsAg biosynthesis.

2.2. Protein synthesis and assembly in the secretory pathway

Proteins to be targeted for secretion in eukaryotic cells, such as HBsAg, are transported by bulk flow along the secretory pathway, an endomembrane system consisting of successive compartments between which the vectorial traffic is thought to be mediated by transport vesicles (Kelly, 1985; Palade, 1975). The first compartment in the secretory pathway is the ER from which the newly synthesized secretory proteins and the proteins destined for plasma membrane or for the lysosomal system are transported to the Golgi complex. The next station after the Golgi is the *trans*-Golgi network (TGN, Griffiths & Simons, 1986). In the TGN, the proteins to be directed to regulated or constitutive secretion and to the plasma membrane, or to the prelysosomes and lysosomes, are sorted to the vesicles mediating the corresponding transport.

Some proteins can be refolded without cellular factors after denaturation *in vitro*. However, it is now well established that the folding and assembly of most proteins is promoted by protein factors in the living cell. Being the principal site of protein folding and assembly in the secretory pathway, the ER contains a number of enzymes for post-translational modifications, and proteins which are thought to promote the folding and assembly by active catalytic mechanisms or by acting as molecular chaperones. The ER is the major secretory compartment hosting these cellular folding factors, many of which belong to evolutionarily highly conserved protein families (Gething & Sambrook, 1992; Schmid, 1991). The cytosol, mitochondria, chloroplasts and the nucleus are the other cellular compartments which contain molecular chaperones or enzymes which catalyze the chemical reactions involved in the folding process ("foldases") (Gething & Sambrook, 1992).

2.2.1. Post-translational modifications in the endoplasmic reticulum

The most important functions of the ER are to promote the correct

folding and assembly of newly synthesized proteins, and to prevent the secretion of incorrectly folded or assembled, or immature proteins. Post-translational modifications that occur within the ER include the cleavage of the signal sequence, N-glycosylation and initial trimming of N-linked glycones, and *cis-trans* isomerization of proline peptide bonds, as well as disulphide bond formation and rearrangements.

In general, polypeptides which are translocated to the ER lumen or inserted into the ER membrane, contain an N-terminal signal sequence which interacts with the translocation apparatus at the rough ER membrane. After the translocation of the N-terminal part of the nascent polypeptide chain into the ER lumen, and often before the translation is completed, the signal sequence is cleaved by the ER signal peptidase (Blobel & Dobberstein, 1975). The transfer of the core oligosaccharide from the lipid carrier to the amido nitrogen of certain asparagine residues in the polypeptides occurs at a very early stage of protein biosynthesis, as a rule co-translationally (Kornfeld & Kornfeld, 1985).

Disulphide bond formation is a very common, early event in protein folding in the ER. Disulphide bonds begin to form very rapidly, often during translation, but the rearrangements of the non-native disulphides to form the native disulphide complement is one of the rate-limiting steps in the folding process (Freedman, 1984).

Isomerization of the peptide bonds between certain prolines and the preceding residues is also a rate limiting step in many cases of *in vitro* folding. Since the nascent polypeptides are thought to be in all-*trans* configuration, and the final proline peptide bonds are often in *cis* configuration, the *cis-trans* isomerization has also been suggested to be an important step in protein folding *in vivo* (Bruckner et al., 1981; Bruckner & Eikenberry, 1984; Schmid, 1991; Stewart et al., 1990). Various peptidyl-prolyl *cis-trans* isomerases have been identified, and some of them reside in the ER (Price et al., 1991; Stamnes et al., 1991).

HBsAg is an unusual secretory protein in that it does not

have a cleavable N-terminal signal scquence. It does contain two internal signal sequences, one of which is located near the N-terminus. This signal directs the correct translocation of the N-terminus into the ER lumen, but no proteolytic processing of HBsAg occurs during its biosynthesis (Dubois et al., 1980; Laub et al., 1983; Liu et al., 1982; Moriarty et al., 1981; Patzer et al., 1984,1986; Simon et al., 1988). In contrast, other secreted proteins are generated by proteolytic cleavage from a larger transmembrane precursor (e. g., transforming growth factors α and β (Derynck et al., 1986; Lee et al., 1985), epidermal growth factor (Gray et al., 1983; Scott et al., 1983), and polymeric Ig receptor (Mostov & Blobel, 1982).

N-glycosylation is an essential covalent modification necessary for the correct folding of many glycoproteins (Paulson, 1989), but not for all. For HBsAg folding or assembly, the N-glycosylation is obviously not required. First, the majority of HBsAg is not glycosylated. Secondly, the domain which contains the N-glycosylation site (Asn146; Fig. 1A, Fig. 2), can be replaced with heterologous domains which are not glycosylated, without affecting the assembly or secretion of HBsAg particles. This is also supported by the observation that tunicamycin treatment does not inhibit HBsAg assembly or secretion (Patzer et al., 1984). The HBsAg N-glycosylation site is conserved in the mammalian hepadnaviruses, but the avian viruses (DHBV, HHBV) lack the entire domain containing this site (Fig. 2).

The most remarkable feature of the mature HBsAg particles is that their structure is stabilized by extensive disulphide cross-linking. All 14 cysteine residues are reported to take part in disulphide bonds (Peterson, 1987; Peterson et al., 1987), which indicates the importance of the disulphide rearrangements during the assembly process. Our knowledge of disulphide bond formation is based on soluble monomeric proteins, but its role in the biosynthesis of large multimeric complexes, such as the HBsAg particles, has not been described in any detail.

2.2.2. Disulphide bond formation in the secretory pathway

Current understanding of the contribution of disulphide bonds to protein stability is fragmentary. The role of disulphides in small peptides such as somatostatin analoques is relatively well understood: the disulphide bonds restrict the set of solution conformations, hence increasing the effective concentration of the bioactive, native conformer (Veber et al., 1979). From this it might be expected that disulphide bonds stabilize the structure of folded proteins by providing a physical constraint to unfolding. However, it is generally assumed that the basis of structural stabilization by crosslinks is in its effect on the unfolded, not the native state. By decreasing the degrees of freedom available to the unfolded state, crosslinks decrease the amount of entropy which can be gained in the unfolding reaction. Since the increase in polypeptide chain entropy is the predominant destabilizing component of the free energy of stabilization, disulphide crosslinking is expected to shift the unfolding equilibrium towards the native folded state (Wetzel, 1987). Although this appears to be true in most cases it is not strictly correct. In some cases the stability provided by crosslinks is not that expected from their entropic effects on the unfolded state (Creighton, 1988).

To form a disulphide bond the two participating cysteine residues must have their α-carbons within 4 - 9 Å, the distance between the paired sulphur atoms must be about 2.05 Å, and the peptide backbone has to be in a proper orientation to allow the bonds between the side chain atoms to assume the proper conformation (Creighton, 1984; Creighton, 1988). Thus the formation of disulphide bonds is determined largely by the protein conformation. Once formed, the disulphides stabilize the conformation that brought them together. The conformation of a disulphide bridge is defined by five dihedral angles formed by the bonds between the side chain atoms. Each of the five bond rotations can be in a favorable or an unfavorable, i.e., low or high energy, conformation for the crosslink stability. The bonds, however, do not have to be of optimal low energy geometry. Disulphides of relatively high dihedral energy can

still be quite effective at imparting the increased stability of the protein structure; disulphide bonds in some naturally occurring proteins exist in high energy conformation with dihedral strain energies ranging from 0.5 to 4.8 kcal mol^{-1} (Katz & Kossiakoff, 1986). These energies would be expected to counteract any positive contributions of disulphide bonds, such as the chain entropy effect, to the free energy of protein structure stabilization. This destabilizing effect must be significant, since it is of a similar magnitude to the chain entropy contribution of disulphide crosslinks. Hence, disulphides can manifest high energy dihedral angles as well as unfavorable non-bonded interactions (Katz & Kossiakoff, 1986).

Relatively little is known about disulphide bond formation in living cells. Most of our knowledge is based on *in vitro* studies on refolding and reoxidation of denatured, reduced proteins (reviewed in Creighton, 1984; Freedman, 1984; Freedman & Hillson, 1980). *In vivo* experiments have produced information about the timing and intracellular localization of disulphide formation. Studies with some secretory proteins have shown that in eukaryotic cells the disulphide pairing takes place at early stages of biosynthesis, during the translocation of the nascent chain to the ER or shortly after the translocation, while the newly synthesized polypeptide is within the ER (Bergman & Kuehl, 1979a,b; Braakman et al., 1991; Bruckner et al., 1981; Peters & Davidson, 1982).

The full complement of native disulphide bonds is not formed directly. The classical view (Creighton, 1984; Creighton et al., 1980; Freedman, 1984) states that protein folding follows kinetically and thermodynamically defined pathways with a defined set of intermediates containing non-native disulphides. The native disulphide bond configuration, which corresponds to thermodynamically most stable protein conformation, or to the most stable conformation which is kinetically accessible, is formed after a series of rearrangements, formation and breakage, of non-native disulphide bonds. These rearrangements occur very slowly *in vitro*. The reactions in the ER are accelerated by protein disulphide isomerase (PDI, EC 5.3.4.1), an abundant soluble protein which resides in the ER lumen of higher eukaryotic cells, catalyzing the formation, the

reduction, and the isomerization of disulphide bonds (Freedman, 1984).

It has been demonstrated that PDI does not change the pathway of this rate-limiting process in the folding of disulphide-containing proteins *in vitro*. Rather, it catalyses thiol/disulphide interchange in proteins, thus accelerating the formation of the native, lowest energy set of disulphide bonds in the ER (Creighton et al., 1980; Freedman, 1984). Bulleid & Freedman (1988) showed that PDI depleted microsomes were defective in cotranslational di-sulphide bond formation. Reconstitution of the microsomes with PDI restored the catalyzing properties. This study demonstrated that PDI is indeed required for correct folding and disulphide pairing during protein biosynthesis. The correct disulphides are formed rapidly in the cells, whereas the uncatalyzed process *in vitro* is very slow (Freedman, 1984). For example, refolding of reduced rat serum albumin takes hours *in vitro*, while in living cells the native con-formation with 17 correct intramolecular disulphide bonds is formed within 2 minutes of polypeptide synthesis (Peters & Davidson, 1982; Peters & Reed, 1980). Care must be taken when the *in vitro* re-folding data is extrapolated to a biological situation. However, the study of Braakman et al. (1992) demonstrated that when the fully folded, newly synthesized influenza hemagglutinin (HA) polypep-tides are reduced and denatured in the ER, they regain efficiently the correct native structure after removal of the reducing agent from the cell culture, indicating that at least for this protein the *in vitro* data are biologically relevant.

Newly synthesized proteins which fail to fold correctly, have been demonstrated to form aggregates containing aberrant di-sulphide linkages that are retained in the ER, and are eventually degraded (Gibson et al., 1979; Hurtley & Helenius, 1989; Marquardt & Helenius, 1992). Another example of disulphide linked aggregate formation in the ER are the intracisternal granules (ICGs), originally described by Palade (1956). Secretory proteins can be sorted, within the ER, by condensation into these large complexes (Tooze et al., 1989). The assembly of ICGs is associated with the exclusion of resident ER proteins, notably PDI. The exclusion of PDI is especially

interesting, since these complexes are heavily crosslinked by disulphides. Formation of the crosslinks was proposed to be a consequence of the absence of PDI which otherwise would dissolve these linkages. Eventually the ICGs are autophagosomally degraded. The assembly mechanism of ICGs is obviously different from that of HBsAg particles, but it indicates that such large disulphide crosslinked multimeric complexes may not be stable in the presence of PDI.

Aggregation in the ER is not restricted to irreversibly misfolded proteins. It has been demonstrated that during the normal folding process some polypeptides form transient complexes which contain intermolecular disulphide bridges (Doms et al., 1989; Kim & Arvan, 1991). Clearly, proteins can form large aggregates in the ER, but they are not stable within that compartment. Indeed, no structurally stable large disulphide crosslinked complex other from the HBsAg has been suggested to form within the ER.

Although the ER evidently is the primary site of disulphide bond formation in the secretory pathway, there are examples of disulphide crosslinking outside the ER. The final assembly of immunoglobulins A and M, and crosslinking of mucins seem to occur at later stages of secretion, and covalent aggregation of fibronectin may occur at the plasma membrane (reviewed in Freedman & Hillson, 1980). The best characterized example of such post-ER crosslinking is the biosynthesis of von Willebrand factor (vWF; Wagner, 1990). This glycoprotein which functions in blood vessel injury repair, in hemostasis, and probably in promoting endothelial cell adhesion on blood vessel walls, is found in plasma as a series of disulphide crosslinked multimers of a single subunit. The large multimers form in *trans*- or post-Golgi compartments. Interestingly, vWF dimerizes in the ER, and only the correctly assembled dimers are transported from this compartment.

The observation that HBsAg particles are extensively crosslinked raises a question since the HBsAg assembly is classically believed to occur in the ER, and hence in the presence of a high levels of PDI which should not allow the formation of such

crosslinked complexes. No other large disulphide linked aggregates are known to be stable in the ER. They are formed later in the secretory pathway or, as in the case of ICGs, after the exclusion of PDI.

2.2.3. Molecular chaperones

Misfolding and irreversible aggregation are often a major problem with folding experiments *in vitro*. In cells, on the other hand, usually more than 95 % of newly synthesized wild type polypeptides fold to their correct, native three-dimensional structure (Gething et al., 1986). In the ER the efficiency and fidelity of folding and assembly are achieved by the action of enzymatic catalysts such as PDI, and of the chaperone-like function of at least three resident ER proteins , BiP (heavy chain binding protein, also known as GRP78), GRP94 and CD-ω/TRAP. Molecular chaperones (Ellis & Hemmingsen, 1989; Ellis & van der Vies, 1991), also called polypeptide chain binding proteins (Rothman, 1989), are defined as cellular proteins that mediate the correct folding of other proteins and their assembly to oligomeric structures, but that are not themselves components of the final structures. The chaperones do not appear to posses steric information for the assembly. They have been suggested to function by inhibiting unproductive assembly pathways, which would otherwise act as dead-end kinetic traps (Gatenby & Ellis, 1990). Thus the molecular chaperone concept supports the self-assembly hypothesis, which Gatenby & Ellis (1990) extended by introducing the concept of assisted self-assembly.

Folding and assembly intermediates of several proteins have been isolated in complexes with BiP (Bole et al., 1986; Doms et al., 1987; Gething et al., 1986; Kim & Arvan, 1991; Machamer et al., 1990; Ng et al., 1989). BiP, a resident soluble ER protein, is thought to stabilize unfolded and partially folded polypeptides and to prevent inappropriate intra- and interchain interactions (Pelham, 1989). BiP is related to the heat shock protein family HSP70. The HSP70 proteins in the cytosol have been shown to assist protein folding and assembly, and mediate the targeting of cytosolic proteins for lysosomal degradation (Beckman et al., 1990; Chiang et al., 1989;

Sheffield et al., 1990). The ER also contains an HSP90 analogue, GRP94, also known as ERp99 and endoplasmin (Koch et al., 1988; Lewis et al., 1985; Macer & Koch, 1988; Mazzarella & Green, 1987). It was recently reported to associate with unassembled immunoglobulin heavy and light chains (Melnick et al., 1991). During the T cell receptor (TCR) assembly, no association between BiP and unassembled TCR subunits has been observed, but another protein, CD-ω or TRAP (TCR Associated Protein), has been shown to bind to the subunits transiently in the ER prior to the assembly (Alarcon et al., 1988; Bonifacino et al., 1988).

While the enzymes which catalyze the rate-limiting reactions (PDI and peptidyl-prolyl *cis-trans* isomerases), accelerate protein folding, the molecular chaperones are not folding catalysts by kinetic criteria. They help the folding and assembly by trapping folding intermediates and preventing their non-specific aggregation. Hence, the chaperones slow down the overall folding rate, but increase the yield of correctly folded proteins.

2.2.4. Quality control in the endoplasmic reticulum

Although the folding and assembly in the ER are generally very efficient, a fraction of newly synthesized polypeptides misfold and aggregate irreversibly (Marquardt & Helenius, 1992). An important function of the ER is to prevent the exit of these incorrect proteins and so to prevent potential damage caused by the transport of defective products. The concept of this quality control (Hurtley & Helenius, 1989) indicates that only properly folded and assembled proteins are allowed to exit the ER and continue their way along the secretory pathway.

The mechanisms of quality control are poorly understood. It is not known what structural properties of newly synthesized polypeptides determine the transport incompetence and lead to retention. Misfolded proteins are usually retained as aggregates, and the aggregation has been suggested as one possible retention mechanism (De Silva et al., 1990; Hurtley & Helenius, 1989; Rose &

Doms, 1988). The aggregates might be retained by interactions with a meshwork of resident ER proteins. This meshwork could either restrict the diffusion of aggregates above a critical size, or it could interact with misfolded proteins directly or with BiP bound to them (De Silva et al., 1990). Weak interactions with such an "ER skeleton" have also been suggested for retention of unassembled subunits and assembly intermediates of protein oligomers (Hu & Tsou, 1991; Hurtley & Helenius, 1989; Pfeffer & Rothman, 1987). Sitia et al. (1990) suggested that exposed free cysteine thiols may have a role in ER retention.

It is not known whether specific protein factors are involved in the retention. BiP has been shown to bind stably to misfolded proteins and it has been suggested to be involved in their ER retention (Bole et al., 1986; Dorner et al., 1987, 1988; Hurtley et al., 1989; Kassenbrock et al., 1988; Kozutsumi et al., 1988). BiP might mediate this retention by virtue of its carboxy-terminal ER-retention sequence (Munro & Pelham, 1987). The observation that reduced BiP levels in the ER lead to the secretion of an improperly folded mutant protein which normally is retained and associated with BiP, indicates its role in protein retention (Dorner et al., 1988). On the other hand, De Silva et al. (1990) have obtained evidence that BiP is probably not involved in the retention of misfolded mutant G protein of vesicular stomatitis virus (VSV).

De Silva et al. (1990) also demonstrated another interesting feature of ER quality control function, showing that ER contains factors which catalyze the misfolding of an already folded and transport-competent thermosensitive VSV-G protein mutant ts045. This polypeptide folds correctly at permissive temperature but at nonpermissive temperature it misfolds in the ER. However, after exit from the ER, probably before reaching the Golgi complex, the protein becomes thermostable, i. e., it does not misfold when the temperature is raised to a nonpermissive level. Interestingly, when the already thermostable ts045 in the Golgi was relocated back to the ER by brefeldin A (BFA) treatment, it misfolded at the nonpermissive temperature. *In vitro* experiments showed that this misfolding was mediated by unidentified protein factors larger than 30 kD. Hence,

an active unfolding mechanism may be associated with the quality control system in the ER. This misfolding was independent of PDI function and is very likely to be independent of BiP function.

Transport-incompetent proteins which are retained in the ER are eventually degraded. The mechanism by which the retained proteins are targeted for degradation, and how and where the degradation takes place, remains undefined. It is now clear that there are several pathways for the degradation of ER proteins, as was proposed by Hurtley & Helenius (1989). Some resident ER proteins, including cytochrome P-450, are degraded by autophagy, a mechanism related to the lysosomal system (Masaki et al., 1987). The breakdown of incompletely assembled or misfolded proteins has been demonstrated to occur by an ER-related process which is insensitive to all known drugs that prevent lysosomal proteolysis (reviewed by Bonifacino & Lippincott-Schwartz,1991; Klausner & Sitia, 1990). This proteolytic system remains poorly characterized. One remarkable feature of this degradation which occurs with very rapid kinetics, in contrast to autophagy, is its selectivity. Certain newly synthesized abnormal proteins are degraded rapidly, whereas others are more stable (Bonifacino & Lippincott-Schwartz, 1991). In some cases there is a lag period between the polypeptide synthesis and proteolysis, which was may represent a transport step from the synthesis site to the degradation site (Lippincott-Schwartz et al., 1988).

The exact location of ER-related protein breakdown is not clear. The recent study of Tsao et al. (1992) elucidates this point. These authors expressed two variants of a resident ER membrane protein ribophorin I in cultured HeLa cells, a membrane bound form and a soluble lumenal form. Both of were degraded relatively rapidly after their synthesis. The membrane bound form was degraded within the ER itself. The degradation of the soluble form showed biphasic kinetics, with an initial breakdown identical to that of the membrane bound form, followed by a transport to a post-ER, pre-Golgi compartment where the final proteolysis took place.

These results support the model of Bonifacino & Lippincott-

Schwartz (1991) who posit two alternative mechanisms. One type of breakdown is proposed to involve sorting and delivery to a specific region of the ER or to a closely related compartment. The final breakdown of the soluble ribophorin appears to occur in agreement of this suggestion, evidently taking place in a compartment distinct from the ER. The alternative is selective degradation accomplished by the recognition of specific determinants on proteins, mediating the binding to a proteolytic complex within the ER, in analogy to cytosolic ubiquitin system. Studies on the initial phase of ribophorin variant breakdown seem to be consistent with this. The molecular details of the degradative system are not known.

A mature HBsAg particle contains approximately 100 protein subunits, each of them with 14 cysteines which all are involved in disulphide bonds. Hence about 700 correct disulphide bonds must be formed during the particle maturation. Thus HBsAg assembly should be a slow and complicated process and multimeric disulphide linked assembly intermediates could be recognized by the ER quality control system and at least to some extent degraded in the ER. However, HBsAg is known to be assembled and secreted very efficiently, albeit slowly, without significant proteolysis. Hence, the possibility that HBsAg particles may assemble in a later compartment should be considered.

2.3. Could HBsAg mature in an ER-Golgi intermediate compartment?

Given the problems of HBsAg particle assembly in the ER, and the observation that this assembly is likely to take place before the Golgi complex, a relevant question is whether this assembly process could occur in an intermediate compartment. Several lines of morphological and biochemical evidence support the existence of a post-ER, pre-Golgi compartment or compartments operating on the secretory pathway. Saraste & Kuismanen (1984) showed that at 15°C the transport of viral membrane glycoproteins from the ER to the Golgi complex in BHK cells was blocked reversibly. The protein could leave the ER but failed to enter the Golgi, accumulating in a distinct

tubulovesicular compartment. Similar results have since been obtained by a number of other investigators (Bonatti et al., 1989; Lodish et al., 1987; Morré et al., 1989; Saraste & Svensson, 1991; Schweitzer et al., 1990; Tartakoff, 1986; Vaux et al., 1990). Recently, Schweitzer et al. (1991a) isolated an intermediate compartment with properties different from the ER and the *cis*-Golgi, using a two-step density gradient procedure. Interestingly, it has been suggested that some viral proteins indeed oligomerize in this compartment (Schweitzer et al., 1990; Tooze et al., 1988).

A number of cellular functions have been proposed to occur in ER-Golgi intermediate compartments. One defining function is the retrieval of ER proteins. ER contains several soluble proteins in its lumen. These resident ER proteins, such as PDI, BiP and GRP94, contain a common retention signal. This signal is the carboxy-terminal tetrapeptide, which in animal cells is usually -Lys-Asp-Glu-Leu (KDEL in one-letter amino acid code; Munro & Pelham, 1987; Pelham, 1990; Pelham, 1989). It is thought to be recognized by a membrane bound receptor that binds to the escaped ER proteins in a post-ER salvage compartment and returns them to the ER (Lippincott-Schwartz ct al., 1990; Pclham, 1988; Pelham, 1989; Vaux et al., 1990; Warren, 1987). The salvage compartment has been suggested to be the same as the 15°C compartment (Pelham, 1989; Vaux et al., 1990). Schweitzer et al. (1991a) concluded from the studies of MHC class I molecules (Cox et al., 1990; Nuchtern et al., 1989; Yewdell & Bennink, 1989) that an ER-Golgi intermediate compartment may be their site of interaction with exogenous antigens. The first step in O-glycosylation of proteins, addition of N-acetylgalactosamine, has been suggested to occur in the same post-ER, pre-Golgi compartment in which the budding of the viral particles takes place in coronavirus infected cells (Tooze et al., 1988). Conversion of a temperature sensitive mutant VSV-G protein to a thermostable form has also been reported to occur in this 15°C compartment (De Silva et al., 1990).

Other functions attributed to ER-Golgi intermediate compartment(s) are palmitoylation of proteins (Bonatti et al., 1989; Rizzolo et al., 1985; Rizzolo & Kornfeld, 1988), the initial step in the

formation of the lysosomal mannose-6-phosphate targeting signal, which is the transfer of N-acetylglucosamine-1-phosphate from UDP-N-glucosamine-1-phosphate to one or more mannose residues on newly synthesized lysosomal enzyme precursors, and protein degradation (Kornfeld & Mellman, 1989; Lazzarino & Gabel, 1988; Pelham, 1988; Tsao et al., 1992).

Although often referred to as "the" intermediate compartment, the diversity of the proposed functions leaves open the possibility that this compartment is heterogeneous. In fact, Bonatti et al. (1989) have demonstrated that protein palmitoylation takes place in a compartment distal to the 15°C block but prior to the site where mannosidase I trims oligosaccharides in cultured Vero cells. Similar observation of Hobman et al. (1992) supports this. In transfected CHO cells, the site at which the exocytic transport of rubella virus (RV) E1 glycoprotein is arrested in the absence of RV E2 protein, is the same as, or distal to the compartment in which RV E1 is palmitoylated, but proximal to the 15°C block. Thus, more than one distinct compartment may operate in the region between the classical ER and the classical Golgi. Furthermore, there seem to be cell type specific differences in the organization of the early secretory pathway.

The nomenclature of the secretory pathway from the ER to the Golgi is somewhat confusing in the literature. The term *cis*-Golgi network (CGN; originally *cis*-Golgi system, Huttner & Tooze, 1989) refers to the whole system between the ER and the Golgi, including the transitional region of the ER, the intermediate compartment(s) and the *cis*-most face of the classical Golgi complex (Mellman & Simons, 1992). Hauri and coworkers use the term ERGIC (ER-Golgi Intermediate Compartment) for the proximal part of the Mellman-Simons CGN, and define the CGN differently (Hauri & Schweizer, 1992; Schweitzer et al., 1991b). The most inclusive definition is that of Rothman & Orci (1992) which equates the Golgi with everything from transitional ER to TGN. In the following, any post-ER, pre-Golgi component of the secretory pathway is denoted as an intermediate compartment.

There are now several markers for intermediate compartments. As mentioned above, there is evidence for intermediate compartment localization of UDP-N-acetylglucosamine:lysosomal enzyme-N-acetylglucosaminc-1-phosphotransferase, the enzyme responsible for the initial phosphorylation of newly synthesized lysosomal enzymes (reviewed by Kornfeld & Mellman, 1989). The recently identified 72 kD membrane protein, a mammalian KDEL-receptor candidate, is concentrated in a compartment distinct from both the ER and the Golgi (Vaux et al., 1990). A member of the low molecular weight GTP binding protein family, rab2, which has been suggested to play a role in transport from the intermediate compartment, has been shown to be associated with an ER-Golgi intermediate compartment (Chavrier et al., 1990). Also a 58 kD glycoprotein (Saraste & Svensson, 1991), a 53 kD protein (p53, Schweitzer et al., 1988) and a 63 kD protein (p63, Schweitzer et al., 1991b) of unknown function have been localized to this compartment. The p63 has been suggested to be a stationary marker for the intermediate compartment (Schweitzer et al., 1991b) whereas the others seem to recycle in the secretory pathway, spending only a fraction of the time in the compartment.

BFA is a fungal metabolite which has been shown to have profound effects on protein secretion and on the distribution of Golgi components. Also known as decumbin, cyanein, or ascotoxin, this macrocyclic lactone is produced by a number of fungi the *Penicillium* family. Takatsuki & Tamura (1985) reported that BFA prevented the transport of newly synthesized G-protein of VSV to the cell surface, causing the glycoprotein to accumulate in the cells in endoglycosidase H sensitive form. This indicated that the transport from the ER to the Golgi was perturbed. Subsequently, Fujiwara et al. (1988) showed that the Golgi complex disappeared morphologically in the presence of BFA. Since then, all the characterized *cis*- and medial Golgi marker proteins, and at least one *trans*-Golgi enzyme have been shown to redistribute to the ER in the presence of BFA, and to return to their normal location upon removal of the drug. Sialyltransferase, a resident TGN enzyme, does not redistribute into the ER during BFA treatment, suggesting that the TGN is a separate compartment distinct from the Golgi and insensitive to BFA

(Chege & Pfeffer, 1990). However, Ivessa et al. (1992) reported recently that ribophorins I and II, resident transmembrane proteins of rough ER, are exposed to TGN enzymes (i.e., sialylated) during BFA treatment.

The earliest observed effect of BFA is a rapid redistribution of two peripheral Golgi membrane proteins, β-coatomer protein (ß-COP) and a recently described 200 kD protein, from the membrane to the cytoplasm (Donaldson et al., 1990; Narula et al., 1992). ß-COP is homologous to clathrin adaptin β and a component of coatomers, cytoplasmic protein complexes which form the coat of non-clathrin-coated vesicles. These have been proposed to mediate the vesicular transport between successive Golgi cisternae (Duden et al., 1991; Waters et al., 1991). This effect precedes all the other observed effects caused by BFA. The results obtained by Donaldson et al. (1991) and Orci et al. (1991) led the authors to propose a model for BFA action on the early secretory pathway. According to the model, BFA prevents the assembly of non-clathrin-coated vesicles from cytoplasmic precursors (coatomers), thus preventing the formation of vesicles mediating the transport from the ER to the Golgi and between the successive Golgi compartments. The anterograde transport is blocked while the retrograde recycling pathway, which has been suggested to occur via smooth non-coated tubules from Golgi to the ER (Lippincott-Schwartz et al., 1990), continues and is even induced by BFA.

The recent observations of the effects which BFA has on the endosomal system and the TGN (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991) extend this model and suggest a general mechanism for BFA's interaction with the formation of coated transport vesicles at different steps of intracellular vesicular traffic. This is supported by the observation that BFA causes a rapid redistribution of coat proteins associated with clathrin-coated vesicles which bud from the TGN (Robinson & Kreis, 1992). Orci et al. (1991) have suggested that the "receptor" of BFA is either a subunit of coatomers, coatomer binding site at the (Golgi) membrane, or a factor that catalyses the assembly of coatomers to the coats. If this model is correct, BFA should not affect the

distribution of the intermediate compartment because the vesicular export from the ER and from the intermediate compartment should be blocked by the drug. Indeed, the distribution of intermediate compartment markers has been reported to be insensitive to BFA (Schweitzer et al., 1991b; Vaux et al., 1990).

2.4. Characterization of the Intermediate Compartment

A clear morphological description of the intermediate compartment is not yet established. This reflects the difficulties in correlating the functional description of this compartment, as for example the compartment involved in the salvage of ER proteins, with morphological data. Two interesting questions are the size of the compartment and whether it is physically continuous with the classical ER. Uncertainty about these issues has led some authors (Mellman & Simons, 1992) to describe the intermediate compartment as a specialization of the ER and to difficulties in understanding its separate functional role. The biochemical characterization of the HBsAg pathway in this work provided a basis for overcoming previous problems with morphological characterization of intermediate compartments. The aggregating intermediates of HBsAg provide a morphological marker of an intermediate compartment which is depleted of ER components, and which is functionally active since essentially all of the intracellular HBsAg is eventually secreted. These intermediate compartment forms of HBsAg are easily distinguished from the ER forms which do not form aggregates. In this thesis, a combination of electron microscopy immunocytochemistry and stereology have been used to provide the first morphometric quantitation of an intermediate compartment. The results show that this intermediate compartment is not only discontinuous from the ER but is also a major element of the secretory pathway.

2.5. Summary

The ER is an organelle with many important functions which make it

unique amongst the cellular organelles. It is the entry site of newly synthesized proteins to the secretory pathway, housing a set of factors facilitating protein folding and assembly, and accomplishing a series of post-translational modifications unique to this compartment. It provides a quality control machinery which takes care that only correctly folded and assembled proteins can be transported further along the secretory pathway, thus acting as a checkpoint for correct expression of genetic information at the protein structure level.

The classical model of HBsAg biosynthesis localized the entire assembly process to the ER. Although the mechanisms of the ER quality control remain poorly understood, some criteria are known. Large protein aggregates are not stable in the ER, and they are eventually degraded in this compartment or by another proteolytic system. The assembly of HBsAg particles is a slow process, and thus the multimeric disulphide linked assembly intermediates could be recognized by the quality control system and degraded. However, HBsAg is known to assemble into the particles and to be secreted efficiently. Further, the ER contains a high concentration of PDI which catalyses thiol/disulphide exchange and hence could resolve extensive crosslinks such as those present in HBsAg particles and it might not allow the maturation of HBsAg. In fact, other characterized large disulphide linked assemblies like vWF form after leaving the ER or, as in the case of ICGs, after exclusion of PDI within the ER. Thus, it seemed unlikely that HBsAg maturation is completed in the ER.

3. AIMS THE PRESENT STUDY

The aim of this thesis is the definition the HBsAg maturation pathway. The specific issues addressed are:

1) the classical hypothesis that HBsAg assembly occurs entirely in the ER, which was tested by using specific ER markers;

2) the new hypothesis that an intermediate compartment might play an important role in HBsAg assembly, which was examined by following the effect of drug treatments on the kinetics of interchain disulphide bond formation and on intracellular distribution of HBsAg;

3) the importance of a special non-ER environment for HBsAg assembly, which was tested by *in vitro* experiments in which mature HBsAg particles were exposed to recombinant PDI;

4) the relationship of the intermediate compartment involved in the assembly of HBsAg to other exocytic compartments, which was studied by conventional and immunoelectron microscopy, and by immunofluorescence.

4. SUMMARY OF METHODS

All of the methods used in the present study are described in Articles I and II. A brief summary of the relevant points is presented here. Details of the drug and enzyme treatments are described in Article I.

4.1. Cells

Stably transfected cell mouse L fibroblast cell line SV24 expressing HBsAg subtype adw under the control of SV40 early promoter (Simon et al., 1988) was a gift from Dr. Don Ganem (University of California, San Francisco, CA). Sf9, a clonal isolate of *Spodoptera frugiperda* IPLB-Sf21-AE cells, was a gift from Dr. Max Summers (Center for Advanced Invertebrate Molecular Sciences, College Station, TX). Rat-PDI-cDNA (obtained from Dr. George Banting, University of Bristol) was cloned into pVL1393 expression vector, and further inserted to the wild type *Autographa californica* polyhedrosis virus genome, and the Sf9 cells were infected with this recombinant virus by Dr. Astrid Eder (EMBL, Heidelberg). Cells were grown as described in Articles I and II.

4.2. Antibodies used for immunocytochemistry and biochemical experiments

The primary antibodies used in the present study are listed in the Table 1. More detailed description of the antibodies can be found in

the references given in the table. The ER marker antibodies were characterized by Western blotting against SV24 lysate and by immunofluorescence on SV24 cells as described in Article I.

Table 1. Antibodies used in the study

Antibody	Produced in[1] (mc=monocl.)	Ig class	Against[2]	Obtained from/ reference
1D3	M (mc)	G	PDI	Vaux et al., 1990
10C3	M (mc)	G	BiP	Buck et al., in preparation
5D3	M (mc)	M	72 kD[3]	Vaux et al., 1990
anti-ManII	R	G	ManII[4]	B. Burke, Harvard (Louvard et al., 1981)
anti-KX_5	R	G	KDEL[5]	Buck et al., in preparation
anti-HBsAg	R	G	HBsAg	Calbiochem
anti-HBsAg	Sh	G	HBsAg	M. Nassal, ZMBH (Behringwerke #61WG20)
anti-HBsAg	M (mc)	G	HBsAg	Behringwerke
anti-rab2	R	G	rab2	M. Zerial, EMBL (Chavrier et al., 1990)
M3A5	M (mc)	G	β-COP	T. Kreis, EMBL (Allan & Kreis, 1986)

[1]M=mouse, R=rabbit, Sh=sheep; [2]for more detailed description see Articles I and II; [3]72 kD KDEL-binding protein (Vaux et al., 1990); [4]Golgi mannosidase II; [5]raised against a mixture of peptides having a general sequence KXXXXXKDEL, where X positions were synthesized from a mixture of alanine, aspartic acid, histidine, glutamine, leucine, tyrosine, and lysine.

4.3 Immunocytochemistry

Indirect double immunolabeling for conventional and confocal fluorescence microscopy on SV24 cells was done as described in Articles I and II. Normal fluorescence microscopy was done using Zeiss Photomicroscope III and Zeiss Axiophot microscope with selective filters for fluorescein, rhodamine and Hoechst 33258. Confocal microscopy was done with the modular laser scanning confocal

microscope developed by the EMBL Light Microscopy Group, using a single exitation wavelength (514 nm) and selective filters for fluorescein and rhodamine.

SV24 cells were prepared for cryosectioning using standard protocols (Green et al., 1981; Griffiths et al., 1984). Thin frozen sections were cut using Reichert Ultracut 4E, Reichert FCS, and LKB Nova ultramicrotomes with cryo-equipment, with a cryo-diamond (Diatome) or tungsten coated glass knives prepared using LKB Knifemaker II. The sections were thawed and double labeled using a standard immuno-gold labeling procedure (Geuze et al., 1981), with the modification that the blocking after the first protein A-gold was done by incubating the sections on 1 % glutaraldehyde (Slot et al., 1991).

4.4. Conventional electron microscopy and morphometry

Conventional thin sections were cut from Epon embedded, glutaraldehyde fixed, osmium tetroxide stained SV24 cell blocks prepared using a standard protocol. The sections were placed on non-coated grids and further contrasted with uranyl acetate and lead citrate (Article II). Sections were examined with Philips 400 and Philips 400T transmission electron microscopes at 80 kV.

The quantitation of electron microscopy data was done using standard stereological methods (Weibel, 1979; Article II).

4.5. Pulse chase and immunoprecipitation experiments

To study the time course of HBsAg secretion and intermolecular disulphide bond formation, SV24 cells were pulse labeled with radioactive methionine and after various chase times lysed in the presence of 20 mM N-ethylmaleimide (NEM) to block free thiol groups. HBsAg was immunoprecipitated from the culture media and cell lysates as described in Article I. The secretion time course was

measured as immunoprecipitable radioactivity in the culture media as a function of time. Immunoprecipitated intracellular HBsAg was quantitatively analysed from the digitized SDS-PAGE autoradiographs (Article I). HBsAg after PDI incubations was immunoprecipitated and analysed as in pulse chase experiments, but the precipitates were not subjected to extensive washing (Article I).

4.6. Digital image analysis

Autoradiographs from pulse chase and other experiments were digitized by using a CCD camera and frame grabber into TIFF-format files and analysed in Image 1.40 program (Article I).

The colocalization of the various antigens was analysed at the immunofluorescence level using an algorithm introduced by Dr. Jean Davoust (Humbert et al., submitted for publication) and implemented as a series modules written in C by Dr. Stephen Fuller (EMBL) for use with the Application Visualisation System (AVS, Stardent Computer) on a Stardent 2000GS computer. Each pixel in the original image consists of a red (R) and a green (G) intensity at a position (X, Y). The analysis generates a two dimensional histogram of the number of occurences of each pair of fluorescence intensity values in the double fluorescence image. The pixels with intensities R and G at all positions are added to the bin of the two dimensional histogram at the position (R, G) to generate this occurence plot. The occurence plot shows how often each combination of fluorescence intensities occurs. Colocalization of red and green labels shows up as a tilted line of while a lack of colocalization shows up as either a vertical or horizontal line. Particular areas of the resulting occurrence graph are then selected and a new image composed to visualize different populations of pixels corresponding to given fluorescence ratio (Article I) for example composing the image from a vertical line of occurence values would reveal the locations of all pixels in which the red and green intensities are not correlated.

5. REVIEW OF THE RESULTS

5.1. Comparison of the alternative hypoteheses for HBsAg assembly (Aims 3.1 and 3.2)

5.1.1. HBsAg particles assemble after the exit from the ER

Indirect immunofluorescence showed that a fraction of HBsAg in SV24 cells was localized in a compartment which excluded soluble resident ER proteins, often in the perinuclear region. Quantitation of the three dimensional double fluorescence data obtained by confocal laser scanning microscopy revealed two subpopulations of pixels. The bulk of the pixels showed a relatively constant PDI to HBsAg ratio, representing a localisation of HBsAg to the ER. The other population consisted of pixels showing a strong HBsAg label but negligible label for PDI. This subpopulation was well separated from the bulk of the pixels, indicating a separate compartment rather than a continuous variation of the label intensity corresponding to either PDI or HBsAg. This argues strongly that a fraction of HBsAg is localized to a compartment separate from the ER (Article I).

This was confirmed by electron microscopy of immuno-labeled SV24 cryosections and conventional embedded SV24 sections (Article II). HBsAg was shown to be sorted to a smooth membraned tubulovesicular compartment excluding the ER marker proteins. Mature HBsAg particles could be seen in the lumen of this compartment, whereas no particles could be seen in the ER, confirming that the assembly of HBsAg particles occurs in this compartment. No continuities between the membranes of the HBsAg

assembly compartment and the ER were observed. Hence HBsAg matures in a compartment which is distinct from and discontinuous with the ER.

The temporal relationship between the occurrence of HBsAg in the cellular regions excluding PDI and in the ER was studied by blocking the protein synthesis with cycloheximide (Article I). The relative fraction of HBsAg localized to the PDI excluding regions increased during the cycloheximide chase, indicating that the later stages of HBsAg maturation take place in a post-ER compartment. After 90 min of cycloheximide treatment HBsAg still colocalized with PDI, although to a lesser extent than at the normal steady state. After 4 hours the colocalization of HBsAg with PDI was negligible and after 6 hours essentially all HBsAg label was found in the PDI excluding compartment while no HBsAg could be detected in the ER. The PDI distribution and staining intensity remained unchanged during the cycloheximide treatment. These data show that HBsAg is transported from the ER to a post-ER compartment, and that the rate limiting step in its secretion (i. e., the particle assembly) corresponds to the exit from the ER.

The relative volumes and surface to volume ratios of some cellular compartments were estimated stereologically from electron microscopy data (Article II). The intermediate compartment in which HBsAg assembly occurs, occupied about 10 % of the total cell volume (12 % of the cytoplasm). This was twice as much as the relative volume of the ER. Surface to volume ratio of the intermediate compartment was about 5 times smaller than that of the ER, similar to that of mitochondria, reflecting its tubulovesicular appearance.

5.1.2. Time course of the interchain disulphide formation

Intermolecular disulphide bond formation during the maturation process was analyzed by pulse chase immunoprecipitation experiments (Article I). SV24 cells were metabolically pulse-labeled, chased for various times with an excess of cold methionine, and

HBsAg was immunoprecipitated from the cell extracts and the culture media, and analysed using SDS-PAGE under non-reducing and reducing conditions. The gels were processed for autoradiography, digitized, and quantitated.

A fraction of HBsAg aggregated at very early chase times, presumably reflecting the association of initially (reversibly) misfolded proteins, in agreement with other studies (Doms et al., 1989; Kim & Arvan, 1991). These aggregates were very rapidly converted to monomers. The monomer concentration then decreased monotonically. Disulphide linked dimers began to form during short (2 min) pulses, and then increased until reaching a maximum at approximately 60 minutes with concomitant decrease of monomers. After this peak, the dimers decreased with corresponding increase in crosslinked oligomers. Analysis of the reduced samples showed that no HBsAg was subject to detectable proteolysis. Both the non-glycosylated and glycosylated forms disappeared from the cells and were secreted to the culture medium with identical rates.

The half-time for the secretion was approximately 2 hours. HBsAg was secreted only in the particle form, in agreement with other published studies (Dubois et al., 1980; Laub et al., 1983; Liu et al., 1982; Moriarty et al., 1981). The lag between the formation of dimers and the secretion of mature crosslinked particles indicates that the oligomer crosslinks are formed from the disulphide linked dimers with a half-time of roughly 1 hour.

5.2. Exit from the ER is required for the covalent oligomerization of HBsAg: Possible role of PDI exclusion (Aim 3.3)

The data discussed above do not tell whether the exit from the ER is a requirement for the particle assembly. To address this question, the time course of interchain disulphide bond formation was followed in SV24 cells by pulse chase analysis in the presence of

BFA (Article I). In SV24 cells, BFA prevented the transport of HBsAg from the ER to the maturation compartment (Article I). In BFA treated cells, newly synthesized HBsAg dimerized with similar initial kinetics as in non-treated cells. However, in the BFA treated cells disulphide linked dimers accumulated in the ER and did not oligomerize further. Thus, the formation of oligomeric disulphide crosslinks, and hence the particle formation, requires the exit from the ER.

This observation suggests that the environment in the intermediate compartment, which allows the oligomeric crosslinks to form, is different from that in the ER. A potential factor contributing to this could be the absence of PDI. The effect of PDI on the oligomeric crosslinks was studied by incubating secreted HBsAg particles isolated from SV24 culture medium with different concentrations of a lysate of PDI-overexpressing baculovirus-infected Sf9 cells. Increasing the concentration of the lysate, i. e., the concentration of PDI, resulted in the conversion of crosslinked oligomeric forms to dimers. This showed that the oligomeric disulphide crosslinks are unstable in the presence of PDI, whereas the dimer formation is promoted. No such increase was seen with control lysates. This indicates that the absence of PDI from the HBsAg assembly compartment may contribute to the process which leads to the formation of mature disulphide stabilized particles.

5.3. Characteristics of the HBsAg assembly compartment

5.3.1. HBsAg assembly occurs in a pre-Golgi compartment

The mostly perinuclear localization of HBsAg which has left the ER resembles that of the Golgi complex in many cell types. Hence a relevant question was whether this compartment is indeed a part of the Golgi apparatus. Patzer et al. (1984) have demonstrated that all detectable oligosaccharides associated with intracellular HBsAg in transfected CHO cells are sensitive to endoglycosidase H digestion.

This was shown to be the case also in SV24 cells (Article I), indicating that no intracellular HBsAg had yet encountered the enzymes of the medial Golgi. Hence the HBsAg particles must form prior to the medial Golgi.

The remaining possibility that the particles assemble in the *cis*-Golgi could be readily tested with BFA. All the characterized *cis*- and medial Golgi markers and, at least in some cell types, the *trans*-Golgi enzymes have been shown to redistribute to the ER in the presence of BFA. The same effect was seen in SV24 cells for the *cis*-Golgi enzyme mannosidase II (Article I). However, BFA did not cause HBsAg in the post-ER compartment to recycle back to the ER, indicating that the HBsAg maturation compartment is not the *cis*-Golgi (Article I). Double immunofluorescence using Golgi marker antibodies together with anti-HBsAg on SV24 cells showed no overlap between the two labels (Article II), confirming that the maturation of HBsAg particles occurs in a pre-Golgi compartment.

5.3.2. Identity of the HBsAg maturation compartment

Double immunofluorescence on SV24 cells from which the free cytoplasmic rab2 was removed, showed that a fraction of rab2 was localized in the maturation compartment (Article I). This was confirmed by immunoelectron microscopy of double labeled cryo-sections (Article II). This indicated that HBsAg matures in the compartment described by Chavrier et al. (1990).

The distribution of HBsAg in relation to the 72 kD KDEL-binding protein was studied by immunofluorescence and electron microscopy (Article II). In the double labeling experiments, an anti-idiotypic monoclonal mouse antibody 5D3 was used together with anti-HBsAg. The KDEL-binding protein was colocalized with HBsAg to the maturation compartment similarly to rab2. This all suggests that the intermediate compartment in which HBsAg matures corresponds to the 15°C compartment (Saraste & Kuismanen, 1984).

6. DISCUSSION

HBsAg particle assembly was classically believed to occur entirely in the ER. This had been based on EM morphology and to the sensitivity of intracellular HBsAg to endoglycosidase H digestion. Considering the recent advances in the cell biology of the secretory pathway, this consensus view needed to be re-examined.

Studies of the biosynthesis of membrane and secretory proteins have resulted in the concept of quality control (Hurtley & Helenius, 1989). Although quality control is not yet completely understood, some of its basic characteristics at the ER level have been established. As a general rule, large aggregates are not stable in the ER, but are dissolved, or are degraded proteolytically in the ER or in another compartment. As discussed above, this could be true also for the assembly intermediates of HBsAg if they formed in the ER.

The ER contains a high concentration of soluble enzyme PDI (>1 mM; Gilbert, 1989; Vaux et al., 1990) which is an efficient catalyst of thiol/disulphide exchange. Given that any other stable disulphide crosslinked aggregates are not formed in the ER, and that the exclusion of PDI has been proposed as a requirement for the formation of crosslinked complexes (Tooze et al., 1989), the presence of PDI may not allow the assembly of such extensively crosslinked structures as HBsAg particles. These can be formed in later exocytic compartments.

The endoglycosidase H sensitivity of intracellular HBsAg might support its ER localization, but it is also consistent with the possibility that the maturation takes place in a post-ER compart-

ment, in the *cis*-Golgi or in an intermediate compartment. The present study shows that transport from the ER precedes the assembly of HBsAg particles, and that the assembly takes place in an intermediate compartment located between the ER and the Golgi complex in the secretory pathway.

6.1. Maturation pathway of HBsAg

The conversion of monomeric, transmembrane HBsAg to soluble liporotein particles is an intricate series of events in which the protein exploits specific properties of the constitutive secretory pathway for its maturation. Extensive work by other laboratories has resulted in a model which explains many details of the process (reviewed by Ganem, 1991 and Ganem & Varmus, 1987). The model localizes the rate-limiting stage in the maturation, the formation of the particles and their extrusion from the membrane, to the ER. The present study extends this model by providing evidence that the rate-limiting step occurs in a post-ER, pre-Golgi compartment, and by identifying the compartments in which the events occur more precisely. It also elucidates the role of disulphide pairing in the maturation process.

Nascent HBsAg is translocated to the ER membrane and a fraction of newly synthesized HBsAg is N-glycosylated (Eble et al., 1986; Simon et al., 1988). The pulse chase data indicates that within 2 minutes of synthesis, probably in part cotranslationally, the newly synthesized polypeptides begin to form disulphide linked dimers in the ER. As Hurtley & Helenius (1989) have pointed out, it is sterically impossible that dimers form between two nascent chains of the size of HBsAg because of the spacing of ribosomes at the ER membrane, but they may form between a nascent and an already finished protein. Dimer formation is relatively rapid and the dimer concentration increases until 60 minutes of the chase time, after which the dimers begin to disappear with a corresponding increase in disulphide linked higher oligomers (Article I). Interestingly, this coincides with the conversion of HBsAg to a protease resistant form

(Simon et al., 1988) which was proposed to reflect a remarkable conformational change and/or aggregation in the membrane.

Immunofluorescence on cycloheximide treated SV24 cells indicates that the bulk of HBsAg is segregated from the ER at the time when dimers become the majority of the population (Article I). BFA treatment of SV24 cells provides evidence that the dimers must be transported out of the ER in order to form higher oligomers. In the presence of BFA, HBsAg forms dimers with similar kinetics as in non-treated cells, but the dimers accumulate in the ER and do not further oligomerize (Article I). Double immunofluorescence (Article I) and immunoelectron microscopy (Article II) show that HBsAg is sorted to a compartment which excludes soluble ER proteins, such as PDI or BiP. It has been demonstrated that the rate-limiting step in the maturation process is the formation and extrusion of HBsAg particles from the membrane (Patzer et al., 1984; Simon et al., 1988). The cycloheximide data (Article I) show that this step takes place in a post-ER compartment.

BFA does not change the distribution of HBsAg in this post-ER compartment. Therefore the assembly compartment is located before the Golgi complex in the constitutive secretory pathway (Article I). This is consistent with endoglycosidase H sensitivity of intracellular HBsAg-glycones (Patzer et al., 1984; Article (I)). A fraction of rab2 is colocalized with HBsAg to this compartment, verifying that the compartment is a part of the normal secretory pathway (Articles I and II), presumably corresponding to the 15°C compartment.

The colocalization of the 72 kD KDEL binding protein raises the possibility that this compartment may be the salvage compartment (Article II). So far, two candidates for mammalian KDEL-receptor have been suggested (Semenza et al., 1990; Vaux et al., 1990) but the 72 kD protein is the only one which has been shown to bind KDEL proteins (Vaux et al., 1990). Further characterization of both candidates is required for the definitive conclusion about their possible role in the retention of soluble ER proteins. The fact that the HBsAg maturation compartment must have a different lumenal en-

vironment from that of the ER (Article I) is consistent with the proposal that the KDEL binding properties of the receptor may be regulated by environmental factors (Vaux et al., 1990).

Simon et al. (1988) have reported that HBsAg is secreted as disulphide stabilized lipoprotein particles with very similar kinetics as it becomes alkaline carbonate extractable. HBsAg particles are secreted rapidly, immediately after their extrusion into the lumen of the maturation compartment. Hence the lag between dimer formation and particle secretion indicates that the rate-limiting step, the formation of extensively disulphide crosslinked particles in the inter-mediate compartment from the dimers formed in the ER, occurs with a half time of roughly 60 minutes.

6.2. Role of disulphide bonds in the assembly

The present study discloses a relationship between HBsAg particle maturation and disulphide bond formation by showing that highly crosslinked oligomers are an intermediate in HBsAg assembly, but it leaves open the question of whether the crosslinking is required for the particle formation. HBsAg purified from recombinant yeast has been shown to contain very few intermolecular disulphides (Hitzeman et al., 1983; McAleer et al., 1984; Wampler et al., 1985), Therefore it has been claimed that disulphides play no essential role in the particle formation. When crude yeast extract was subjected to velocity sedimentation through a sucrose gradient, HBsAg sedimented at similar density to that of human plasma HBsAg. SDS-PAGE analysis of these yeast aggregates under nonreducing conditions revealed that they were assembled of monomeric and dimeric HBsAg. During further immunoaffinity purification highly crosslinked particles were formed (McAleer et al., 1984; Wampler et al., 1985), presumably because the method includes elution of HBsAg with a high concentration of thiocyanate which promotes interchain disulphide formation.

Although HBsAg can be purified from yeast in a form morphologically and physically similar to HBsAg secreted by animal

cells, the process of their formation appears to be quite different. Particles are not secreted by yeast but must be released by lysis and then purified. Hitzeman et al. (1983) have proposed that the resultant particles are formed as a consequence of cell extraction. Yeast cells may actually be defective in initiating the budding process and hence these results do not speak to the normal pathway of HBsAg assembly. Further support for this conclusion comes from the recent work of Kuroda et al. (1991), who showed that the expression of the large protein of ORF-S (pre-S_1) results in the formation of particles in yeast cells, although the expression of this protein in mammalian cells blocks the secretion of all forms of HBsAg (Persing et al., 1986).

Very recent results indicate that intermolecular disulphide bond formation is required for HBsAg assembly. Mutation of any of the cysteines C48, C65, or C69 of HBsAg (Fig. 2) prevents the the particle assembly and secretion (C. Mangold, personal communication). We have analysed these mutants in transfected cells using immunofluorescence, and the results show that the mutants are not transported out of the ER and accumulate in that compartment (A. Huovila, S. Fuller, C. Mangold, unpublished results). These mutants do not form disulphide linked oligomers.

The formation of covalent dimers appears to be the first step in the maturation process. In the presence of BFA the newly synthesized monomers are converted to a stable population of dimers but further oligomerization via crosslinks is blocked (Article I). The transport of dimers from the ER allows this further maturation. The incubation of mature crosslinked particles in the presence of recombinant PDI results in the conversion of multimers to dimers, which suggests that the free energy of dimer crosslinks is lower than that of oligomer crosslinks. Hence PDI which catalyses the formation of the lowest energy disulphide configuration, may not allow the oligomerization of the dimers. Thus, exclusion of PDI may contribute to the particle assembly but also other factors, such as the concentration of HBsAg in the membrane, are obviously important.

Mishiro et al. (1980) have shown that disulphide linked dimers may serve as building blocks for mature HBsAg. When these authors incubated purified human HBsAg particles in the presence of 1 % SDS, dimeric HBsAg was released. During a 2 hour incubation only 2 % of the total HBsAg was released, but this suggests that dimers may be basic structural components of HBsAg particles. Interestingly, these dimers were very immunogenic and antigenic, and it was concluded that the conformation maintained by dimers is required for the full antigenicity of the particles. The present data support the conclusion that the dimer formation is a prerequisite for the transport out of the ER, and hence for the particle formation. However, it is not possible to say whether the further oligomerization is a condition of particle formation or a consequence of it. The expression studies in yeast also indicate that the covalent dimer formation precedes aggregation.

6.3. Quality control and the HBsAg assembly compartment

The results represented in this thesis have implications for the general concept of quality control. The hypothesis of quality control posits that only properly disulphide linked and oligomerized proteins will be allowed to leave the ER (Hurtley & Helenius, 1989). This has been ably demonstrated for simple oligomeric proteins but the case of HBsAg is more complex and the concept has to be extended. The present data show that the initial properly disulphide linked dimers clearly meet the quality control criteria for transport competence at the ER level, and hence they can exit from the ER. A separate quality control which prevents the further transport of immature assembly intermediates must be maintained in the pre-Golgi compartment where the final particle assembly takes place. This quality control system takes care that only HBsAg in the form of soluble lipoprotein particles can transit the Golgi complex and be secreted.

A similar two-stage quality control seems to function in the

biosynthesis of vWF, which is secreted by endothelial cells and megakaryocytes into the circulation as a series of disulphide cross-linked homo-oligomers. The first control step occurs at the level of the ER, from which the protein is exported only after it has dimerized, similarly to HBsAg. The next step occurs in *trans*- or post-Golgi structures from which the mature crosslinked complexes are formed prior to secretion (Wagner, 1990). In both cases the exit from the ER precedes the formation of oligomeric disulphide cross-links. HBsAg is not the first secretory protein suggested to form disulphide bonds outside of the ER, but the present study provides the first demonstration of the exit from the ER as a requirement for the assembly of extensively disulphide crosslinked protein structures

6.4. The HBsAg assembly compartment and its relation to other exocytic compartments

The data presented above support the model schematically represented in Figure 3. In the ER, the dimers form rapidly in the presence of PDI. Formed dimers are transported, presumably via vesicular transport, to an intermediate compartment (IC) where the different lumenal environment allows the dimers to aggregate further to higher covalent oligomers. The host membrane proteins are excluded from these aggregates, perhaps as a result of tight and specific interaction between the dimers. It has been suggested that a substantial reorganization of host lipid may be associated with the particle formation, and that the lipid in the particles may not be in a conventional bilayer (Ganem & Varmus, 1987; Simon et al., 1988; Simon et al., 1988). After the extrusion to the IC lumen, the particles are transported by bulk flow rapidly through the Golgi complex and the TGN where the HBsAg associated glycones become endoglycosidase H resistant. After this the particles are secreted from the cell.

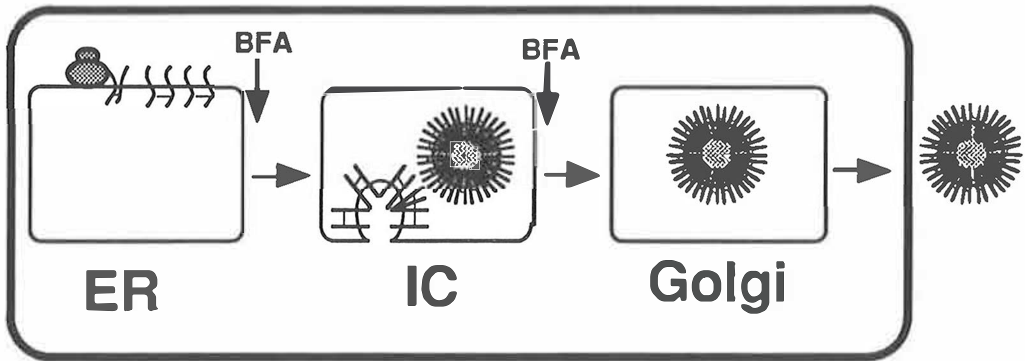

Figure 3. A model for HBsAg assembly along the secretory pathway. Newly synthesized HBsAg is translocated from ribosomes to the ER membrane and begins to form dimers. In the ER, HBsAg is transported to the transitional elements corresponding to the RV E1 transport arrest site (see the text) which is continuous with the ER (hence, in this scheme the ER and the RV E1 compartment are represented as ER). Here the dimers are sorted into transport vesicles which are targeted to the intermediate compartment (IC) where they oligomerize and assemble into HBsAg particles. This intermediate compartment is not continuous with the ER. Immediately after their extrusion to the IC lumen, the particles are rapidly transported through the Golgi complex and secreted. BFA prevents the transport of HBsAg from the ER to the IC and from the IC to the Golgi complex.

The observations of Bonatti et al. (1989) and Hobman et al. (1992) indicate the existence of more than one intermediate compartment. Hobman et al. (1992) showed that the RV E1 glycoprotein accumulated in a compartment which lies on the secretory pathway between the ER and the 15°C compartment. This compartment was distinguishable from the ER but physical continuities were demonstrated between the two, and lumenal ER proteins were shown to have access to this compartment.

The intermediate compartment in which HBsAg particles assemble is clearly distinct from the RV E1 compartment. HBsAg maturation compartment is not continuous with the ER, and it excludes the lumenal ER proteins, as judged by immunofluorescence and electron microscopy. The localization of rab2 and the 72 kD KDEL binding protein to this compartment indicate that it is the same as the 15°C compartment (Chavrier et al., 1990; Vaux et al., 1990). Hobman et al. (1992) suggested that the traffic from RV E1 compartment to the 15°C compartment is mediated by vesicular traffic.

The transport of HBsAg from the ER to the assembly compartment was blocked by BFA (Article I), which is thought to prevent the formation of non-clathrin coated vesicles mediating the traffic from the ER to the Golgi. The RV E1 compartment, which is hypertrophied in E1 overexpressing cells, has been suggested to represent the transitional elements related to the exit sites of the ER in normal cells (Hobman et al., 1992). Thus, the formation of transport vesicles at this compartment is depicted as the target of BFA action which prevents HBsAg from reaching its maturation compartment. Because BFA did not change the distribution HBsAg that had already reached the compartment, the exit from there, presumed to occur via vesicle formation, must be another target for BFA.

The exocytic traffic from the ER to the Golgi complex may occur via two distinct post-ER, pre-Golgi compartments. One corresponds to the RV E1 compartment and is continuous with the ER, whereas the later station, corresponding to the HBsAg assembly compartment, is both functionally distinct and physically discontinuous with the ER. Because of the physical connection and apparently similar lumenal environment, the ER and the RV E1 compartment are represented as one (the ER) in Figure 3. The HBsAg assembly compartment is linked to the preceding (RV E1) and to the next compartment (*cis*-Golgi) via vesicular traffic.

The stereological data presented here provides the first quantitation of an intermediate compartment. The HBsAg maturation occupies approximately one tenth of the total cell volume in SV24 cells, about twice as much as the ER. This presumably reflects the hypertrophy due to expression of HBsAg and its accumulation in this compartment, which would be reminiscent of RV E1 compartment in the E1 overexpressing cells. By its lower surface to volume ratio and structure, this compartment resembles endosomal compartments also operating in protein traffic and sorting. These characteristics reflect the dynamic nature of this compartment, and the high level of membrane traffic through it.

7. CONCLUSIONS

The classical HBsAg biosynthesis model left open the possibility that the assembly takes place after the ER and before medial Golgi. The recent developments in the biology of intracellular dynamics have opened new views, both of organization and function of the eukaryotic secretory pathway, providing circumstantial evidence for post-ER assembly. In this context, the localization of the stages in HBsAg maturation demanded reinvestigation. The results presented in this thesis demonstrate that the later stages of the process in fact occur after the exit from the ER, in an intermediate compartment located between the ER and the Golgi complex. This work further defines the model for HBsAg biosynthesis.

My main conclusions are:

1. HBsAg particles assemble after the exit from the ER, in an intermediate compartment between, and distinct from, the ER and the Golgi complex;

2. HBsAg particles do not mature in the ER, and hence the transport from the ER is a requirement for the assembly;

3. the exclusion of PDI, which is retained in the ER, is required for HBsAg maturation;

4. HBsAg assembly occurs in two stages: a rapid formation of disulphide linked dimers in the ER, and a slower formation of higher oligomeric crosslinks in the intermediate compartment;

5. HBsAg assembly compartment is a functionally distinct compartment which is connected to the preceding and to the subsequent exocytic compartments via vesicle traffic;

6. "the" intermediate compartment is heterogeneous containing at least one component in which HBsAg assembles, and which is not continuous with either the ER or the Golgi.

ACKNOWLEDGEMENTS

I wish to thank Stephen Fuller for all the four years I was in his laboratory. I am very grateful for his support and friendship which made my time enjoyable and most rewarding. I would like to acknowledge all the effort he spent on my supervision but it is impossible to do it here.

Very special thanks are also due to Brent Gowen. His skilful assistance and advise in electron microscopy were invaluable. I am very grateful also to other members of our group Patricia Buck, Astrid Eder, Anna Cyrklaff, Rob Hendriks and Hartmut Krafft-Czepa, for teaching me a number of techniques and for always helping me when I asked. I also want to thank all other people at EMBL who were always helpful, who advised and assisted me in numerous experiments, and who showed almost unnatural patience and understanding for my tendency to "borrow" things, especially Avi Epstein, Charles Ferguson, Ruth and Michael Hollinshead, Heinz Horstman, Pekka Hänninen, Andreas Merdes, and Hilkka Virta.

I also acknowledge the generous gifts of SV24 cells from Don Ganem (University of California at San Francisco) as well as antibodies to rab2 from Marino Zerial (EMBL) and Cecilia Bucci (Università di Napoli), to β-cop from Thomas Kreis (EMBL) and Rainer Duden (University of California at Berkeley), to HBsAg from Michael Nassel (Zentrum für Molekularbiologie in Heidelberg), and to a 135 kD Golgi protein (mannosidase II) from Brian Burke (Harvard University Medical School).

I am grateful for the helpful discussions with and comments from Carl-Henrik von Bonsdorff (University of Helsinki), Thomas Creighton (EMBL), Rainer Duden (University of California at Berkeley), Don Ganem (University of California at San Francisco), Ari Helenius (Yale University Medical School), Rob Hendriks (EMBL), Gareth Griffiths (EMBL), Matti Saraste (EMBL), Kai Simons (EMBL), Sharon Tooze (EMBL), and David Vaux (Sir William Dunn School of Pathology, Oxford University). Matti and David read the drafts of this thesis very carefully and gave a number of excellent comments

and suggestions which led to its remarkable improvement.

I am indebted to my wife Seija and to my parents for all their support and care.

I also want to acknowledge the help I got from my friends at the Department of Cell Biology of the University of Jyväskylä and at the Department of Biochemistry of the University of Helsinki to overcome the difficulties arising from the geographical distance and bureaucracy to get my studies finished, especially Markku Kulomaa, Varpu Marjomäki and Martti Tolvanen.

The work described in this thesis was done at European Molecular Biology Laboratory (EMBL) in Heidelberg. I was supported by an EMBL predoctoral fellowship and by a grant from the Academy of Finland.

SELOSTUS

Hepatiitti B virus-infektoituneet maksasolut erittävät verenkiertoon infektiivisten virusten lisäksi ei-infektiivisiä partikkeleita, jotka koostuvat viruksen pääasiallisesta vaippaproteiinista, hepatiitti B pinta-antigeenista (HBsAg) ja isäntäsoluperäisestä lipidistä. Näiden partikkelien rakenne on voimakkaasti stabiloitu disulfidi-ristisidoksilla. HBsAg-geenillä transfektoidut viljelmäsolut tuottavat ja erittävät HBsAg-partikkeleita, jotka ovat identtisiä potilasseerumeista eristettyjen partikkeleiden kanssa.

HBsAg-partikkeleiden on uskottu rakentuvan solun endoplasmakalvostossa (ER). Tämä rakentumismalli osoittautui kyseenalaiseksi muun muassa siksi, että ER sisältää runsaasti proteiinidisulfidi-isomeraasia (PDI), jonka läsnäollessa voimakkaasti ristisidottujen proteiinikompleksien muodostuminen vaikutti epätodennäköiseltä.

Tässä työssä HBsAg:n biosynteesiä tutkittiin transfektoidun solulinjan avulla. Kineettinen analyysi osoitti, että disulfidisidottuja dimeerejä alkoi muodostua polypeptiditranslaation kuluessa tai välittömästi sen jälkeen. Dimeerit lisääntyivät nopeasti, kunnes niiden pitoisuus saavutti maksiminsa noin tunnin kuluttua polypeptidisynteesistä, minkä jälkeen niiden pitoisuus aleni tasaisesti korkeampien oligomeerien muodostumisen myötä. Useat immunohistokemialliset ja biokemialliset kokeet osoittivat, että dimeerinen HBsAg kuljetetaan morfologisesti ja toiminnallisesti erilliseen solun osaan, sekretorisella reitillä ER:n ja Golgin laitteen välissä sijaitsevaan "väliosastoon". Tämä osasto ei sisällä ER:lle tai Golgille tyypillisiä proteiineja, eivätkä sitä rajoittavat kalvot ole jatkuvia ER:n kalvojen kanssa. Kahden väliosastoproteiinin paikantaminen tähän samaan osastoon vahvisti edellä mainitut tulokset.

HBsAg:n poistuminen ER:stä osoittautui partikkelien rakentumisen edellytykseksi. Lisäksi PDI hajoitti disulfidisidotut multimeerit dimeereiksi, osoittaen että PDI:n poissaolo HBsAg:n rakentumispaikalta saattaa olla tärkeä edellytys partikkelien muodostumiselle.

Elektronimikroskooppikuvien stereologinen analyysi osoitti tämän osaston olevan kooltaan merkittävän ja dynaamisen sekretorisen reitin osan.

REFERENCES

Aggerbeck, L. P. & Peterson, D. L. 1985: Electron microscopic and solution X-ray scattering observations on the structure of hepatitis B surface antigen. - Virology 141:155-161.

Alarcon, B., Berkhout, B., Breitmeyer, J. & Terhorst, C. 1988: Assembly of the human T cell receptor-CD3 complex takes place in the endoplasmic reticulum and involves intermediary complexes between the CD3-gamma, delta, epsilon core and single T cell receptor alpha or beta chains. - J. Biol. Chem. 263:2953-2961.

Bayer, M. E., Blumberg, B. S. & Werner, B. 1968: Particles associated with Australia antigen in the sera of patients with leukaemia, Down's Syndrome and hepatitis. - Nature 218:1057-1059.

Beasley, R. P. & Hwang, L.-Y. 1984: Epidemiology of hepatocellular carcinoma. - In: Vyas, G. H., J. L. Dienstag & J. H. Hoofnagle (eds.), Viral hepatitis and liver disease:209-224. Orlando.

Beasley, R. P., Hwang, L.-Y., Lin, C.-C. & Chien, C.-S. 1981: Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. - Lancet 2:1129-1132.

Beckman, R. P., Mizzen, L. A. & Welch, W. J. 1990: Interaction of Hsp70 with newly synthesized proteins: implications for protein folding and assembly. - Science 248:850-854.

Bergman, L. W. & Kuehl, W. M. 1979: Formation of an intrachain disulphide disulphide bond on nascent immunoglobulin light chains. - J. Biol. Chem. 254:8869-8876.

Bergman, L. W. & Kuehl, W. M. 1979: Formation of intermolecular disulfide bonds on nascent immunoglobulin polypeptides. - J. Biol. Chem. 254:5690-5694.

Blobel, G. & Dobberstein, B. 1975: Transfer of proteins across membranes. - J. Cell Biol. 67:852-862.

Bole, D. G., Hendershot, L. M. & Kearney, J. F. 1986: Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. - J. Cell Biol. 102:1558-1566.

Bonatti, S. G., Migliaccio, G. & Simons, K. 1989: Palmitylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. - J. Biol Chem. 264:12590-12595.

Bonifacino, J. S. & Lippincott-Schwartz, J. 1991: Degradation of proteins within the endoplasmic reticulum. - Curr. Opinion Cell Biol. 3:592-600.

Bonifacino, J. S., Lippincott-Schwartz, J., Chen, C., Antusch, D., Samelson, L. E. & Klausner R. D. 1988: Association and dissociation of the murine T cell receptor associated protein (TRAP): early events in the biosynthesis of a multisubunit receptor. - J. Biol. Chem. 263:8965-8971.

Braakman, I., Hoover-Litty, H.,Wagner, K. R. & Helenius, A. 1991: Folding of influenza hemagglutinin in the endoplasmic reticulum. - J. Cell Biol. 114:401-411.

Braakman, I., Helenius, J. & Helenius, A. 1992. Manipulating disulfide formation and protein folding in the endoplasmic reticulum. - EMBO J. 11:1717-1722.

Bruckner, P., Eikenberry, E. F. & Prockop, D. J. 1981: Formation of the triple helix of type I procollagen *in cellulo.* A kinetic model based on *cis-trans* isomerization of peptide bonds. - Eur. J. Biochem. 118:607-613.

Bruckner, P. & Eikenberry, E. F. 1984: Formation of the triple helix of type I procollagen *in cellulo.* Temperature-dependent kinetics support a model based on *cis-trans* isomerization of peptide bonds. - Eur. J. Biochem. 140:391-395.

Bruss, V. & Ganem, D. 1991: The role of envelope proteins in hepatitis B virus assembly. - Proc. Natl. Acad. Sci. U. S. A. 88:1059-1063.

Bulleid, N. J. & Freedman, R. B. 1988: Defective co-translational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes. - Nature 335:649-651.

Chavrier, P., Parton, R. G., Hauri, H.-P., Simons, K. & Zerial, M. 1990: Localization of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. - Cell 62:317-329.

Chege, N. W. & Pfeffer, S. R. 1990: Compartmentation of the Golgi complex: brefeldin-A distinguishes trans-Golgi cisternae from the trans-Golgi network. - J. Cell Biol. 111:893-899.

Chiang, H.-L., Terlecky, S. R., Plant, C. P. & Dice, J. F. 1989: A role a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. - Science 246:382-385.

Cossart, Y. E. & Keirnan, E. 1984: Hepadnaviruses of Australian animals. - In: Vyas, G. H., J. L. Dienstag & J. H. Hoofnagle (eds.), Viral hepatitis and liver disease:647. Orlando.

Cox, J. H., Yewdell, J. W., Eisenlohr, L. C., Johnson, P. R. & Bennink J. R. 1990: Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. - Science 247:715-718.

Creighton, T. E. 1984: Disulfide bond formation in proteins. - Methods Enzymol. 107:305-329.

Creighton, T. E. 1988: Disulfide bonds and protein stability. - BioEssays 8:57-63.

Creighton, T. E., Hillson, D. A. & Freedman R. B. 1980: Catalysis by protein-disulphide isomerase of the unfolding and refolding of proteins with disulphide bonds. - J. Mol. Biol. 142:43-62.

Crowley, C. W., Liu, C.-C. & Levinson, A. D. 1983: Plasmid directed synthesis of hepatitis B surface antigen in monkey cells. - Mol. Cell. Biol. 3:44-55.

Dane, D. S., Cameron, C. H. & Briggs, M. 1970: Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. - Lancet 1:695-698.

De Silva, A. M., Balch, W. E. & Helenius, A. 1990: Quality control in the endoplasmic reticulum: Folding and misfolding of vesicular stomatitis virus G protein in cells and in vitro. - J. Cell Biol. 111:857-866.

Delpeyroux, F., Chenciner, N., Lim, A., Malpiece, Y., Blondel, B., Crainic, R., van der Werf, S. & Streeck, R. E. 1986: A poliovirus neutralization epitope expressed on hybrid hepatitis B surface antigen particles. - Science 233:472-475.

Derynck, R., Jarret, J., Chen, E. & Goeddel, D. 1986: The murine transforming growth factor-β precursor. - J. Biol. Chem. 261:4377-4379.

Doms, R. W., Keller, D. S., Helenius, A. & Balch, W. E. 1987: Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. - J. Cell Biol 105:1957-1969.

Doms, R. W., Russ, G. & Yewdell, J. W. 1989: Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. - J. Cell Biol. 109:51-72.

Donaldson, J. G., Lippincott, S. J., Bloom, G. S., Kreis, T. E. & Klausner, R. D. 1990: Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. - J. Cell Biol. 111:2295-2306.

Donaldson, J. G., Lippincott-Schwartz, J. & Klausner, R. D. 1991: Guanine nucleotides modulate the effects of brefeldin A in semipermeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. - J, Cell Biol. 112:579-588.

Dorner, A. J., Bole, D. G. & Kaufman, R. J. 1987: The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. - J. Cell Biol. 107:89-99.

Dorner, A. J., Krane, M. G. & Kaufman, R. J. 1988: Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. - Mol. Cell. Biol. 8:4063-4070.

Dreesman, G. R., Hollinger, F. B., Suriano, J. R., Fujioka, R. S., Brunschwig, J. P. & Melnick, J. L. 1972: Biophysical and biochemical heterogeneity of purified hepatitis B antigen. - J. Virol. 10:469-476.

Dubois, M. F., Pourcel, C., Rousset, S., Chany, C. &Tiollais, P. 1980: Excretion of hepatitis B surface antigen from mouse cells transformed with cloned viral DNA. - Proc. Natl. Acad. Sci. U. S. A. 77:4549-4553.

Duden, R., Griffiths, G., Frank, R., Argos, P. & Kreis, T. E. 1991: β-COP, a 110 kD protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. - Cell 64:649-665.

Eble, B. E., Lingappa, V. R. & Ganem, D. 1986: Hepatitis B surface antigen: an unusual secreted protein initially synthesized as a transmembrane polypeptide. - Mol. Cell. Biol. 6:1454-1463.

Eble, B. E., MacRae, D. R., Lingappa, V. R. & Ganem, D. 1987: Multiple topogenic sequences determine the transmembrane orientation of hepatitis B surface antigen. - Mol. Cell. Biol. 7:3591-3601.

Ellis, R. J. & Hemmingsen, S. M. 1989: Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. - Trends Biochem. Sci. 14:339-342.

Ellis, R. J. & van der Vies, S. M. 1991: Molecular chaperones. - Annu. Rev. Biochem. 60:321-347.

Feitelson, M. A., Millman, I. & Blumberg, B. S. 1986: Tree squirrel hepatitis B virus: antigenic and structural characterization. - Proc. Natl. Acad. Sci. U. S. A. 83:2994-2997.

Freedman, R. B. 1984: Native disulphide band formation in protein biosynthesis: evidence for the role of protein disulphide isomerase. - Trends Biochem. Sci. 9:438-441.

Freedman, R. B. & Hillson, D. A. 1980: Formation of disulphide bonds. - In: Freedman, R. B. & H. C. Hawkins (eds.), The enzymology of post-translational modifications of proteins:157-212. London.

Fujiwara, T., Oda, K., Yokota, A., Takatsuki, A. & Ikehara, Y. 1988: Brefeldin A causes disassembly of the Golgi complex and accumulation of of secretory proteins in the endoplasmic reticulum. - J. Biol. Chem. 265:18545-18552.

Galibert, F., Chen, T. N. & Mandart, E. 1982: Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence. - J. Virol. 41:51-65.

Ganem, D. 1991: Assembly of hepadnaviral virions and subviral particles. - Curr. Topics Microbiol. Immunol. 168:61-83.

Ganem, D. & Varmus, H. E. 1987: The molecular biology of the hepatitis B viruses. - Annu. Rev. Biochem. 56:651-693.

Gatenby, A. A. & Ellis, R. J. 1990: Chaperone function: the assembly of ribulose bisphophate carboxylase-oxygenase. - Annu. Rev. Cell Biol. 6:125-149.

Gavilanes, F., Gonzales-Ros, J. M. & Peterson, D. L. 1982: Structure of hepatitis B surface antigen. - J. Biol. Chem. 257:7770-7777.

Gerber, M. A., Hadziyannis, S., Vissoulis, C., Schaffner, F., Paronetto, F. & Popper, F. 1974: Electron microscopy and immuno-electron microscopy of cytoplasmic hepatitis B antigen in hepatocytes. - Am. J. Pathol. 75:489-502.

Gerber, M. A., Schaffner, F. & Paronetto, F. 1972: Immuno-electron microscopy of hepatitis B antigen in liver. 1. - Proc. Soc. Exp. Biol. Med. 140:1334-1339.

Gething, M.-J., McGammon, K. & Sambrook, 1986: Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. - Cell 46:939-950.

Gething, M.-J. & Sambrook, J. 1992: Protein folding in the cell. - Nature 355:33-45.

Geuze, H. J., Slot, J. W.,van der Ley, P. A. & Scheffer, R. C. T. 1981: Use of colloidal gold particles in double-labeling immunoelectron microscopy on ultrathin frozen sections. - J. Cell Biol. 89:653-665.

Gibson, R., Schlesinger, S. & Kornfeld, S. 1979: The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature sensitive and undergoes intracellular aggregation at elevated temperatures. - J. Biol. Chem. 254:3600-3007.

Gilbert, H. 1989: Catalysis of thiol/disulfide exchange: single-turnover reduction of protein disulfide-isomerase by glutathione and catalysis of peptide disulfide reduction. - Biochemistry 28:7298-7305.

Gray, A., Dull, T. A. & Ullrich, A. 1983: Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. - Nature 303:722-725.

Green, J., Griffiths, G., Louvard, D., Quinn, P. & Warren, G. 1981: Passage of viral membrane proteins through the Golgi complex. - J. Mol. Biol. 152:663-698.

Griffiths, G., McDowall, A., Back, R. & Dubochet, J. 1984: On the preparation of cryosections for immunocytochemistry. - J. Ultrastruct. Res. 89:65-78.

Griffiths, G. & Simons, K. 1986: The trans Golgi network: sorting at the exit site of the Golgi complex. - Science 234:438-443.

Hauri, H.-P. & Schweizer, A. 1992: The endoplasmic reticulum-Golgi intermediate compartment. - Curr. Opinion Cell Biol. 4:600-608.

Hitzeman, R. A., Chen, C. Y., Hagie, F. E., Patzer, E. J., Liu, C.-C., Estell,D. A, Miller J. V., Yaffe, A., Kleid, D. G., Levinson , A. D.& Opperman, H. 1983: Expression of hepatitis B virus surface antigen in yeast. - Nucl. Acid Res. 11:2745-2763.

Hobman, T. C., Woodward. L. & Farquhar, M. G. 1992: The rubella virus E1 glycoprotein is arrested in a novel post-ER, pre-Golgi compartment. - J. Cell Biol. 118:795-811.

Hu, C. H. & Tsou, C. L. 1991: Formation of enzyme-substrate disulfide linkage during catalysis by protein disulfide isomerase. - FEBS Lett. 290:87-89.

Hunziker, W., Whitney, J. A. & Mellman, I. 1991: Selective inhibition of transcytosis by brefeldin A in MDCK cells. - Cell 67:617-27.

Hurtley, S. M., Bole, D. G.,Hoover-Litty, H., Helenius, A. & Copeland, C. S. 1989: Interactions of misfolded influenza hemagglutinin with binding protein (BiP). - J. Cell Biol. 108:2117-2126.

Hurtley, S. M. & Helenius, A. 1989: Protein oligomerization in the endoplasmic reticulum. - Annu. Rev. Cell Biol. 5:277-307.

Huttner, W. B. & Tooze, S. A. 1989: Biosynthetic protein transport in the secretory pathway. - Curr. Opinion Cell Biol. 1:648-654.

Imai, M., Gotoh, K., Nishioka, K., Kurashina, S., Miyakawa, Y. & Mayumi, M. 1974: Antigenicity of reduced and alkylated Australia antigen. - J. Immunol. 112:416-419.

Ivessa, N. E., De Lemos-Chiarandini, C., Tsao, Y. S., Takatsuki, A., Adesnik, M., Sabatini, D. D. & Kreibich, G. 1992: O-glycosylation of intact and truncated ribophorins in brefeldin A-treated cells: newly synthesized ribophorins are only transiently accessible to the relocated glycosyltransferases. - J. Cell Biol. 117:949-958.

Kassenbrock, C. K., Carcia, P. D., Walter, P. & Kelly, R. B. 1988: Heavy chain binding protein recognizes aberrant polypeptides translocated *in vitro*. - Nature 333:90-93.

Katz, B. A. & Kossiakoff, A. A. 1986: The crystallographically determined structures of atypical strained disulfides engineered into subtilisin. - J. Biol. Chem. 261:15480-15485.

Kelly, R. B. 1985: Pathways of protein secretion in eukaryotes. - Science 230:25-32.

Kim, C. Y. & Tilles, J. G. 1973: Purification and biophysical characterization of hepatitis B antigen. - J. Clin. Invest. 52:1176-1186.

Kim, P. S. & Arvan, P. 1991: Folding and assembly of newly synthesized thyroglobulin occurs in a pre-Golgi compartment. - J. Biol. Chem. 266:12412-12418.

Klausner, R. D. & Sitia, R. 1990: Protein degradation in the endoplasmic reticulum. - Cell 62:611-614.

Koch, G. L. E., Macer, D. R. J. & Wooding, F. B. P. 1988: Endoplasmin is reticuloplasmin. - J. Cell Sci. 90:485-491.

Kornfeld, R. & Kornfeld, S. 1985: Assembly of asparige-linked oligosaccharides. - Annu. Rev. Biochem. 54:631-644.

Kornfeld, S. & Mellman, I. 1989: The biogenesis of lysosomes. - Annu. Rev. Cell Biol. 5:483-525.

Kozutsumi, Y., Segal, M., Normington, K., Gething, M.-J. & Sambrook, J. 1988: The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-related proteins. - Nature 332:462-464.

Kuroda, S., Otaka, S., Miyazaki, T., Nakao, M. & Fujisawa, Y. 1991: Hepatitis B virus envelope L protein particles: Synthesis and assembly in *Saccharomyces cerevisiae*, purification and characterization. - J. Biol. Chem. 267:1953-1961.

Laub, O., Rall, L. B., Truett, M., Shaul, Y., Standring, D. N., Valenzuela, P. & Rutter, W. J. 1983: Synthesis of hepatitis B surface antigen in mammamlian cells: expression of the entire gene and the coding region. - J. Virol. 48:271-280.

Lazzarino, D. A. & Gabel, C. A. 1988: Biosynthesis of the mannose-6-phosphate recognition marker in transport-impaired mouse lymphoma cells: demonstration of a two-step phosphorylation. - J. Biol. Chem. 263:10118-10126.

Lee, D., Rose, T., Webb, N. & Todaro, G. J. 1985: Cloning and sequence analysis of a cDNA for rat transforming growth factor alpha. - Nature 313:489-491.

Lewis, M. J., Mazzarella, R. A. & Green, M. 1985: Structure and assemnbly of endoplasmic reticulum. - J. Biol. Chem. 260:3050-3057.

Lippincott-Schwartz, J., Bonifacino, J. S., Yuan, L. & Klausner, R. D. 1988: Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. - Cell 54:209-229.

Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H., Yuan, P. L. C. & Klausner, R. D. 1990: Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. - Cell 60:821-836.

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. & Klausner, R. D. 1991: Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. - Cell 67:601-616.

Liu, C.-C., Yansura, D. & Levinson, A. D. 1982: Direct expression of hepatitis B surface antigen in monkey cells from an SV40 vector. - DNA 1:213-221.

Lodish, H. F., Kong, N., Hirani, S. & Rasmussen, J. 1987: A vesicular intermediate in the transport of hepatoma secretory proteins from the rough ER to the Golgi complex. - J. Cell Biol. 104:221-230.

Macer, D. R. J. & Koch, G. L. E. 1988: Identification of a set of calcium binding proteins in reticuloplasm, the luminal content of the endoplasmic reticulum. - J. Cell Sci. 91:61-70.

Machamer, C. E., Doms, R. W., Bole, D. G., Helenius, A. & Rose, J. K. 1990: Heavy chain binding protein recognizes incompletely disulfide-bonded forms of vesicular stomatitis virus G protein. - J. Biol. Chem. 265:6879-6883.

Mandart, E., Kay, A. & Galibert, F. 1984: Nucleotide sequence of cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. - J. Virol. 49:782-792.

Marion, P. L., Oshiro, L. S., Regnery, D. C., Scullard, G. H. & Robinson W. S. 1980: A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. - Proc. Natl. Acad. Sci. U. S. A. 77:2941-2945.

Marquardt, T. & Helenius, A. 1992: Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. - J. Cell Biol. 117:503-513.

Masaki, R., Yamamoto, A. & Tashiro, Y. 1987: Cytochrome P-450 and NADPH-cytochrome P-450 reductase are degraded in the autolysosomes in rat liver. - J. Cell Biol. 104:1207-1215.

Mason, W. S., Seal, G. & Summers, J. 1980: Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. - J. Virol. 36:829-836.

Mazzarella, R. A. & Green, M. 1987: ERp99, an abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90-kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (GRP94). - J. Biol. Chem. 260:6926-6931.

McAleer, W. J., Buynak, E. B., Maigetter, R. Z., Wampler, D. E., Miller, W. J. & Hilleman, M. R. 1984: Human hepatitis B vaccine from recombinant yeast. - Nature 307:178-180.

Mellman, I. & Simons, K. 1992: The Golgi complex: In vitro veritas? - Cell 68:829-840.

Melnick, J. R., Aviel, S. & Argon, Y. 1991: A 100 kD protein is associated in the endoplasmic reticulum with both BiP/GRP78 and newly-synthesized proteins. - J. Cell Biol. 115:1a.

Michel, M.-L., Mancini, E., Sobczak, E., Favier, V., Guétard, D., Bahraoui, E.-M. & Tiollais, P. 1988: Induction of anti-human immunodeficiency virus (HIV) neutralizing antibodies in rabbits immunized with recombinant HIV-hepatitis B surface

antigen particles.
- Proc. Natl. Acad. Sci. U. S. A. 85:7957-7961.

Michel, M.-L., Mancini, M., Riviere, Y., Dormont, D. & Tiollais, P. 1990: T- and B-lymphocyte responses to human immunodefiency virus (HIV) type 1 in macaques immunized with hybrid HIV/hepatitis B surface antigen particles.
- J. Virol. 64:2452-2455.

Mishiro, S., Mitsunobu, I., Takahashi, K., Machida, A., Gotanda, T., Miyakawa, Y. & Mayumi, M. 1980: A 49,000-dalton polypeptide bearing all antigenic determinants and full immunogenicity of 22-nm hepatitis B surface antigen particles. - J. Immunol. 124:1589-1593.

Moriarty, A. M., Hoyer, B. H., Shih, J. W.-K., Gerin, J. L. & Hamer, D. H. 1981: Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector.
- Proc. Natl. Acad. Sci. U. S. A. 78:2606-2610.

Morré, D. J., Minnifield, N. & Paulik, M. 1989: Identification of the 16° compartment of the endoplasmic reticulum in rat liver and cultured hamster kidney cells. - Biol. Cell 67:51-60.

Mostov, K. & Blobel, G. 1982: A transmembrane precursor of secretory component, the receptor for intracellular of polymeric immunoglobulin. - J. Biol. Chem. 257:11816-11821.

Munro, S. & Pelham, H. R. B. 1987: A C-terminal signal prevents the secretion of luminal ER proteins. - Cell 48:899-907.

Narula N., McMorrow, I., Plopper, G., Doherty, J., Matlin, K. S., Burke, B. & Stow, J. L. 1992: Identification of a 200-kD, brefeldin-sensitive protein on Golgi membranes.
- J. Cell Biol. 117:27-38.

Ng, D. T. W., Randall, R. E. & Lamb, R. A. 1989: Intracellular maturation and transport of the SV5 type II glycoprotein hemagglutinin-neuraminidase: specific and transient association with GRP78-BiP in the endoplasmic reticulum and extensive internalization from the cell surface.
- J. Cell. Biol. 109:3273-3289.

Nuchtern, J. G., Bonifacino, J. S., Biddison,W. E. & Klausner, R. D. 1989: Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. - Nature 339:223-226.

Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J. G., Lippincott,-Schwartz J., Klausner, R. D & J. E. Rothman. 1991: Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae.
- Cell 64:1183-1195.

Palade, G. E. 1956: Intracisternal granules in the exocrine cells of the pancreas. - J. Biophys. Biochem. Cytol. 2:417-422.

Palade, G. E. 1975: Intracellular aspects of the process of protein secretion. - Science 189:347-358.

Patzer, E. J., Nakamura G. R., Simonsen, C. C., Levinson, A. D. & Brands, R. 1986: Intracellular assembly and packaging of hepatitis B surface antigen particles occur in the endoplasmic reticulum. - J. Virol. 58:884-892.

Patzer, E. J., Nakamura, G. R. & Yaffe, A. 1984: Intracellular transport and secretion of hepatitis B surface antigen in mammalian cells. - J. Virol. 51:346-353.

Paulson, J. C. 1989: Glycoproteins: what are the sugar chains for? - Trends Biochem. Sci. 14:272-275.

Pelham, H. R. B. 1989: Control of protein exit from the endoplasmic reticulum. - Annu. Rev. Cell Biol. 5:1-23.

Pelham, H. R. 1990: The retention signal for soluble proteins of the endoplasmic reticulum. - Trends Biochem. Sci. 15:483-486.

Pelham, H. R. B. 1988: Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. - EMBO J. 7:913-918.

Persing, D. H., Varmus, H. E. & Ganem, D. 1985: A frameshift mutation in the preS region of the human hepatitis B virus genome allows production of surface antigen particles but eliminates binding to polymerized albumin. - Proc. Natl. Acad. Sci. U. S. A. 82:3440-3444.

Persing, D. H., Varmus, H. E. & Ganem, D. 1986: Inhibition of the secretion of hepatitis B surface antigen by a related presurface polypeptide. - Science 234:1388-1391.

Peters, T. J. & Davidson, L. K. 1982: The biosynthesis of rat serum albumin. In vivo studies on the formation of the disulfide bonds. - J. Biol. Chem. 257:8847-8853.

Peters, T. J. & Reed, R. G. 1980: The biosynthesis of rat serum albumin. Composition and properties of the intracellular precursor, proalbumin. - J. Biol. Chem. 255:3156-3163.

Peterson, D. L. 1981: Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. - J. Biol. Chem. 256:6975-6983.

Peterson, D. L. 1987: The structure of hepatitis B surface antigen and its antigenic sites. - BioEssays 6:258-262.

Peterson, D. L., Gavilanes, F., Paul, D. A. & Achord, D. T. 1987: Hepatitis B surface antigen: protein structure and development of alternative hepatitis B virus vaccines. - In: Chisari, F. V. (ed.), Advances in hepatitis research:30-39. New York.

Pfeffer, S. R. & Rothman, J. E. 1987: Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. - Annu. Rev. Biochem. 56:829-852.

Price, E. R., Zudowsky, L. D., Baker, C. H. & McKeon, F. D. 1991: Human cyclophilin B: a second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. - Proc. Natl. Acad. Sci. U. S. A. 88:1903-1907.

Rizzolo, L. J., Finidori, J., Gonzales, A., Arpin, M., Ivanov, I. E., Adesik, M. & Sabatini, D. D. 1985: Biosynthesis and intracellular sorting of growth hormone-viral envelope protein hybrids. - J. Cell Biol. 101:1351-1362.

Rizzolo, L. J. & Kornfeld, R. 1988: Post-translational protein modification in the endoplasmic reticulum: Demonstration of fatty acylase and deoxymannojirimycin-sensitive α-mannosidase activities. - J. Biol. Chem. 263:9520-9525.

Robinson, M. S. & Kreis, T. E. 1992: Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. - Cell 69:129-138.

Robinson, W. S. & Lutwick, L. I. 1976: The virus of hepatitis, type B (first of two parts). - N. Engl. J. Med. 295:1168-1175.

Rogler, C. E. 1991: Cellular and molecular mechanisms of hepatocarcinogenesis associated with hepadnavirus infection. - Curr. Topics Microbiol. Immunol. 168:103-140.

Rose, J. K. & Doms, R. W. 1988: Regulation of protein export from the endoplasmic reticulum. - Annu. Rev. Cell Biol. 4:257-288.

Rothman, J. E. 1989: Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. - Cell 59:591-601.

Rothman, J. E. & Orci, L. 1992: Molecular dissection of the secretory pathway. - Nature 355:409-415.

Rutgers, T., Gordon, D., Gathoye, A.-M., Hollingdale, M., Hockmeyer, W., Rosenberg, M. & De Wilde, M. 1988: Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circumsporozoite protein of Plasmodium falciparum. - Bio/Technology 6:1065-1070.

Saraste, J. & Kuismanen, E. 1984: Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. - Cell 38:535-549.

Saraste, J. & Svensson, K. 1991: Distribution of the intermediate elements operating in ER to Golgi transport. - J. Cell Sci. 100:415-430.

Schmid, F. X. 1991: Catalysis and assistance of protein folding. - Curr. Topics Struct. Biol. 1:36-41.

Schweitzer, A., Fransen, J., Bächi, T., Ginsel, L. & Hauri, H.-P. 1988: Identification, by a monoclonal antibody, of a 53 kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. - J. Cell Biol. 107:1643-1653.

Schweitzer, A., Fransen, J. A. M., Matter, K., Kreis, T. E., Ginsel, L. & Hauri,H.-P. 1990: Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. - Eur. J. Cell Biol. 53:185-196.

Schweitzer, A., Griffiths, G., Bächi, T. & Hauri, H.-P. 1991b: Organization of the exocytic pathway from ER to Golgi. - J. Cell Biol. 115:65a.

Schweitzer, A., Matter, K., Ketcham, C. M. & Hauri, H.-P. 1991a: The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and *cis*-Golgi. - J. Cell Biol. 113:45-54.

Scott, J., Urdea, M., Quiroga, M., Sanchez-Pescador, R., Fong, N., Selby, M., Rutter, W. & Bell, G. 1983: Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. - Science 221:236-240.

Seeger, C., Ganem, D., Varmus, H. 1984: Nucleotide sequence of an infectous molecularly cloned genome of ground squirrel hepatitis virus. J. Virol. 51: 367-375.

Semenza, J. C., Hardwick, K. G., Dean, N. & Pelham, H. R. 1990: ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. - Cell 61:1349-1357.

Sheffield, W. P., Shore, G. C. & Randall, S. K. 1990: Mitochondrial precursor protein. Effects of 70-kilodalton heat shock protein on polypeptide folding, aggregation and import competence. - J. Biol. Chem. 265:11069-11076.

Sherker, A. H. & Marion, P. L. 1991: Hepadnaviruses and hepatocellular carcinoma. - Annu. Rev. Microbiol. 45:475-508.

Shibayama, T., Watanabe, T., Kojima, H., Yoshikawa, A., Watanabe, S., Kamimura, T., Suzuki, S. & Ichida, F. 1984: Studies by immune electron microscopy of hepatitis B surface antigen in PLC/PRF/5 cells. - J. Med. Virol. 13:205-214.

Simon, K., Lingappa, V. R. & Ganem, D. 1988: A block to intracellular transport and assembly of hepatitis B surface antigen polypeptides in *Xenopus* oocytes. - Virology 166:76-81.

Simon, K., Lingappa, V. R. & Ganem, D. 1988: Secreted hepatitis B surface antigen polypeptides are derived from a transmembrane precursor. - J. Cell Biol. 107:2163-2168.

Sitia, R., Neuberger, M., Alberini, C., Bet, P., Fra, A., Valetti, C., Williams, G. & Milstein, C. 1990: Developmental regulation of gM secretion: the role of the carboxy-terminal cysteine. - Cell 60:781-790.

Slagle, B. L., Lee, T.-H. & Butel, J. S. 1992: Hepatitis B virus and hepatocellular carcinoma. - Prog. Med. Virol. 39:167-203.

Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. & James, D. E. 1991: Immuno-localization of the rat insulin regulatable glucose transporter in brown adipose tissue of the rat. - J. Cell Biol. 113:123-135.

Sprengel, R., Kaleta, E. F. & Will, H. 1988: Isolation and characterization of a hepatitis B virus endemic in herons. - J. Virol. 62:3832-3839.

Stamnes, M. A., Shieh, B.-H., Chuman, L., Harris, G. L. & Zuker, C. S. 1991: The cyclophilin homolog ninaA is a tissue-specific integral membrane required for the proper synthesis of a subset of Drosophila rhodopsins. - Cell 65:219-227.

Stewart, D. E., Sarkar, A. & Wampler, J. E. 1990: Occurrence and role of cis-peptide bonds in protein structures. - J. Mol. Biol. 214:253-260.

Sukeno, N., Shirachi, R., Yamaguchi, J. & Ishida, N. 1972: Reduction and reoxidation of Australia antigen: loss and reconstitution of particle structure and antigenicity. - J. Virol. 9:182-183.

Summers, J., Smolec, J. M. & Snyder, R. 1978: A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. - Proc. Natl. Acad. Sci. U. S. A. 75:4533-4537.

Szmuness, W., Stevens, C. E., Harley, E. J., Zang, E. A., Oleszko, W. R., William, D. C., Sadovsky, R. & Morrison, J. M. 1980: Hepatitis B vaccine. - N. Engl. J. Med. 303:833-841.

Takatsuki, A. & Tamura, G. 1985: Brefeldin A, a specific inhibitor of intracellular translocation of vesicular stomatitis virus G protein: Intracellular accumulation of high mannose type einn and inhibition of its cell surface expression. - Agric. Biol. Chem. 45:899-902.

Tartakoff, A. M. 1986: Temperature and energy dependence of the secretory protein transport in the exocrine pancreas. - EMBO J. 5:1477-1482.

Tiollais, P. & Buendia, M. A. 1991: Hepatitis B virus. - Sci. Am. 264:116-123.

Tiollais, P., Pourcel, C. & Dejean, A. 1985: The hepatitis B virus. - Nature 317:489-495.

Tooze, J., Kern, H., Fuller, S. D. & Howell, K. E. 1989: Condensation-sorting event in the rough endoplasmic reticulum of exocrine pancreatic cells. - J. Cell Biol. 109:35-50.

Tooze, S. A., Tooze, J. & Warren, G. 1988: Site of addition of N-acetylgalactosamine to the E1 glycoprotein of a mouse hepatitis virus-A59. - J. Cell Biol. 106:1475-1487.

Tsao, Y. S., Ivessa, N. E., Adesnik, M., Sabatini, D. D. & Kreibich, G. 1992: Carboxy terminally truncated forms of ribophorin I are degraded in pre-Golgi compartments by a calsium dependent process. - J. Cell Biol. 116:57-67.

Valenzuela, P., Gray, P., Quiroga, M., Zaldivar, J., Goodman, H. M. & Rutter,W. J. 1979: Nucleotide sequence of the gene coding for the major protein of hepatitis B surface antigen. - Nature 280:815-819.

Valenzuela, P., Medina, A. & Rutter, W. J. 1982: Synthesis and assembly of hepatitis B surface antigen particles in yeast. - Nature 298:347-351.

Vaux, D., Tooze, J. & Fuller, S. 1990: Identification by anti-idiotype antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal. - Nature 345:495-502.

Veber, D. F., Holly, F. W., Nutt, R. F., Bergstrand, S. J., Brady, S. F., Hirschmann, R., Glitzer, M. S. & Saperstein, R. 1979: Highly active cyclic and bicyclic somatostatin analogues of reduced ring size. - Nature 280:512-514.

Vyas, G. N., Rao, K. R. & Ibrahim, A. B. 1972: Australia antigen (hepatitis B antigen): a conformational antigen dependent on disulfide bonds. - Science 178:1300-1301.

Wagner, D. D. 1990: Cell biology of von Willebrand factor. - Annu. Rev. Cell Biol. 6:217-246.

Wampler, D. E., Lehman, E. D., Boger, J., McAleer, W. J. & Scolnick, E. M.. 1985: Multiple chemical forms of hepatitis B surface antigen produced in yeast. - Proc. Natl. Acad. Sci. U. S. A. 82:6830-6834.

Warren, G. 1987: Signals and salvage sequences. - Nature 327:17-18.

Waters, M. G., Serafini, T. & Rothman, J. E. 1991: "Coatomer": a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. - Nature 349:248-251.

Weibel, E. W. 1979: Stereological methods. Practical methods for biological morphometry. - 415 pp. Orlando.

Wetzel, R. 1987: Harnessing disulfide bonds using protein engineering. - Trends Biochem. Sci. 12:478-482.

Wood, S. A., Park, J. E. & Brown, W. J. 1991: Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosomes. - Cell 67:591-600.

Yewdell, J. W. & Bennink, J. R. 1989: Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. - Science 244:715-718.

I

HEPATITIS B SURFACE ANTIGEN ASSEMBLES IN A POST-ER, PRE-GOLGI COMPARTMENT

Journal of Cell Biology 118:1305-1320, 1992

b y

Ari Huovila, Astrid Eder & Stephen Fuller

https://doi.org/10.1083/jcb .118.6.1305

Reproduced from the Journal of Cell Biology (118:1305-1320, 1992)
by copyright permission of the Rockefeller University Press

II

AN ER-GOLGI INTERMEDIATE COMPARTMENT IS NOT CONTINUOUS WITH ENDOPLASMIC RETICULUM

(Submitted)

b y

Ari Huovila & Stephen Fuller