

# Hepatitis B Surface Antigen Assembles in a Post-ER, Pre-Golgi Compartment

Ari-Pekka J. Huovila, Astrid M. Eder, and Stephen D. Fuller

Biological Structures and Biocomputing Programme, European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

**Abstract.** Expression of hepatitis B surface antigen (HBsAg), the major envelope protein of the virus, in the absence of other viral proteins leads to its secretion as oligomers in the form of disk-like or tubular lipoprotein particles. The observation that these lipoprotein particles are heavily disulphide crosslinked is paradoxical since HBsAg assembly is classically believed to occur in the ER, and hence in the presence of high levels of protein disulphide isomerase (PDI) which should resolve these higher intermolecular crosslinks. Indeed, incubation of mature, highly disulphide crosslinked HBsAg with recombinant PDI causes the disassembly of HBsAg to dimers. We have used antibodies against resident ER proteins in double immunofluorescence studies to study the stages of the conversion of the HBsAg from individual protein subunits to the secreted, crosslinked, oligomer. We show that HBsAg is rapidly sorted to a post-ER, pre-Golgi compartment which excludes PDI and other major soluble resident ER proteins although it overlaps

with the distribution of rab2, an established marker of an intermediate compartment. Kinetic studies showed that disulphide-linked HBsAg dimers began to form during a short (2 min) pulse, increased in concentration to peak at 60 min, and then decreased as the dimers were crosslinked to form higher oligomers. These higher oligomers are the latest identifiable intracellular form of HBsAg before its secretion ( $t_{1/2} = 2$  h). Brefeldin A treatment does not alter the localization of HBsAg in this PDI excluding compartment, however, it blocks the formation of new oligomers causing the accumulation of dimeric HBsAg. Hence this oligomerization must occur in a pre-Golgi compartment. These data support a model in which rapid dimer formation, catalyzed by PDI, occurs in the ER, and is followed by transport of dimers to a pre-Golgi compartment where the absence of PDI and a different luminal environment allow the assembly process to be completed.

ENVELOPED animal viruses have been major tools in the characterization of the secretory pathway and in advancing our understanding of the mechanisms of protein sorting. Frequently, particular features of the pathways of protein traffic are exploited in the maturation pathways of different viruses so that a study of virus assembly leads to unexpected rewards in terms of an understanding of intracellular transport. This paper presents a study of the assembly of the lipoprotein particle formed by the transmembrane glycoprotein of hepatitis B virus. This particle has the unusual feature of being stabilized by extensive intermolecular crosslinks and here we show that this reflects its assembly in an intermediate compartment between the ER and the Golgi.

Hepatitis B virus (HBV)<sup>1</sup> is a small (440 Å) DNA virus

1. *Abbreviations used in this paper:* BfA, Brefeldin A; BiP, Ig heavy chain binding protein; HBsAg, hepatitis B surface antigen protein; HBV, hepatitis B virus; PDI, protein disulphide isomerase; Sf9, a clonal isolate of *Spodoptera frugiperda* IPLB-Sf21-AE cells; anti-KX<sub>5</sub>KDEL a polyclonal antibody developed in rabbits against a mixture of peptides with the sequences KXXXXXXKDEL where X indicates a position filled by a mixture of alanine, aspartic acid, histidine, glutamine, leucine, tyrosine, and lysine.

which is the type virus of the hepadnaviridae (hepatotropic DNA viruses). HBV consists of a nucleoprotein core containing a relatively small DNA genome (3,200 bp) surrounded by a protein capsid composed of the core protein, hepatitis B core antigen. The nucleocapsid is surrounded by an envelope in which the major protein component is the 226 amino acid hepatitis B surface antigen (HBsAg) which exists in nonglycosylated 24-kD (P24) and in N-glycosylated 27-kD (GP27) forms. In addition to being an envelope protein of the infectious virion, HBsAg is assembled into coreless, lipoprotein particles that are secreted from HBV infected cells and form the bulk of HBsAg in the circulation. These noninfectious lipoprotein particles occur in two morphological forms: 22-nm diameter particles which appear as spherical when negatively stained for EM and filamentous particles of the same diameter and variable length (Bayer et al., 1968; Robinson and Lutwick, 1976; Dane et al., 1970). The "spherical" forms appear disk-like when visualized by cryoelectron microscopy and could represent fragments of the tubular forms (Huovila, A.-P. J., and S. D. Fuller, manuscript in preparation).

The round 22-nm HBsAg particles isolated from the sera

of HBV infected patients are complex macromolecular assemblies with a molecular weight between 2 and  $4 \times 10^6$  (Dreesman et al., 1972; Kim and Tilles, 1973), containing ~75% protein, 25% lipid, and N-linked carbohydrate (Gavilanes et al., 1981; Peterson, 1987). One lipoprotein particle contains ~100 HBsAg polypeptide monomers (Aggerbeck and Peterson, 1985; Ganem and Varmus, 1987; Peterson, 1987). HBsAg particles are unique among lipoproteins in that their structure is stabilized by extensive intermolecular disulphide crosslinking (Sukeno et al., 1972; Vyas et al., 1972; Imai et al., 1974; Peterson, 1987). It is the site of formation of these extensive crosslinks which is the subject of this paper.

Previous studies have provided much information on the biogenesis and the secretion of HBsAg. Eble et al. (1986) demonstrated that in a cell-free translation system HBsAg is initially synthesized as a transmembrane polypeptide that spans the lipid bilayer at least twice. Cultured cells which are transfected with the coding region for HBsAg secrete 22-nm particles that are morphologically and antigenically indistinguishable from those in serum (Crowley et al., 1983; Dubois et al., 1980; Liu et al., 1982; Moriarty et al., 1982; Patzer et al., 1984, 1986; Simon et al., 1988). Using such a system, Simon et al. (1988) showed that HBsAg becomes carbonate extractable with a very similar half-time to that of particle secretion. This indicated that the rate-limiting step in the export process is the budding of the lipoprotein particles from the membrane. All of the oligosaccharides on the secreted HBsAg lipoprotein particles are of the N-linked complex type. However Patzer et al. (1984) showed that all of the glycans on cellular HBsAg were of the high mannose type. Hence, the rate limiting step in secretion must lie prior to the medial Golgi. EM of HBsAg in infected or in stably transfected cells reveals particles within the lumen of smooth intracellular membranes (Gerber et al., 1974; Shibayama et al., 1984; Patzer et al., 1986). These regions have been interpreted as elements of the ER and contributed to a consensus view that particle assembly in the membrane and budding which is the rate limiting step in lipoprotein secretion, occurs in the ER (reviewed in Ganem and Varmus, 1987).

The model that the assembly of HBsAg occurs in the ER has been based on morphological and immunocytochemical observations and on the assumption that endoglycosidase H sensitivity is restricted to this compartment. Unfortunately, specific markers for the ER have not been used in previous studies and hence the possibility that assembly may take place in an intermediate compartment between the ER and the Golgi has not been addressed. There is biochemical evidence that N-linked oligosaccharides on glycoproteins are still sensitive to endoglycosidase H digestion in such a compartment (Pelham, 1988; Schweizer et al., 1990). It has also been suggested that oligomerization of some viral proteins may indeed occur in this compartment (Schweizer et al., 1990; Tooze et al., 1988).

We felt that the consensus view led to a paradox since it posits that the complex disulphide crosslinks which stabilize the secreted particle are formed in the ER and hence in the presence of high levels of PDI. The current model for intracellular disulphide bond formation is based on studies of intramolecular disulphide pairing in soluble monomeric proteins (Freedman and Hillson, 1980; Creighton, 1984; Freedman, 1984). The rate limiting step in the folding of

many disulphide containing proteins is the exchange of disulphide bonds to generate the final, lowest free energy arrangement of the native state. Protein disulphide isomerase (PDI), an abundant, soluble resident protein of the ER, accelerates this process by catalyzing disulphide bond exchange and so causes dissociation of disulphide-linked aggregates and allows refolding of proteins to their native, properly disulphide-linked conformation. Two classes of disulphide bonds can be distinguished operationally in our studies. The first class comprises the crosslinks within an HBsAg dimer. We hypothesized that the formation of these bonds would be promoted by PDI since they are analogous to the bonds linking small oligomers such as influenza haemagglutinin. We shall refer to this class as dimer crosslinks. The second set of crosslinks are those that bind dimers together to form the highly crosslinked oligomer. We felt that it was unlikely that this second class would be stable in the presence of PDI since they resemble the aggregated crosslinks resolved by this enzyme. The formation of these oligomer crosslinks cannot be completed without particle assembly since they stabilize the oligomeric lipoprotein particle.

Immunofluorescence and pulse-chase studies were performed in a mouse L cell line stably expressing HBsAg and no other viral proteins to localize the steps in the formation of these disulphide bonds. We show here that HBsAg is transported to a compartment which is intermediate between the ER and the Golgi and which excludes the major resident soluble ER proteins such as PDI but overlaps with rab2, a marker of an intermediate compartment. We also show that the formation of the dimer crosslinks is completed in the ER while the formation of oligomer crosslinks occurs after exit from the ER and coincides with the budding of particles into the lumen of this intermediate compartment. We also demonstrate directly that the two classes of disulphide crosslinks have different energies since incubation of isolated, highly crosslinked particles with PDI causes the conversion of oligomers to dimers.

## Materials and Methods

### Cells and Reagents

The stably transfected mouse L fibroblast cell line SV24, expressing the short form (S) of hepatitis B surface antigen, subtype adw, under the control of the SV-40 early promoter (Simon et al., 1988), was a kind gift from Dr. Don Ganem (University of California, San Francisco, CA). The cells were grown in DME containing 2.2 g/liter sodium bicarbonate, 10% (vol/vol) FCS, 10 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a standard cell incubator at 37°C in 5% CO<sub>2</sub> atmosphere. Rat PDI-cDNA was obtained from Dr. George Banting (Bristol University, UK). Pansorbin and polyclonal rabbit antiserum against human HBsAg were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Affinity-purified rabbit antibody against the COOH-terminus of rab2 (Chavrier et al., 1990) was kindly provided by Dr. Marino Zerial (EMBL, Heidelberg, Germany). Sheep antibody against human HBsAg (#61WG20, Behringwerke, Marburg, Germany) was a generous gift from Dr. Michael Nassal (Zentrum für Molekularbiologie, Heidelberg, Germany) and it was used with permission from Behringwerke. Rhodamine-conjugated goat-anti-rabbit-IgG antibodies were from Jackson Immunoresearch Laboratories Inc. (West Grove, PA) and from Southern Biotechnology Associates (Birmingham, AL). Fluorescein-conjugated goat antibodies against mouse IgG (gamma chain specific) were from Zymed (San Francisco, CA). Hoechst 33258 DNA stain was from Hoechst (Frankfurt a/M, Germany). Alkaline phosphatase-conjugated goat-anti-rabbit-IgG (whole molecule) and goat-anti-mouse-IgG (gamma chain specific) were purchased from Zymed.

Fluorescence-conjugated donkey antibody against sheep IgG and teleostean gelatin (from cold water fish skin) and cycloheximide was purchased from Sigma Chemical Co. (St. Louis, MO). Falcon (Beckton and Dickinson, Lincoln Park, NJ) and Nunc (Roskilde, Denmark) plasticware were used in cell culture.  $^{35}\text{S}$ -labeling L-methionine was purchased from Amersham (Braunschweig, Germany). *Streptomyces splicatus* Endo- $\beta$ -N-acetylglucosaminidase H (endoglycosidase H), produced in recombinant *E. coli*, was purchased from Boehringer-Mannheim (Mannheim, Germany). Brefeldin A (BFA) was purchased from Epicenter Technologies (Madison, WI). Molecular weight markers used in PAGE were the prestained molecular mass marker mixture for range 20–180 kD (Sigma Chemical Co.) and  $^{14}\text{C}$ -molecular mass standards for the range 14.3–200 kD (Amersham).

### Western Blot

SV24 total cell lysate corresponding to 1.5 mg protein as measured by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) was loaded into a 18-cm well and separated by electrophoresis on an SDS-polyacrylamide gel (10%). The proteins were then electrotransferred from the gel to a nitrocellulose sheet (Schleicher and Schuell, Dassel, Germany) using a Gene blotter apparatus (Idea Scientific Company, Corvallis, OR) cooled with ice for 1 h at 24 V constant voltage. Proteins were fixed to the nitrocellulose in 3% TCA containing 0.2% Ponceau S protein stain (Serva, Heidelberg, Germany). Strips cut from the nitrocellulose were blocked with 10% (wt/vol) fat-free instant milk powder in PBS (blotto; Johnson et al., 1984) and incubated with primary antibodies diluted in blotto. After washing with 0.2% Triton X-100 in PBS the nitrocellulose strips were incubated with alkaline phosphatase-conjugated secondary antibodies diluted in blotto. After washing with 0.2% Triton X-100 in PBS, the strips were incubated with alkaline phosphatase substrate solution (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 5 mM  $\text{MgCl}_2$ , 0.33  $\mu\text{g}/\text{ml}$  nitroblue tetrazolium, 0.165  $\mu\text{g}/\text{ml}$  5-bromo-4-chloro-indolylphosphate). All the steps, except the electrotransfer, were performed at room temperature.

### Immunofluorescence

Subconfluent layers of SV24 cells were grown on glass coverslips or multiwell glass slides (Dunn Labor Technik GmbH, Asbach, Germany), rinsed in PBS and fixed with 3% (wt/vol) paraformaldehyde in PBS for 15 min. After blocking the unreacted aldehyde groups with 50 mM  $\text{NH}_4\text{Cl}$  in PBS, the cells were permeabilized with 0.1% (wt/vol) Triton X-100 in PBS for 5 min. To prevent unspecific binding of antibodies, the cells were incubated for 30 min with 10% (vol/vol) goat serum in PBS. Primary antibodies were diluted in 10% (vol/vol) goat serum in PBS, and incubated with the cells for 20 min. After extensive washing with PBS the cells were incubated again for 30 min with 10% (vol/vol) goat serum in PBS followed by fluorescent labeled secondary antibodies diluted in 10% (vol/vol) goat serum in PBS. The secondary antibodies were used at the manufacturer's recommended dilution. The cell nuclei were stained with Hoechst 33258 stain at dilution 1/1,000 in PBS, to aid in finding the cells in the fluorescence microscope. Cycloheximide chase was done by incubating the cells on coverslips with 20  $\mu\text{g}/\text{ml}$  cycloheximide in normal growth medium before the fixation. To see the effect of BFA on the distribution of HBsAg within the cells, the subconfluent cell layers on coverslips were incubated in normal medium supplemented with 10  $\mu\text{g}/\text{ml}$  BFA for 2 h, and the cells were fixed and labeled for immunofluorescence as described above. The immunolabeled cells were examined with Zeiss Photomicroscope III with a Planapo 63 $\times$  oil immersion objective and Zeiss Axiophot microscope with 63 $\times$  Planapo oil immersion objective. Photographs were recorded on Kodak TMAX400 black and white negative film.

Double immunofluorescence labeling to study the distribution of rab2 and HBsAg in SV24 cells was performed essentially as described by Chavrier et al. (1990). Free cytoplasmic rab2 was removed from the cells by incubating them for 4 min with 0.1% (wt/vol) saponin in 80 mM Pipes, pH 6.8, containing 5 mM EGTA and 1 mM  $\text{MgCl}_2$ . After fixation with 3% paraformaldehyde as described above, the cells were incubated with 0.1% (wt/vol) saponin in PBS for 5 min and quenched with 50 mM  $\text{NH}_4\text{Cl}$  in PBS/saponin. The primary antibodies and the fluorescent labeled secondary antibodies were diluted in PBS/saponin and incubated as described by Chavrier et al. (1990).

For confocal microscopy, subconfluent SV24 cell layers on coverslips were fixed with 3% paraformaldehyde in PBS as for normal immunofluorescence. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. Free aldehyde groups were quenched with 75 mM  $\text{NH}_4\text{Cl}$  and 20 mM glycine in PBS. To prevent unspecific binding of the antibodies the cells were incubated with 0.2% fish skin gelatin (wt/vol) in PBS for 10 min. The pri-

mary antibodies were diluted in PBS containing 0.2% fish skin gelatin and incubated with the cells for 30 min. After washing with PBS the cells were incubated with 0.2% fish skin gelatin in PBS for 10 min, followed by fluorescent labeled secondary antibodies diluted in PBS containing 0.2% fish skin gelatin. After washing with PBS, the cells were postfixed for 30 min with 4% paraformaldehyde in cacodylate buffer (pH 7.4). Postfixation was necessary because 1,4-diazabicyclo[2.2.2]octane could cause marked changes in cell morphology and redistribute the antibodies unspecifically. The coverslips were mounted on glass slides with 50% glycerol in PBS, containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane to prevent fluorescein quenching during microscopy. The mounted cells were examined with laser scanning confocal microscope (EMBL Light Microscopy Group) using a single excitation wavelength (514 nm) and selective filters for fluorescein and rhodamine.

### Pulse-Chase Experiments

SV24 cells were grown in 35-mm dishes to form subconfluent layers. The cells were depleted of methionine in methionine-free MEM (MEM-met) containing 2.2 g/liter sodium bicarbonate, 10% FCS (dialyzed against PBS), 2 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 10 mM Hepes. The cells were pulsed for 10 min with 300  $\mu\text{l}$  of the same medium containing 500  $\mu\text{Ci}/\text{ml}$   $^{35}\text{S}$ -labeled L-methionine (Amersham), and after the radioactive pulse, 2 ml of normal growth medium supplemented with 1.5 mg/ml nonradioactive L-methionine was added to prevent any further incorporation of [ $^{35}\text{S}$ ]methionine into newly synthesized proteins. After various times the chase was stopped by placing the dish on an ice cooled metal slab and washing the cells with ice-cold PBS after removing the medium. There was no increase in dimer concentration when a 10-min pulsed (not chased) dish was allowed to stand for 1 h on an ice cooled metal slab before processing (data not shown). This control showed that cooling the samples to 0°C stopped any PDI catalyzed or spontaneous disulphide bond formation. All of the pulse-chase incubations described above were done at 37°C in 5%  $\text{CO}_2$ .

For short radioactive pulses, the experiments were done in a water bath in the room atmosphere. Media were as above, but with 0.35 g/liter sodium bicarbonate instead of the normal 2.2 g/liter. The cells were pulsed for 2 min and chased for various times as described above.

The pulse-chased cells were lysed in cold RIPA buffer (10 mM Tris, pH 7.4, 1% [wt/vol] Triton X-100, 0.1% [wt/vol] SDS, 0.1% [wt/vol] sodium deoxycholate, 150 mM NaCl) (Tooze et al., 1987). 20 mM N-ethylmaleimide was added to lysis buffer to prevent spontaneous disulphide bond formation after cell lysis. Lysis was effected by passing the cells through a 21-gauge needle several times. The cell extracts and the media were spun for 5 min at 14,000 rpm in an Eppendorf microcentrifuge (Netheler and Hinz GmbH, Hamburg, Germany) to remove the insoluble debris. 100  $\mu\text{l}$  of RIPA-washed Pansorbin was added to each sample to remove proteins that bind to Pansorbin. After overnight rotation at 4°C the Pansorbin was spun out and 5  $\mu\text{l}$  of rabbit antiserum against HBsAg was added to each of the samples, and the mixture was rotated overnight at 4°C. 100  $\mu\text{l}$  of Pansorbin, which had been preincubated overnight with nonradioactive RIPA extract of SV24 cells to block the nonspecific binding sites and washed three times with RIPA, was added to each sample and the samples were rotated overnight at 4°C. The Pansorbin was spun out and the immunoprecipitates were washed three times with cold RIPA and three times with each of the wash buffers used by Tooze et al. (1987): with cold buffer A (10 mM Tris-HCl, pH 7.4, 0.1% [wt/vol] SDS, 1 mM EDTA), with cold buffer B (10 mM Tris-HCl, pH 7.4, 0.01% [wt/vol] SDS, 1 mM EDTA), with cold buffer C (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 500 mM NaCl), and finally with cold buffer D (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). Between all the washes the immunoprecipitates were spun 3 min at 8,000 rpm in an Eppendorf microcentrifuge.

Washed immunoprecipitates were suspended in 50  $\mu\text{l}$  of electrophoresis sample buffer containing 200 mM Tris, pH 8.8, 5 mM EDTA, 1% SDS, 10% (wt/vol) sucrose and 0.1% (vol/vol) bromophenol blue, with 40 mM DTT (to reduce the disulphide bonds) or without (for nonreduced samples), and incubated at 95°C for 5 min. It is necessary to use this concentration of DTT to fully reduce mature HBsAg. After heating the samples were cooled on ice and 10  $\mu\text{l}$  of iodoacetamide (500 mM) was added to each of the samples to block free thiols by acetylation. The samples were immediately mixed well on a vortex, and spun for 3 min at 14,000 rpm in an Eppendorf centrifuge before preparing the supernatant for electrophoresis.

The immunoprecipitated proteins were analyzed by SDS-PAGE using 10–15% polyacrylamide gradient gels. After electrophoresis the gels were fixed with 10% (vol/vol) acetic acid and 50% (vol/vol) ethanol in  $\text{H}_2\text{O}$  for

1 h at room temperature and processed for autoradiography using Entensify autoradiography enhancer (NEN Research, Boston, MA) according to the manufacturer's instructions. The gels were dried and exposed on Kodak X-OMAT AR film at  $-80^{\circ}\text{C}$ . The film was pre-exposed according to Laskey and Mills (1975) before autoradiography to obtain greater sensitivity and optimal linear response to the radioactivity on the gels. The exposed films were developed with Kodak RP X-OMAT Processor M6B.

The pulse chase in the presence of BfA was performed as described above for 10 min pulse, but 5  $\mu\text{g}/\text{ml}$  BfA was present in the medium during methionine depletion, pulse, and the chases. The immunoprecipitation and the washes were done as described above and the samples were analyzed by SDS-PAGE and autoradiography.

### **Incubation of HBsAg with Baculovirus Expressed PDI**

The *Spodoptera frugiperda* cells (Sf9—a clonal isolate of *Spodoptera frugiperda* IPLB-Sf21-AE cells) and baculovirus expression vectors were obtained from Dr. M. Summers (Center for Advanced Invertebrate Molecular Sciences, College Station, TX). Rat PDI-cDNA (obtained from Dr. G. Banting, University of Bristol) was cloned between the SmaI and XbaI sites of the pVL1393 expression vector. This construct was used together with linearized *Autographa californica* nuclear polyhedrosis virus wild type-DNA to cotransfect Sf9 cells. The transfection was done with Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) according to manufacturer's instructions. Recombinant virus was plaque purified and infected cells were screened for PDI expression by immunofluorescence and Western blotting using a polyclonal antibody raised against purified rat PDI. For the overexpression of PDI, Sf9 cells were infected with the recombinant virus ( $9 \times 10^6$  cells, MOI = 10). As a control, Sf9 cells were infected with wild type virus (Luckow and Summers, 1988; Summers and Smith, 1988). Lysates were prepared from cells harvested at 48 h after infection. To prepare the lysates, cells were washed with PBS, resuspended in 500  $\mu\text{l}$  PBS, passed through a ball bearing cell cracker (20 passes with 18  $\mu\text{m}$  clearance) and then subjected to two rounds of freezing and thawing.

HBsAg particles were collected from SV24 medium after 24 h radioactive labeling (100  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine and 10% of normal cold methionine) by spinning 12 h at 34,000 rpm in a Beckman SW40Ti rotor at  $4^{\circ}\text{C}$ . The pelleted material was suspended in 100 mM potassium phosphate, pH 7.5, and divided into aliquots that were incubated 10 h at  $37^{\circ}\text{C}$  in 100 mM potassium phosphate, pH 7.5, containing 5 mM reduced glutathione, 0.1% (wt/vol) Triton X-100, and 5, 25 or 125  $\mu\text{l}$  of the lysate of the rat PDI-cDNA bearing baculovirus-infected cells or of the wild type baculovirus infected cells (containing 7 mg/ml protein as measured with BioRad protein assay). The total incubation volume was 1.0 ml. After the incubation, 200  $\mu\text{l}$  of 120 mM NEM was added to each sample to block the free thiols. HBsAg was immunoprecipitated as described for the pulse-chase experiments, except the final washes were omitted. Before preparing the immunoprecipitates for SDS-PAGE, they were washed once with RIPA-buffer and once with 10 mM Tris-Cl, pH 7.4. After the electrophoresis the gels were processed for autoradiography and quantitated as described for pulse-chase experiments.

### **Digital Quantitation**

Autoradiographs from the pulse-chase experiments were digitized with Sony XC-77CE CCD camera module with a CCTV 12.5-75 F1.9 zoom lens connected to Apple Macintosh IIfx microcomputer via a DT2255-50 Hz card (Data Translation Inc., Marlboro, MA) using Image 1.40 image processing and analysis program (Wayne Rasband, National Institute of Health, Research Services Branch, Bethesda, MD). 16 frames were averaged into one TIFF-format file. The optical densities of individual protein bands on the autoradiographs were quantitated as peak areas in pixels from column average plots using the Image 1.40 program.

For dual channel correlation analysis of double-labeled immunofluorescence, the separate micrographs taken with rhodamine and fluorescein selective filters were scanned with a Perkin-Elmer Micro D 1010 at a step size of 25  $\mu\text{m}$  or were taken directly as digital images from the modular confocal microscope using  $512 \times 0.093 \mu\text{m}$  by  $512 \times 0.93 \mu\text{m}$  by  $20 \times 0.55 \mu\text{m}$  steps. The image data was processed with a series of networks in the Application Visualization System (AVS, Stardent Computer) on a Stardent 2000 GS. The separate image datasets were aligned by cross correlation and combined. The confocal microscope data could be used without further alignment. The further analysis followed the general procedure introduced by Dr. Jean Davoust (Marseilles) for the treatment of double-

labeled confocal images and implemented by us as a series of AVS modules. The reader is directed to his paper (manuscript submitted for publication) for a complete description of the procedure. Briefly, the two vector valued array of pixel data is replotted as the number of occurrences of pixels with a given ratio of red to green. This second plot reveals the correlation between red and green values for a given pixel and hence the likelihood that the antigen labeled with the fluorescein coupled antibody is found in the same position as the antigen labeled with the rhodamine-coupled antibody. Particular regions of this occurrence graph can then be selected and a new image composed which shows only those pixels corresponding to a given ratio of red to green or a three color image in which the selected pixels are shown in blue and the others are shown in their original red and green color. The AVS modules written by us for this processing are written in C and are available upon request.

### **Endoglycosidase H Digestion of HBsAg**

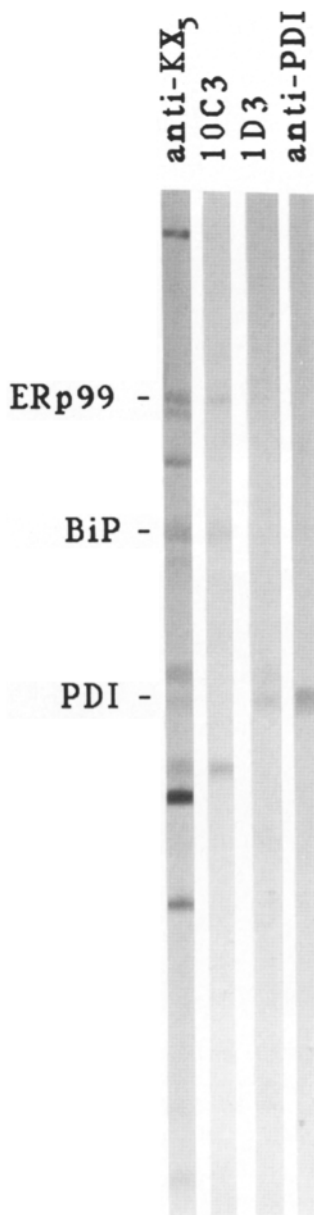
A confluent layer of SV24 cells was labeled with [ $^{35}\text{S}$ ]methionine overnight with 200  $\mu\text{Ci}/\text{ml}$  in MEM made with dialyzed serum and containing 10% of the normal methionine. The labeling medium was removed and the cells were washed with PBS and lysed. HBsAg was immunoprecipitated from aliquots of the lysate, as described above and the washed immunoprecipitates were suspended in 70  $\mu\text{l}$  of 71.7 mM Na-citrate, pH 5.5, containing 0.143 M 2-mercaptoethanol and 0.15% (wt/vol) SDS, and incubated at  $95^{\circ}\text{C}$  for 5 min. After this mild denaturation to increase oligosaccharide accessibility for the enzyme, the samples were cooled to  $37^{\circ}\text{C}$ . 10  $\mu\text{l}$  of 1% (wt/vol) BSA was then added, followed by 20  $\mu\text{l}$  of endoglycosidase H stock (1 mU/ $\mu\text{l}$ ). The final incubation buffer contained 50 mM Na-citrate, 0.1 M 2-mercaptoethanol, 0.1% (wt/vol) SDS and 0.2 mU/ $\mu\text{l}$  endoglycosidase H. To the control sample, 20  $\mu\text{l}$  of water was added instead of the enzyme. After 24 h incubation at  $37^{\circ}\text{C}$ , the samples were prepared for electrophoresis as described for the pulse-chase experiments, and analyzed by SDS-PAGE.

## **Results**

### **Markers for the ER in SV24 Cells**

All of the experiments described below were performed with SV24 cells, a mouse L cell line which had been stably transfected with the coding region for HBsAg. This line which secretes HBsAg lipoprotein particles was generously provided to us by Prof. Don Ganem (University of California at San Francisco). The generation of this cell line and the kinetics of secretion of HBsAg have been described previously (Eble et al., 1986; Simon et al., 1988).

To test the consensus view that HBsAg monomers are assembled and bud as lipoprotein particles in the ER, we needed specific markers for this compartment. Fig. 1 shows a Western blot with four different antibodies specific for proteins residing in the ER against SV24 cell lysate. 1D3 is a mouse monoclonal IgG raised against the peptide KDDQKAVKDEL, corresponding to the carboxy terminus of PDI. It recognizes a major protein band at a relative molecular mass of  $\sim 55$  kD corresponding to PDI and another protein, calreticulin, which is one of the major calcium binding proteins in the ER lumen and migrates slightly more slowly on SDS-PAGE (Macer and Koch, 1988; Vaux et al., 1990; Buck, P., D. J. Vaux, J. Tooze, R. Hendriks, and S. D. Fuller, manuscript in preparation). Anti-PDI is a polyclonal rabbit antiserum raised against purified bovine liver PDI which reacts specifically with PDI on Western blots of SV24 lysate and does not recognize calreticulin. 10C3, raised against the peptide KSEKDEL, which contains the carboxy-terminal six amino acids of BiP, recognizes three major bands at  $\sim 99$ , 75, and 47 kD. The band at an apparent molecular mass of 75 kD is immunoglobulin heavy chain binding protein (BiP). BiP (Haas and Wabl, 1983), also known as GRP78 (Shiu et al., 1977), is an abundant soluble resident



**Figure 1.** Western blot analysis of the ER marker antibodies on SV24 cell lysate. The antibodies used were: anti-KX<sub>5</sub> (anti-KX<sub>5</sub>KDEL raised against a mixture of peptides containing the KDEL recognition sequence at the carboxyterminus), 10C3 (anti-BiP tail), 1D3 (anti-PDI tail), and anti-PDI. The positions of ERp99 (endoplasmic), BiP, and PDI are indicated.

ER protein which has been suggested to dissolve or to prevent the formation of protein aggregates in the ER (Pelham, 1986), to help proteins to fold and oligomerize correctly (Gething et al., 1986), and to prevent incompletely assembled proteins from leaving the ER (Bole et al., 1986). The 99-kD band probably represents ERp99 (Lewis et al., 1985), the mouse homologue of 94-kD glucose-regulated protein (GRP94; Mazzeella and Green, 1987), also known as endoplasmic. (Koch et al., 1988; Macer and Koch, 1988). Anti-KX<sub>5</sub>KDEL is a polyclonal rabbit antiserum raised against a mixture of peptides having the general sequence KXXXXXXKDEL, where the X positions were synthesized from a mixture of alanine, aspartic acid, histidine, glutamine, leucine, tyrosine, and lysine. It specifically recognizes at least 12 different proteins on the Western blot of an SV24 cell lysate, including ERp99, BiP, PDI, and calreticulin (Fig. 1) as well as the unidentified lower molecular weight protein recognized by 10C3. A more complete description of the production of these antibodies and further evidence that they

specifically recognize soluble proteins of the ER will be published elsewhere (Buck, P., D. J. Vaux, J. Tooze, R. Hendriks, and S. D. Fuller, manuscript in preparation).

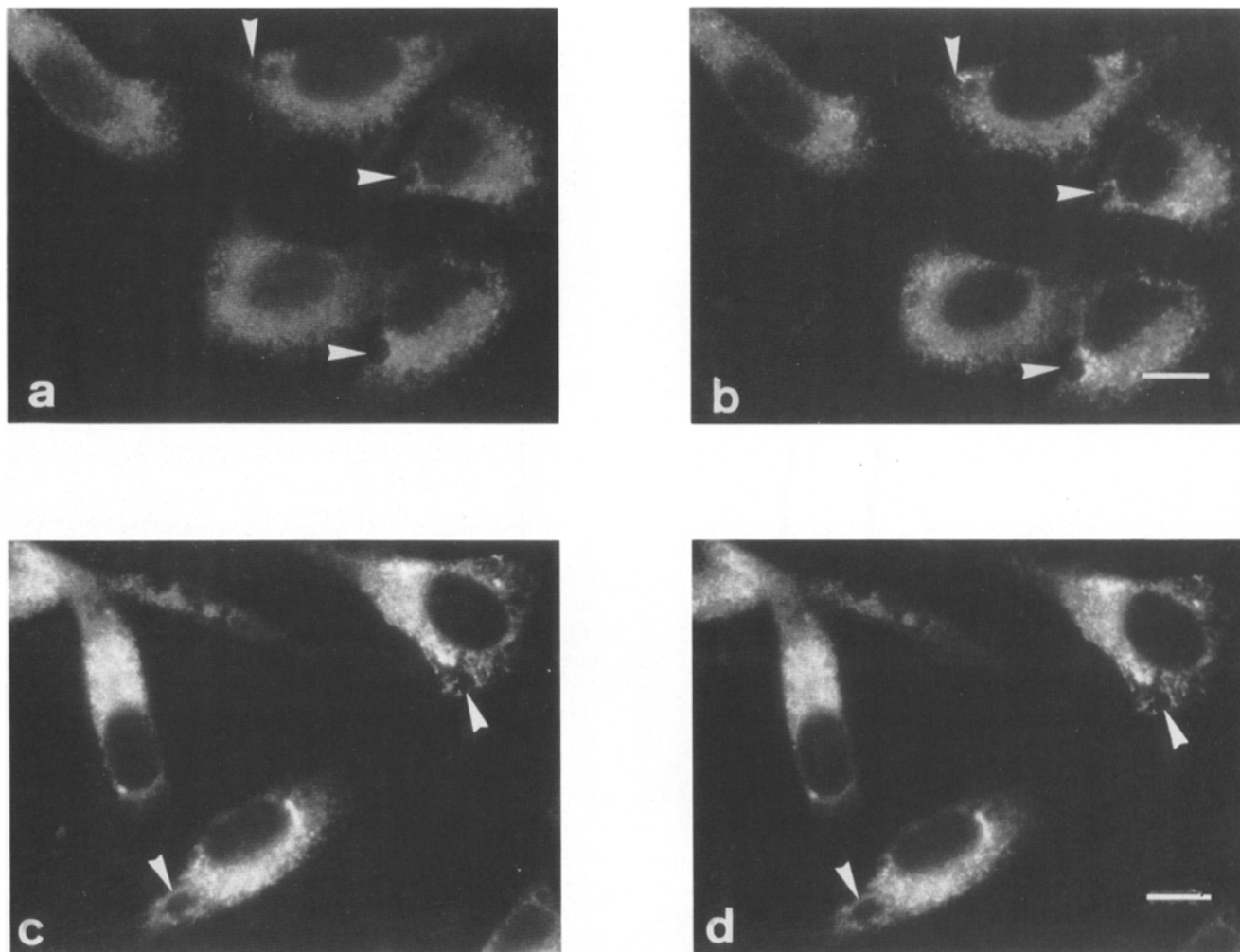
Double immunofluorescence of SV24 cells with polyclonal anti-PDI (Fig. 2 *a*) and 10C3 (Fig. 2 *b*) shows that they reveal the same reticular pattern which is typical for soluble ER proteins. Similarly, anti-KX<sub>5</sub>KDEL (Fig. 2 *c*) and 1D3 (Fig. 2 *d*) yield identical, reticular staining patterns. This result shows that the distributions of PDI, BiP, and that of the bulk of the other KDEL-proteins which are visualized with anti-KX<sub>5</sub>KDEL are identical in SV24 cells and together with previous work with these reagents (Tooze et al., 1989; Vaux et al., 1990) validates their use to test the hypothesis that HBsAg assembles in the ER.

### **Resident Soluble ER Proteins Are Excluded from a Compartment Enriched in HBsAg**

The relationship of the localization of the intracellular pool of HBsAg to that of soluble ER proteins was studied by indirect double immunofluorescence microscopy. Although a large fraction of the HBsAg (Fig. 3 *b*) colocalizes with PDI (Fig. 3 *a*), there are localized HBsAg rich regions which appear to exclude PDI. A similar pattern is revealed with other ER markers such as BiP. All regions that show BiP staining (Fig. 3 *c*) also show staining for HBsAg but localized regions that are enriched in HBsAg (Fig. 3 *d*) appear to exclude the soluble ER protein. The identity of staining of anti-KX<sub>5</sub>KDEL with that of the other ER markers in SV24 cells (Fig. 2) suggests that the other major KDEL-proteins behave similarly.

The relationship of the PDI excluding compartment to the intermediate compartment marked by rab2 was also probed by double immunofluorescence. For this experiment a rabbit polyclonal anti-rab2 (Fig. 3 *e*) and a polyclonal anti-HBsAg developed in sheep (Fig. 3 *f*) were used and the cells treated with saponin to remove cytoplasmic rab2. This saponin treatment results in a slightly altered cell morphology however the fact that the PDI excluding areas contain rab2 is clear.

The immunofluorescence data shown in Fig. 3 suggest that a fraction of HBsAg may exist in a compartment which excludes ER markers, however, it does not allow one to distinguish between a gradual decrease in the concentration of ER marker relative to HBsAg and an abrupt change in the ratio of these proteins which would indicate a separate compartment. We quantitated the relationship between the distribution of HBsAg and ER markers by analyzing three-dimensional double fluorescence data collected with confocal microscopy. Fig. 4 *a* shows a plot of the pixels in a single section of the confocal data resolved into their fluorescein (1D3-PDI-horizontal; shown separately in 4 *c*) and rhodamine (anti-HBsAg-vertical; shown separately in 4 *b*) intensities. The strong, heavily populated diagonal corresponds to the bulk of the pixels in the image for which the ratio of PDI to HBsAg is relatively constant. The pixels which form a separate population at ~15 degrees from the vertical correspond to those regions of the image which are rich in HBsAg but have no detectable PDI. These regions are shown in Fig. 4 *d* in which only the pixels marked in Fig. 4 *a* were used to compose the image. The fact that this subpopulation of pixels is well separated in Fig. 4 *a* from the bulk of the pixels indicates that they correspond to a separate compartment



**Figure 2.** Distribution of ER marker proteins in SV24 cells. Indirect double immunofluorescence shows that the antibodies characterized in Fig. 1 produce typical ER staining patterns in paraformaldehyde-fixed SV24 cells. The antibodies used are anti-PDI (*a*, visualized with rhodamine) which shows an identical reticular staining pattern to 10C3 (*b*, visualized with fluorescein) and anti-KX<sub>3</sub>KDEL (*c*, visualized with rhodamine) which coincides with the staining for 1D3 (*d*, visualized with fluorescein). Structures that are morphologically similar to the HBsAg budding compartment and exclude ER markers are indicated by arrowheads. Bars, 10  $\mu$ m.

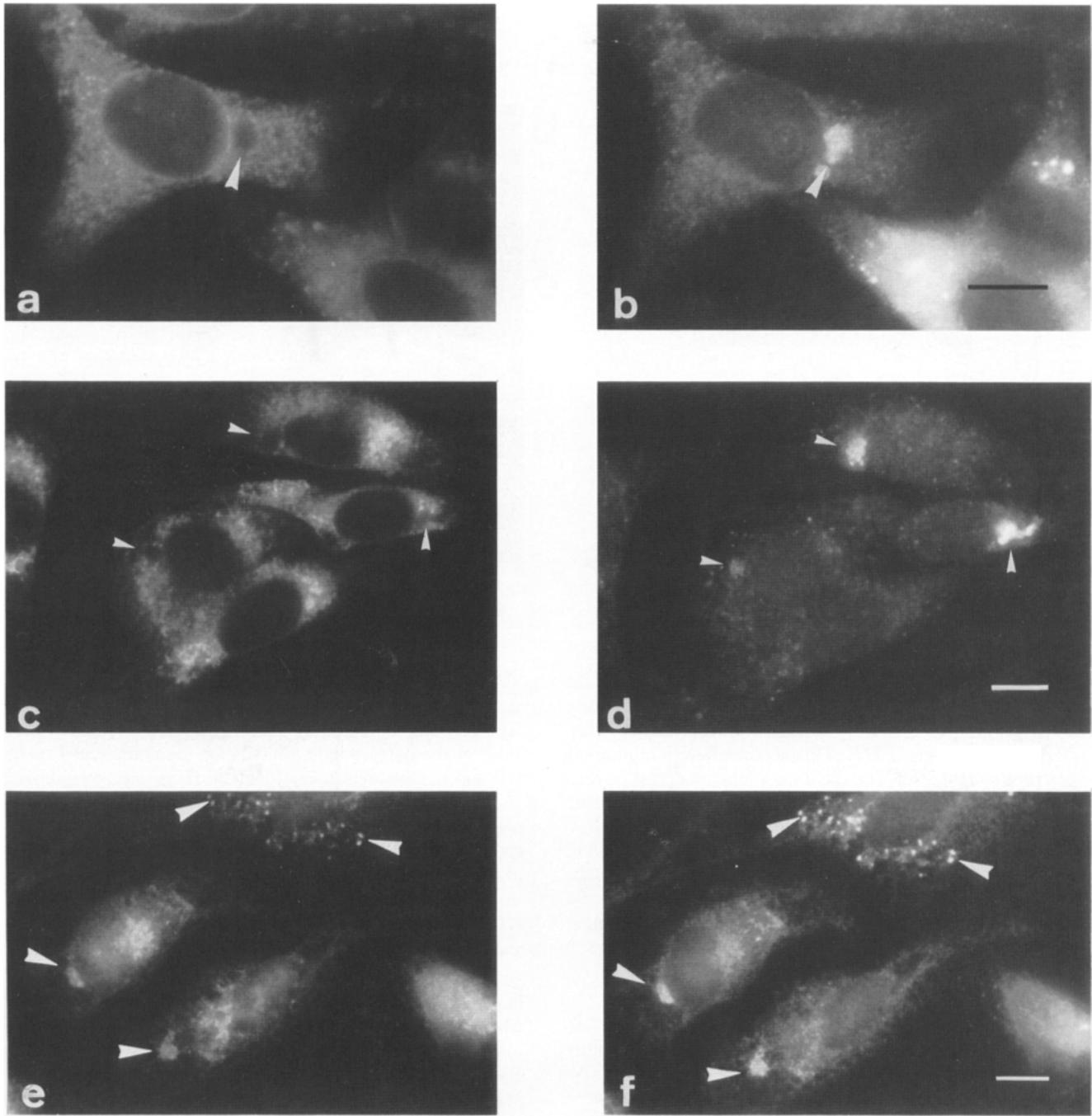
rather than reflecting the result of continuous variation in the intensity of either PDI or HBsAg staining. Processing of many sections of double stained cells revealed the presence of only the two populations of pixels seen in Fig. 4 *a* for transfected cells.

The temporal relationship between the HBsAg found in the PDI excluding regions and that seen in the PDI containing regions was revealed by blocking synthesis of HBsAg with cycloheximide. The fraction of the HBsAg staining seen in the PDI excluding regions increased with time indicating that these regions correspond to later stations in the maturation pathway. After 90 min of cycloheximide treatment (Fig. 5, *A* and *B*) HBsAg still colocalizes with PDI but to a lesser extent than in the normal steady state situation (Fig. 3, *a* and *b*). After 4 h in the presence of cycloheximide (Fig. 5, *C* and *D*) the amount of HBsAg in the ER was markedly decreased, and after 6 h (Fig. 5, *E* and *F*) chase, no detectable HBsAg remained colocalized with PDI. The PDI distribution remained essentially unchanged during these incubations. These data show that HBsAg moves to the PDI excluding re-

gion with increasing chase times and that the rate limiting step in secretion must correspond to exit from this compartment.

#### **Time Course of Intermolecular Disulphide Bond Formation**

We analyzed the maturation process biochemically to separate the early events occurring in the ER from the ones which occur in the PDI excluding compartment. We approached this by studying the time course of intermolecular disulphide bond formation and of particle secretion by pulse-chase immunoprecipitation experiments. SV24 cells were pulsed for either 2 or 10 min with radioactive methionine and chased for various times. HBsAg was then immunoprecipitated from the cell extracts and the culture media and analyzed by SDS-PAGE in reducing (+) or nonreducing conditions (-) (Fig. 6, *A* and *B*). Analysis of the reduced samples in which all of the HBsAg entered the gel and migrated as monomers (M/gp<sup>R</sup> and M/p<sup>R</sup> for glycosylated and non-

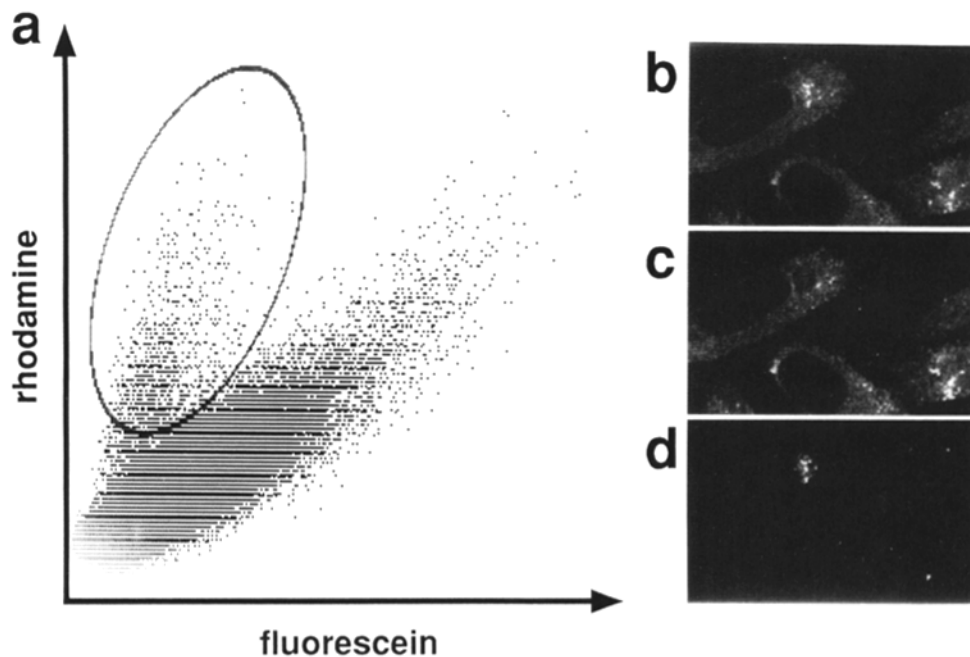


**Figure 3.** Distribution of HBsAg in SV24 cells. Indirect double immunofluorescence with anti-HBsAg and antibodies to ER and intermediate compartment markers show a partial overlap of staining. The paired images are stained with 1D3 (*a*, fluorescein) which reveals the typical ER distribution of PDI, 10C3 (*c*, fluorescein) which shows the distribution of BiP and other ER proteins, anti-rab2 (*e*, rhodamine) which marks the ER and an intermediate compartment, rabbit anti-HBsAg (*b* and *d*, rhodamine) and sheep anti-HBsAg (*f*, fluorescein). Some of the structures in which HBsAg is concentrated and which exclude PDI and other ER markers, are indicated by arrowheads. Bars, 10  $\mu$ m.

glycosylated forms, respectively) showed that newly synthesized HBsAg was not subjected to significant proteolysis, which would have complicated the kinetic analysis. This agrees with the results of previous experiments with the SV24 cell line which showed that it secretes >95% of the synthesized HBsAg as lipoprotein particles within 10 h (Simon et al., 1988). The nonreduced lanes of the gels are conveniently interpreted in terms of our two class description of disulphide bonds in HBsAg. The monomer bands for the gly-

cosylated ( $M/gp^N$ ) and the nonglycosylated ( $M/p^N$ ) disappear with the formation of the dimers showing the occurrence of the dimer crosslinks. The formation of oligomer crosslinks is reflected by the occurrence of material which runs at very high molecular weight or does not enter the gel in the nonreduced state.

The kinetics of dimer crosslink formation were analyzed by digitizing the autoradiographs (Fig. 6 C) of immunoprecipitated pulse-chased cell lysates. A fraction of the HBsAg



**Figure 4.** Dual channel correlation analysis of double immunofluorescence. The intensities of rhodamine and fluorescein fluorescence for individual pixels of a z section of SV24 cells taken with the modular confocal microscope are plotted in *a* with the rhodamine (HBsAg) intensity vertical and the fluorescein (PDI) intensity horizontal. Two populations of pixels are seen, the majority of which have similar intensities of rhodamine and fluorescein and hence contain both PDI and HBsAg and a distinct population (marked by an *ellipse*) which show only rhodamine intensity and correspond to the presence of HBsAg. The rhodamine (*b*, HBsAg) and fluorescein (*c*, PDI) images are shown to the right as is the image of the HBsAg rich, PDI poor pixels (*d*) which are marked in *a*.

is present as aggregated protein at very early times in the chase presumably reflecting the association of improperly folded protein seen at early times in other systems (Marquardt and Helenius, 1992). This is converted to monomers very rapidly (Fig. 6 *A*) and the monomer concentration then begins to decrease monotonically (Fig. 6 *B*). Dimers begin to form during the short (2 min) radioactive pulse (Fig. 6 *A*) and then increased in concentration with short chase times to peak at ~60 min of chase with a concomitant fall in the monomer concentration. The fraction of dimers then decreased with a corresponding increase in high molecular weight oligomers.

#### **Time Course of Particle Formation and Secretion**

Previous work has shown that the time course of secretion matches the time course of HBsAg carbonate extractability which reflects particle budding (Simon et al., 1988). We wanted to compare the rates of these processes to those of dimer and oligomer crosslink formation seen above. Fig. 7 *A* shows an autoradiograph of immunoprecipitates of SV24 culture media after various chase times. These precipitates did not contain any detectable contaminants and hence we quantitated HBsAg secretion by measuring the incorporated radioactivity from the immunoprecipitated secreted particles (Fig. 7 *B*). We and others have shown that HBsAg secreted from SV24 and other transfected cells is particulate, as judged by EM and CsCl<sub>2</sub> centrifugation (Huovila, A.-P. J., and S. D. Fuller, manuscript in preparation; Dubois et al., 1980; Moriarty et al., 1981; Liu et al., 1982; Laub et al., 1983). This is consistent with the fact that none of the secreted HBsAg entered the gel without reduction (data not shown), indicating all of it was crosslinked in the particles. The approximate half-time for the particle secretion from the

SV24 cells in our chase conditions was 2 h. The rates of secretion for the glycosylated and nonglycosylated forms of HBsAg were compared by quantitation of the relative intensities of the corresponding bands for the nonreduced material and can be seen to be identical (Fig. 7 *B*).

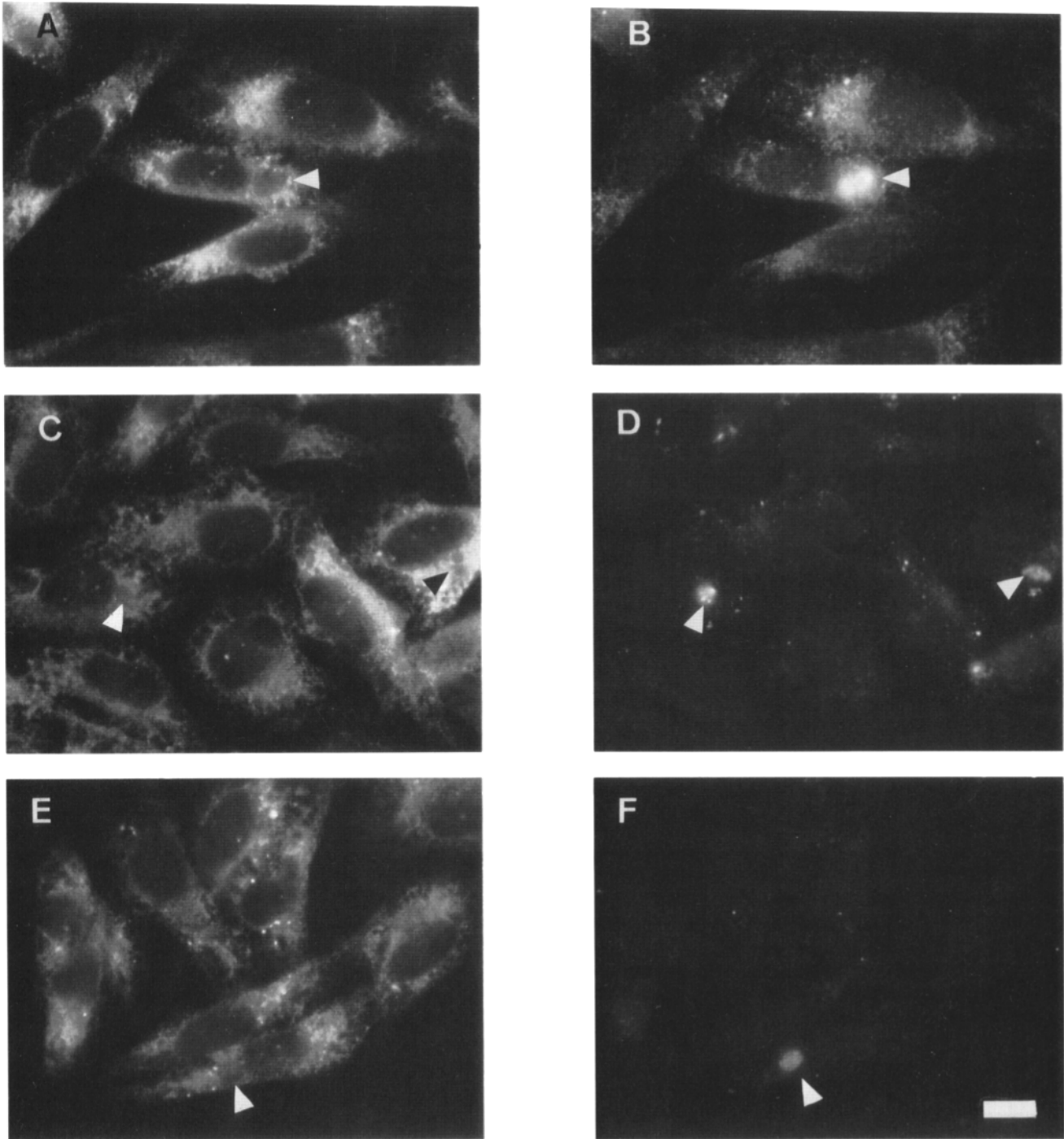
The lag between the formation of dimers and lipoprotein particle secretion allows us to conclude that the oligomer crosslinks are formed with a half-time of <160 min from the disulphide-linked dimers.

#### **HBsAg Assembly Occurs in a Pre-Golgi Compartment**

The immunofluorescence data revealed that the surface antigen migrated to PDI excluding regions with a similar distribution to the Golgi complex after synthesis. We asked whether these regions represent a portion of the Golgi complex. Although the majority of HBsAg in SV24 cells is not glycosylated, the state of the glycosylation on the remainder provides a marker for its location. Patzer et al. (1984) showed that all of the oligosaccharides associated with intracellular HBsAg in stably transfected CHO cells were sensitive to endoglycosidase H digestion. We confirmed that this was also true for HBsAg expressed in SV24 cells (data supplied to referees but not shown). Since the secreted HBsAg particles bear only endoglycosidase H-resistant glycans, this provided evidence that the intracellular pool had not yet encountered the enzymes of the medial Golgi.

BfA is a fungal metabolite which has been shown to have profound effects on the distribution of Golgi components. All characterized *medial*- and *cis*-Golgi markers have been shown to redistribute to the ER in the presence of BfA and to return to their normal distribution upon its removal (Doms et al., 1989; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Takatsuki and Tamura, 1985). Immunofluorescent

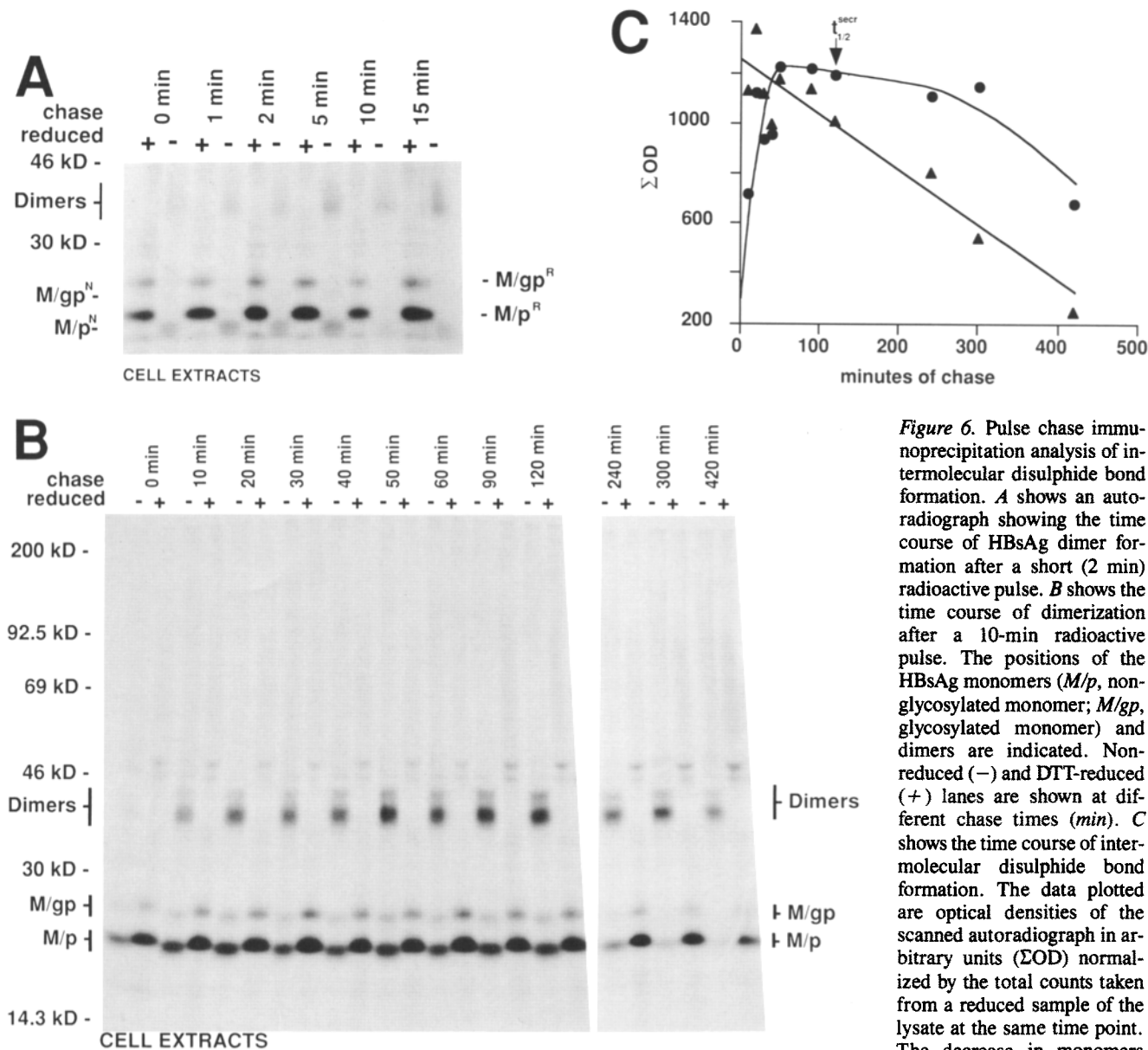




**Figure 5.** Localization of PDI and HBsAg during cycloheximide treatment. Indirect double immunofluorescence with anti-HBsAg and antibodies to ER and intermediate compartment markers showing the change in distribution of the surface antigen during incubation with cycloheximide. The paired stainings are with 1D3 (*A*, *C*, and *E*, fluorescein) and rabbit anti-HBsAg (*B*, *D*, and *F*, rhodamine). The times of incubation with cycloheximide are 90 min (*A* and *B*), 4 h (*C* and *D*), and 6 h (*E* and *F*). Some of the structures in which HBsAg is concentrated and which exclude PDI are marked by arrowheads. The fraction of HBsAg that is found in the PDI-excluding compartment increases during the incubation. For comparison, the half time for secretion is  $\sim 2$  h (see Fig. 7). Bar, 10  $\mu\text{m}$ .

staining with 1D3 and a polyclonal antibody that recognizes mannosidase II (obtained from Brian Burke, Harvard Medical School) shows that BfA has a similar effect on the redistribution of Golgi markers in SV24 cells (data not shown). Double immunofluorescence on BfA-treated SV24 cells (Fig. 8) shows that BfA does not cause HBsAg in the PDI

excluding compartment to recycle back to the ER, indicating that the HBsAg maturation compartment is not part of the Golgi complex. This behavior is reminiscent of the effect of BfA on the region of the intermediate compartment marked by the 72-kD KDEL binding protein (Vaux et al., 1990) or by rab2 (Chavrier et al., 1990). In combination with the ex-



**Figure 6.** Pulse chase immunoprecipitation analysis of intermolecular disulphide bond formation. **A** shows an autoradiograph showing the time course of HBsAg dimer formation after a short (2 min) radioactive pulse. **B** shows the time course of dimerization after a 10-min radioactive pulse. The positions of the HBsAg monomers (*M/p*, non-glycosylated monomer; *M/gp*, glycosylated monomer) and dimers are indicated. Non-reduced (-) and DTT-reduced (+) lanes are shown at different chase times (min). **C** shows the time course of intermolecular disulphide bond formation. The data plotted are optical densities of the scanned autoradiograph in arbitrary units ( $\Sigma OD$ ) normalized by the total counts taken from a reduced sample of the lysate at the same time point. The decrease in monomers ( $\blacktriangle$ ) is followed by an increase in dimers ( $\bullet$ ) which then form oligomers which do not enter the gel. The half-time for particle secretion (2 h) is indicated by an arrow. Pulse-chase experiments, immunoprecipitation, and SDS-PAGE autoradiography were performed as described in Materials and Methods. The mobilities of the molecular weight markers are indicated.

increase in dimers ( $\bullet$ ) which then form oligomers which do not enter the gel. The half-time for particle secretion (2 h) is indicated by an arrow. Pulse-chase experiments, immunoprecipitation, and SDS-PAGE autoradiography were performed as described in Materials and Methods. The mobilities of the molecular weight markers are indicated.

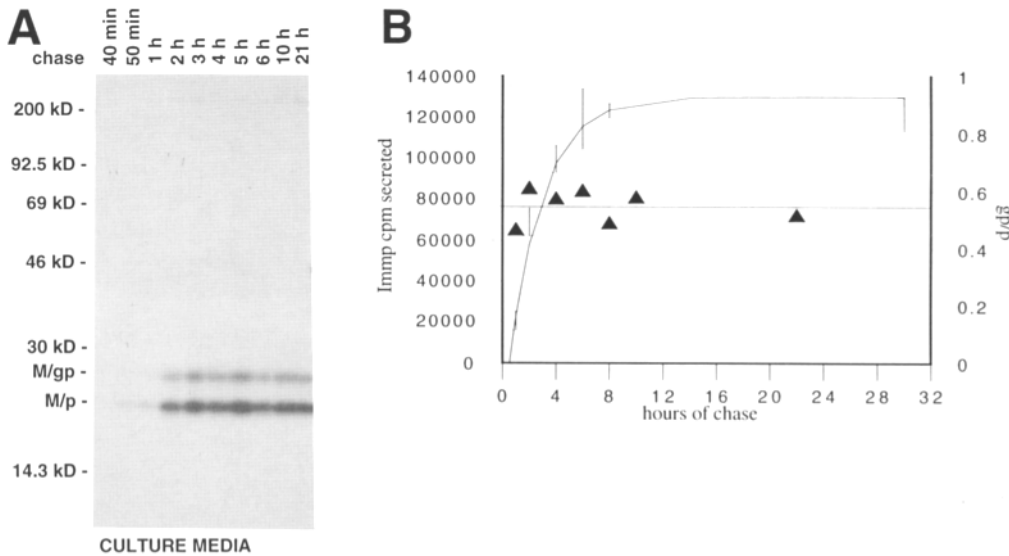
clusion of ER markers, the glycosylation and BfA data indicate that HBsAg migrates to a compartment intermediate between the ER and the Golgi.

#### Formation of Oligomer Cross-links Requires Exit from the ER

The demonstrated ability of BfA treatment to block exit of newly synthesized proteins from the ER while allowing ER functions to continue (Lippincott-Schwartz et al., 1989; Klausner et al., 1992) allowed us to separate the formation of the stages of HBsAg maturation which occur in the ER from later ones. The time course of dimer formation was followed by pulse-chase analysis of cells in the presence of BfA (Fig. 8, *c* and *d*). In the presence of BfA, newly synthesized

HBsAg is converted from monomers to dimers, however, the dimers accumulate and do not proceed to form oligomer crosslinks. The rate of dimer formation is approximately the same as in the non-BfA-treated cells represented in Fig. 6. Hence the formation of oligomer crosslinks in HBsAg requires exit from the ER. This distinguishes the case of HBsAg from that of smaller disulphide-linked oligomers such as influenza haemagglutinin in which all disulphide bond formation is completed in the ER and required for exit from it.

The observation that oligomer crosslinks only form after exit from the ER suggests that there must be something different about the later compartment which allows these oligomer crosslinks to form. The obvious candidate for this difference is the absence of PDI. We tested the effect of PDI



**Figure 7.** The time course of HBsAg secretion from SV24 cells. The autoradiograph in *A* shows the secreted material which was immunoprecipitated from the medium after a 10-min radioactive pulse and the indicated chase times. *B* shows a quantitative analysis of the secretion. The X indicates the immunoprecipitable counts released into the medium as a function of chase time with the nonspecific specifically bound counts subtracted. The fitted curve (---) shows the equation  $16000 \text{ cpm} \cdot (1 - \exp(-t/2.5\text{h}))$  which was the best weighted least squares fit to the data. The corresponding half-time for the appearance of HBsAg par-

ticles in the culture medium is  $\sim 2$  h. The ratios of glycosylated monomers to the nonglycosylated monomers in the particles secreted as a function of chase time ( $\blacktriangle$ ) were measured from the autoradiograph in *A*. The average ratio of glycosylated HBsAg to nonglycosylated HBsAg (*gp/p*) was 0.551 and is indicated by the horizontal line.

on the oligomer crosslinks by incubating secreted, fully crosslinked HBsAg particles with a lysate of Sf9 cells which had been infected with a rat PDI-cDNA bearing recombinant baculovirus and so expressed high levels of this protein (Fig. 9). As a control, lysate from cells infected with wild type baculovirus was used. The effect of increasing amounts of the PDI-containing lysate is the conversion of the oligomeric forms of HBsAg to the dimeric form (Fig. 9 *C*). The level of monomer remains approximately the same. Although secreted HBsAg contains no monomer, the prolonged incubation with glutathione causes the direct conversion of a small fraction of the oligomer to monomer. This can also be observed in incubations in which no cell lysate has been added (data not shown). This experiment shows that the oligomer crosslinks of HBsAg particles are not stable in the presence of PDI although the dimer crosslink formation is promoted.

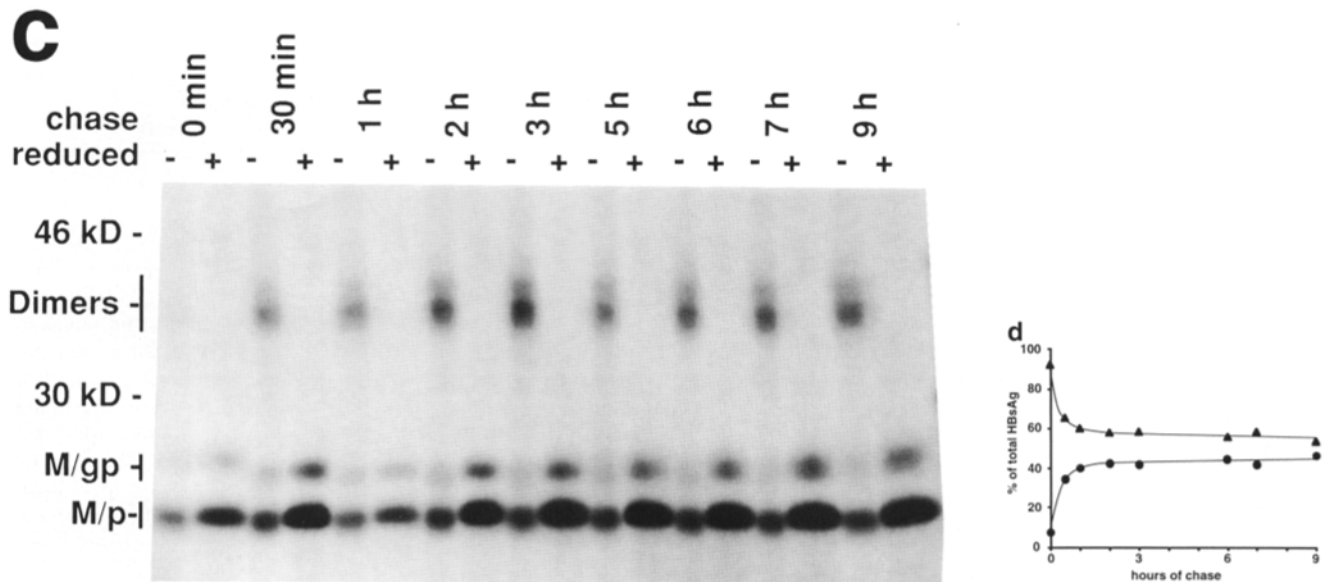
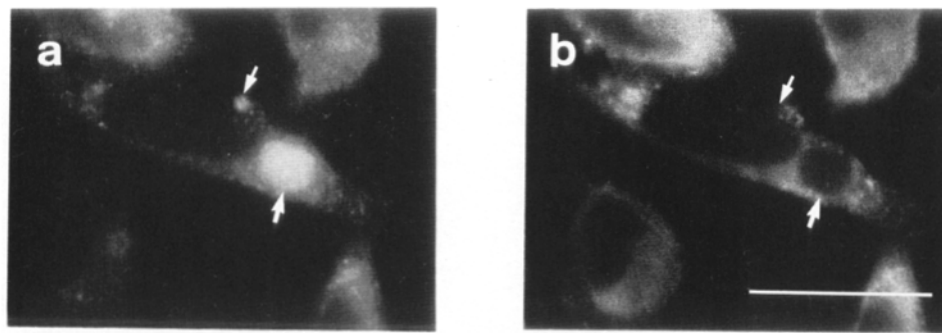
## Discussion

The consensus view of HBsAg assembly localized the processes which convert the monomeric, transmembrane HBsAg protein to the oligomeric, soluble lipoprotein particle to the ER. Here, we present evidence that the later stages of this process, in which the HBsAg lipoprotein particle is assembled from dimeric HBsAg, occurs in an intermediate compartment between the ER and the Golgi. This result expands our understanding of the assembly pathway of this lipoprotein particle and of the role of this intermediate compartment in biosynthesis in that assembly utilizes the special environment in this compartment to promote the extensive disulphide crosslinking of the HBsAg particle. Once again, a study of the way in which a virus exploits the cellular machinery has resulted in a better understanding of that machinery.

The conclusion that lipoprotein particle assembly occurs in an intermediate compartment is based on a combination

of immunocytochemical and biochemical results. Using a cell line that stably expresses HBsAg, we showed that a fraction of the HBsAg was segregated to a region depleted of soluble ER proteins such as BiP and PDI, although it contained an established marker of an intermediate compartment, rab2. Quantitation of confocal microscope data showed that the exclusion of PDI was abrupt and suggested that the HBsAg enriched regions were distinct from the ER. The observation that the fraction of HBsAg segregated to this region increases during incubations with cycloheximide while the amount in the ER decreases shows that this region corresponds to a later and rate limiting step in HBsAg maturation. In agreement with previous work on other cell lines (Patzner et al., 1984), our biochemical data showed that intracellular HBsAg remained endoglycosidase H sensitive and hence must be in a premedial Golgi compartment. This is reinforced by the immunocytochemical observation that treatment of cells with BfA has no effect on the morphology of the PDI excluding region in which HBsAg is concentrated. Since BfA is known to have a dramatic effect on the distribution of Golgi markers including those of the *cis*-Golgi, this shows that HBsAg is concentrated in a pre-Golgi compartment.

Pulse-chase studies revealed the relationship between HBsAg lipoprotein particle assembly and disulphide bond formation. We interpreted our results in terms of two classes of disulphide bonds in HBsAg, dimer crosslinks and oligomer crosslinks. Disulphide-linked dimers are formed early and are converted slowly into high molecular weight, disulphide-linked oligomers. The observation that the peak of dimer concentration (1 h) precedes the half-time for particle budding and secretion (2 h), indicates that disulphide-linked oligomers are an intermediate in the HBsAg assembly. Pulse-chase experiments in the presence of BfA showed that newly synthesized monomers were converted into a stable population of dimers. Dimer crosslinks were formed efficiently in the ER while oligomer crosslink formation was



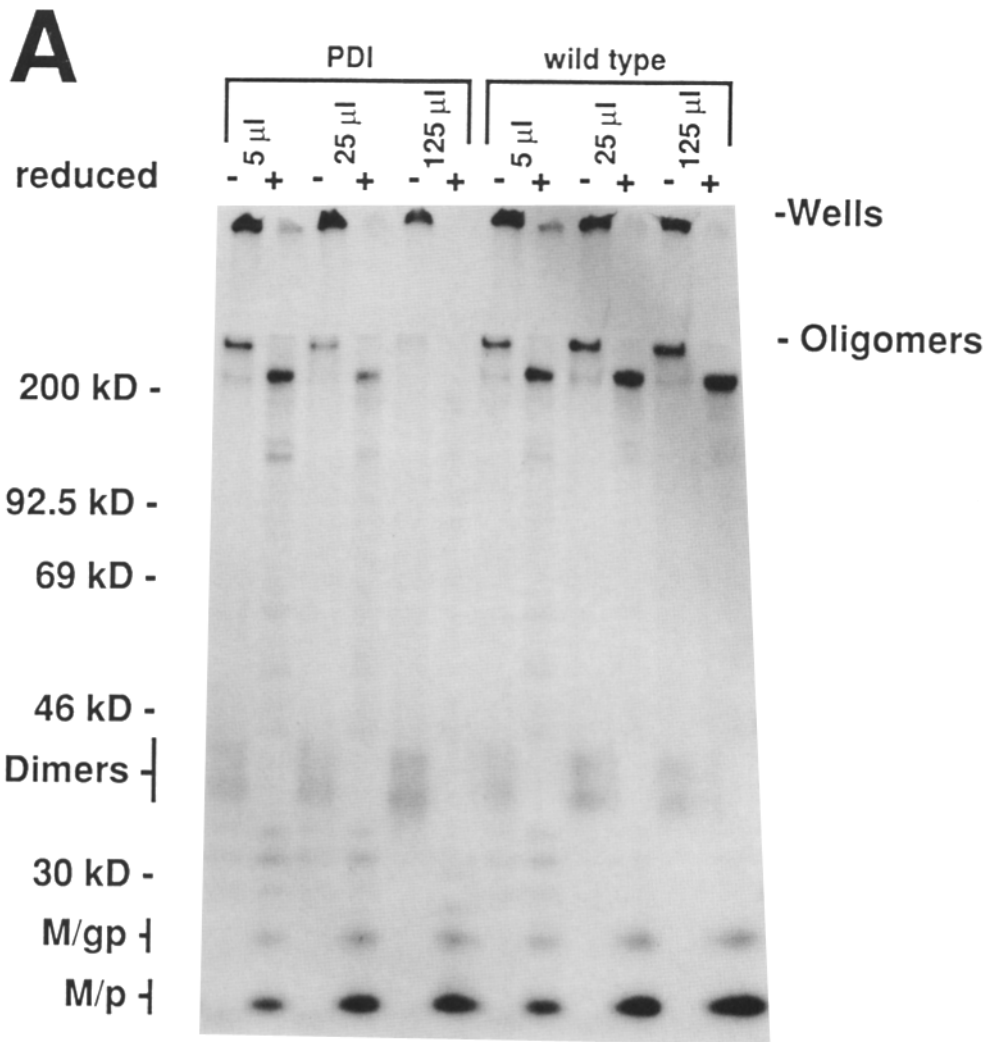
**Figure 8.** Effect of Brefeldin A treatment on HBsAg distribution in SV24 cells. Indirect double immunofluorescence of HBsAg (*a*, rhodamine) and PDI (*b*, labeled with 1D3 followed by fluorescein-labeled second antibody) on BfA-treated SV24 cells shows that Brefeldin A does not cause HBsAg to recycle back to the ER, indicating that the HBsAg maturation compartment is distinct from the Golgi. A region in which HBsAg is concentrated and from which PDI is excluded has been marked by an arrow. *c* shows a pulse-chase of SV24 cells in the presence of BfA. *d* shows the quantitation of the experiment depicted in *c* with the total monomers (▲) and dimers (●) seen in the nonreduced lanes represented as a percentage of total immunoprecipitable HBsAg. Note that disulphide crosslinking halts at the stage of dimer formation. Bar, 10  $\mu$ m.

blocked. The complementary experiment in which mature, highly crosslinked, secreted HBsAg particles were incubated in the presence of PDI containing lysate showed the conversion of oligomers to dimers demonstrating that oligomeric crosslinks are not stable in the presence of PDI. Although we have no evidence concerning which cysteines are involved in the two classes of bonds, this experiment validates our distinguishing them; the free energy of the dimer crosslinks must be lower than that of the oligomer crosslinks since incubation with a catalyst causes the conversion of oligomers to dimers.

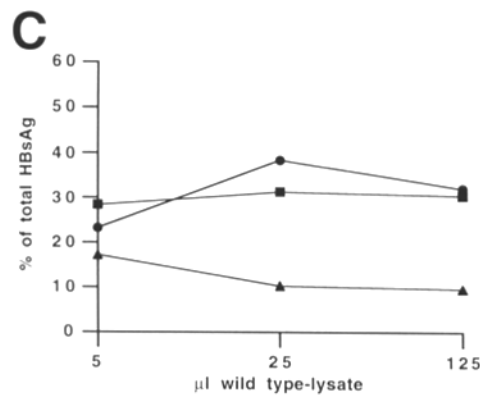
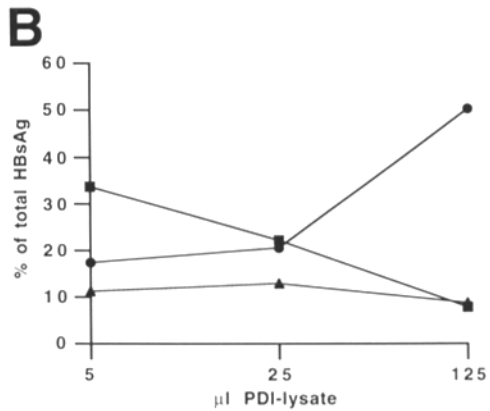
Together these results yield a picture of HBsAg lipoprotein particle assembly in which rapid dimer formation in the ER is followed by segregation of HBsAg to an intermediate compartment in which the absence of PDI allows extensive intermolecular disulphide crosslinking of the dimers and budding of the lipoprotein particle to occur.

### Maturation Pathway of HBsAg

The role of this intermediate compartment in the biosynthesis of the HBsAg lipoprotein particle is indicated in the model (Fig. 10) which extends that of Ganem and co-workers (Eble et al., 1986; Simon et al., 1988) by identifying the compartments in which events occur more precisely. Newly synthesized HBsAg is translocated across to the lumen of the ER (Eble et al., 1986; Simon et al., 1988) where disulphide-linked dimers are formed rapidly (<2 min) in the presence of PDI. The concentration of dimers continues to increase until 60 min after the pulse. Our cycloheximide data indicates that the bulk of the HBsAg is segregated to the PDI excluding region at the time that dimers become the majority of the population. After the transport of dimers to a post-ER compartment (intermediate compartment), the absence of PDI allows the formation of oligomer crosslinks. Concomi-



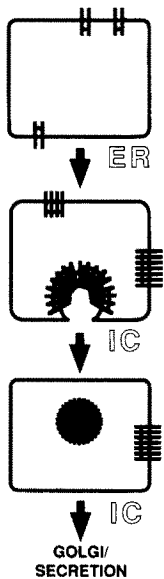
**Figure 9.** Incubation of HBsAg with PDI in vitro. The autoradiograph in *a* shows the effect of incubating secreted,  $^{35}\text{S}$ -labeled HBsAg particles with increasing amounts of Sf9 cell lysate from cells infected with control virus (*wild type*) or PDI-cDNA bearing virus. The plots in *b* (control virus-*wild type*) and *c* (rat PDI-cDNA bearing-*PDI*) show the amounts of the different forms of HBsAg after incubation with varying amounts of cell lysate. The monomer ( $\blacktriangle$ ) and dimer ( $\bullet$ ) components are defined as in Fig. 6. The oligomer ( $\blacksquare$ ) components are the combination of the material that did not enter the separating gel (Wells) and the very high molecular weight material that enters the gel and disappears after DTT reduction. The specific effect of increased PDI expression in the lysate is the conversion of the oligomeric forms of HBsAg to dimers.



tant with oligomer formation, HBsAg becomes resistant to protease digestion from the cytosolic side and becomes carbonate extractable (Simon et al., 1988) reflecting the budding of the oligomer into the lumen. Host membrane proteins are excluded from these oligomers, perhaps as a result of the tight interaction between HBsAg dimers. After budding into this compartment, the crosslinked, oligomeric, lipoprotein particles pass rapidly through the Golgi where

they acquire endoglycosidase H resistance before their secretion into the medium.

Our results also do not directly address the state of the HBsAg in the virion. During viral infection, the bulk of HBsAg follows the pathway which we have described in this paper but a fraction of it forms the envelope of budding virions. Budding of the virion requires interaction with the capsid, or core protein of this DNA virus in a manner which



**Figure 10.** A model for HBsAg particle maturation in the cell. Newly synthesized HBsAg is translocated into the ER lumen (ER) where disulphide-linked dimers form rapidly in the presence of PDI. Dimers are then transported to an ER-Golgi intermediate compartment (IC), where HBsAg forms higher oligomers and assembles into extensively disulphide crosslinked particles. This assembly process is the rate limiting step in the HBsAg particle secretion. The mature particles are then transported rapidly through the Golgi and are secreted. The depiction of the dimer and oligomer crosslinks should be interpreted schematically.

is presumed to be similar to that of other enveloped viruses. Virion formation makes use of all three forms of the glycoprotein, S (24 kD, which we have described in this work) and the two longer forms S2 (31 kD) and S1 (39 kD) which are produced from three in frame ATGs in the S gene (Ganem and Varmus, 1987). S2 and S are found in both the virion and the lipoprotein particles while S1 is found only in the virion. An appealing hypothesis is that the virion incorporates the dimeric forms of the glycoprotein (S, S1, S2, and hybrids) before they exit from the ER while the excess S and S2 dimers are transported to an intermediate compartment in which maturation to lipoprotein particles occurs. This hypothesis is supported by the observation that coexpression of the longer S1 form of the HBsAg glycoprotein blocks the secretion of all forms of HBsAg (Pershing et al., 1986). Since the S1 form has been shown to have a retention signal (Kuroki et al., 1989), formation of oligomers with the other forms of HBsAg would prevent their transport and maturation. The scavenging of the S1 form by budding virions would then allow the other forms to proceed to the intermediate compartment and to lipoprotein particle formation.

### Quality Control and Intermediate Compartments

The relationship revealed in this work between disulphide bond formation and secretion for the HBsAg lipoprotein particle extends that seen in previous studies on simple protein oligomers. Elegant studies of the biosynthesis of a number of membrane and secretory proteins have resulted in the concept of quality control (Hurtley and Helenius, 1989), which posits that only properly disulphide-linked and oligomerized proteins are allowed to leave the ER. Although this has been ably demonstrated for simple oligomers, the case of highly crosslinked particles such as that formed by HBsAg is more complex. The initial disulphide-linked dimers appear to meet the normal requirements of quality control and hence they exit from the ER. The later pre-Golgi step of maturation occurs in a distinct, intermediate compartment. A second quality control step occurs here which prevents the further transport of immature HBsAg; only HBsAg in lipoprotein particles transits the Golgi as shown by the correspondence

of endoglycosidase H resistance, carbonate extractability and secretion. A similar two-stage assembly and control process also seems to occur for the von Willebrand factor (reviewed by Wagner, 1990) which functions in blood vessel injury repair, hemostasis, and in promoting endothelial cell adhesion. This glycoprotein is found in the plasma as a series of disulphide crosslinked multimers built from a single subunit. These large multimers appear to be formed in *trans*- or post-Golgi compartments. The two-stage nature of quality control is reflected in the fact that only after protein has dimerized in the ER is it transported from this compartment and that only crosslinked forms are secreted from the cell. In both cases, exit from the ER precedes the formation of the highly crosslinked oligomers.

Our conclusion that the assembly of HBsAg dimers into crosslinked oligomeric, lipoprotein particles occurs in a separate compartment rests on several assumptions about the nature of compartments along the secretory pathway. We use the word "compartment" to mean a region that lies on the secretory pathway, is distinct in composition from the compartments which precede and follow it, and is morphologically distinct from other compartments. Clearly, the compartment in which oligomer crosslinks are formed in the HBsAg lipoprotein particle lies on the secretory pathway since the overwhelming majority of the HBsAg is eventually secreted from cells. The composition of this compartment is distinct from that of the ER since it lacks soluble ER proteins and from that of the Golgi since it lacks the enzymes responsible for conferring endoglycosidase H resistance. The morphological separation of the compartment is demonstrated by our immunocytochemistry which shows that the region from which PDI is excluded is far larger than that corresponding to an HBsAg particle and by the observation that HBsAg segregation is unaffected by BfA treatment.

The finding that assembly of HBsAg dimers into the secreted lipoprotein particle occurs in an intermediate compartment between the ER and the Golgi ascribes a new role to this compartment in biosynthesis. Saraste and Kuismanen (1984) first identified this compartment by showing 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 tubulovesicular compartment distinct from the ER and the Golgi. An important and, to many workers, defining function is the retrieval of the soluble resident ER proteins containing the KDEL retention sequence at their carboxy termini, such as PDI and BiP (Munro and Pelham, 1987). Munro and Pelham (1987) hypothesized the existence of a salvage compartment from which escaped KDEL terminated proteins could be retrieved by recycling to the ER. Since the extensive disulphide crosslinking is a consequence of the exclusion of PDI it is likely that the compartment in which oligomer crosslinks form is either identical to or later than the salvage compartment. We have also shown here that rab2, an established marker of an intermediate compartment (Chavier et al., 1990) is found in the HBsAg enriched, PDI excluding regions. This salvage compartment has been proposed to be the same as the 15°C compartment (Lippincott-Schwartz, 1990; Pelham, 1988, 1989; Vaux et al., 1990; Warren, 1987), although colocalization with a viral glycoprotein under conditions of 15°C block has not yet been published.

Although a number of authors refer to "the" ER-Golgi intermediate compartment, the diversity of functions that have been attributed to it open the possibility that this compartment is heterogeneous. An ER-Golgi intermediate compartment has been identified as the site of the interaction of MHC class I with antigen (Cox et al., 1989; Nuchtern et al., 1989; Yewdell et al., 1989), and several proteins including p53 and p58, two transmembrane proteins of still unknown function (Schweizer et al., 1988; Saraste and Svensson, 1991), have been localized to it. Tooze et al. (1988) showed that the first step of the O-glycosylation of a coronavirus glycoprotein, the addition of N-acetylgalactosamine, occurred in the same ER-Golgi intermediate compartment as budding of viral particles. Palmitoylation of proteins (Bonatti et al., 1989) and the initial step in the formation of the lysosomal mannose-6-phosphate targeting signal, the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylgalactosamine-1-phosphate to newly synthesized lysosomal enzymes have also been shown to occur in a post-ER pre-Golgi compartment (Rizzolo et al., 1985; Rizzolo and Kornfeld, 1988; Kornfeld and Mellman, 1989; Lazzarino and Gabel, 1988; Pelham, 1988). Recent work shows that these functions may be in distinct compartments between the ER and the Golgi complex. Schweizer et al. (1991) isolated a fraction from Vero cells that is enriched in p53 and has properties different from either the ER or the cis-Golgi. Further, Bonatti et al. (1989) demonstrated that protein palmitoylation occurs in a compartment which is later than the 15°C block but before mannosidase I trimming of glycans. We are pursuing experiments to further characterize the compartment which is involved in the maturation of HBsAg lipoprotein particles and to relate it to other markers of this region.

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#### References

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

Bayer, M. E., B. S. Blumberg, and B. Werner. 1968. Particle associated with

Australia antigen in the sera of patients with leukemia, Down's syndrome and hepatitis. *Nature (Lond.)*. 218:1057-1059.

Bole, D. G., L. M. Hendershot, and J. F. Kearney. 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, and K. Simons. 1989. Palmitoylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. *J. Biol. Chem.* 264:12590-12595.

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

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

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

Crowley, C. W., C.-C. Liu, and Levinson, A. D. 1983. Plasmid directed synthesis of hepatitis B surface antigen in monkey cells. *Mol. Cell Biol.* 3:44-45.

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

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

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

Dubois, M. F., C. Pourcel, S. Rousset, C. Chany, and P. Tiollais. 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with clonal viral DNA. *Proc. Natl. Acad. Sci. USA*. 77:4549-4553.

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

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., and D. A. Hillson. 1980. Formation of disulphide bonds. In *The Enzymology of Post-Translational Modifications of Proteins*. Vol. 1. R. B. Freedman and H. C. Hawkins, editors. Academic Press, London. 157-212.

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

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

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

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

Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (Lond.)*. 306:387-389.

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

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

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

Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Techn.* 1:3-8.

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

Koch, G. L. E., D. R. J. Macer, and F. B. P. Wooding. 1988. Endoplasmic reticulum chaperonin. *J. Cell Sci.* 90:485-491.

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

Kuroda, S., Otaka, S. Miyazaki, T. Nakao, M., and Y. Fujisawa. 1992. Hepatitis B virus envelope L protein particles—Synthesis and assembly in *Saccharomyces cerevisiae*, purification and characterization. *J. Biol. Chem.* 267:1953-1961.

Kuroki, K., R. Russnack, and D. Ganem. 1989. Novel N-terminal amino acid sequence required for retention of a hepatitis B virus glycoprotein in the endoplasmic reticulum. *Mol. Cell Biol.* 9:4459-4466.

Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of <sup>3</sup>H and <sup>14</sup>C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335-341.

Laub, O., L. B. Randall, M. Truett, Y. Schaul, D. N. Standing, P. Valenzuela, and W. J. Rutter. 1983. Synthesis of hepatitis B surface antigen in mammalian cells: expression of the entire gene and the coding region. *J. Virol.*

- 48:271-280.
- Lazzarino, D. A., and C. A. Gabel. 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.
- Lewis, M. J., R. A. Mazzarella, and M. Green. 1985. Structure and assembly of the endoplasmic reticulum. *J. Biol. Chem.* 260:3050-3057.
- Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with Brefeldin A: evidence for the membrane cycling from Golgi to ER. *Cell.* 56:801-813.
- Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 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.
- Liu, C.-C., D. Yansura, and A. D. Levinson. 1982. Direct expression of hepatitis B surface antigen in monkey cells from an SV40 vector. *DNA.* 1:213-221.
- Luckow, V. A., and M. D. Summers. 1988. Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology.* 167:56-71.
- Macer, D. R. J., and G. L. E. Koch. 1988. Identification of a set of calcium binding proteins in reticuloplasm, the luminal content of the endoplasmic reticulum. *J. Cell Sci.* 91:61-70.
- Marquardt, T., and A. Helenius. 1992. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. *J. Cell Biol.* 117:503-513.
- Mazzarella, R. A., and M. Green. 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.* 262:8875-8883.
- McAleer, W. J., E. B. Byunak, R. Z. Maigetter, D. E. Wampler, and M. R. Hilleman. 1984. Human hepatitis B vaccine from recombinant yeast. *Nature (Lond.).* 307:178-180.
- Moriarty, A. M., B. H. Hoyer, J. W.-K. Shih, J. L. Gerin, and D. H. Hamer. 1981. Expression of the hepatitis B virus surface antigen gene in cell culture using a simian virus 40 vector. *Proc. Natl. Acad. Sci. USA.* 78:2606-2610.
- Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899-907.
- Nuchtern, J. G., S. Bonafacino, W. B. Bidison, and R. D. Klausner. 1989. Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. *Nature (Lond.).* 339:223-226.
- Patzer, E. J., G. R. Nakamura, C. C. Simonsen, A. D. Levinson, and R. Brands. 1986. Intracellular assembly and packaging of hepatitis B surface antigen particles occur in the endoplasmic reticulum. *J. Virol.* 58:884-892.
- Patzer, E. J., G. R. Nakamura, and A. Yaffe. 1984. Intracellular transport and secretion of hepatitis B surface antigen in mammalian cells. *J. Virol.* 51:346-353.
- Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose regulated proteins. *Cell.* 46:959-961.
- Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913-918.
- Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1-23.
- Pershing, D. H., H. E. Varmus, and D. Ganem. 1986. Inhibition of the secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science (Wash. D.C.).* 234:1388-1391.
- Peterson, D. L. 1987. The structure of hepatitis B surface antigen and its antigenic sites. *Bioessays.* 6:258-262.
- Peterson, D. L., F. Gavilanes, D. A. Paul, and D. T. Achord. 1987. Hepatitis B surface antigen: protein structure and the development of alternative hepatitis B vaccines. In *Advances in Hepatitis Research*. F. V. Chisari, editor. Masson, New York. 30-39.
- Rizzolo, L. J., and R. Kornfeld. 1988. Post-translational protein modification in the endoplasmic reticulum: Demonstration of fatty acylase and deoxymannojirimycin-sensitive alpha-mannosidase activities. *J. Biol. Chem.* 263:9520-9525.
- Rizzolo, L. J., J. Finidori, A. Gonzales, M. Arpin, I. E. Ivanov, M. Adesik, and D. D. Sabatini. 1985. Biosynthesis and intracellular sorting of growth hormone-viral envelope glycoprotein hybrids. *J. Cell Biol.* 101:1351-1362.
- Robinson, W. S., and L. I. Lutwick. 1976. The virus of hepatitis, type B (first of two parts). *N. Engl. J. Med.* 295:1168-1175.
- Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki forest virus membrane proteins to the cell surface. *Cell.* 38:535-549.
- Saraste, J., and Svensson, K. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415-430.
- Schweizer, A., J. Fransen, T. Bachi, L. Ginsel, and H.-P. Hauri. 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.
- Schweizer, A., J. A. M. Fransen, K. Matter, T. E. Kreis, L. Ginsel, and H.-P. Hauri. 1990. Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. *Eur. J. Cell Biol.* 53:185-196.
- Schweizer, A., K. Matter, C. M. Ketcham, and H.-P. Hauri. 1991. The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and cis-Golgi. *J. Cell Biol.* 113:45-54.
- Shibayama, T., T. Watanabe, H. Kojima, A. Yoshikawa, S. Watanabe, T. Kamimura, S. Suzuki, and F. Ichida. 1984. Studies by immune electron microscopy of hepatitis B surface antigen in PLC/PRF/5 cells. *J. Med. Virol.* 13:205-214.
- Shiu, R. P. C., J. Pouyssegur, and I. Pastan. 1977. Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA.* 74:3840-3844.
- Simon, K., V. R. Lingappa, and D. Ganem. 1988. Secreted hepatitis B surface antigen polypeptides are derived from a transmembrane precursor. *J. Cell Biol.* 107:2163-2168.
- Sukeno, N., R. Shirachi, J. Yamaguchi, and N. Ishida. 1972. Reduction and reoxidation of Australia antigen: loss and reconstitution of particle structure and antigenicity. *J. Virol.* 9:182-183.
- Summers, M. D., and G. E. Smith. 1988. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin. Texas A&M University, College Station, TX. 1555:26-32.
- Takatsuki, A., and G. Tamura. 1985. Brefeldin A, a specific inhibitor of intracellular translocation of Vesicular Stomatitis virus G protein: Intracellular accumulation of high mannose type G protein and inhibition of its cell surface expression. *Agric. Biol. Chem.* 45:899-902.
- Tartakoff, A. M. 1986. Temperature and energy dependence of secretory protein transport in the exocrine pancreas. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1477-1482.
- Tooze, S. A., J. Tooze, and G. Warren. 1988. Site of addition of N-acetylgalactosamine to the E1 glycoprotein of a mouse hepatitis virus-A59. *J. Cell Biol.* 106:1475-1487.
- Tooze, J., S. A. Tooze, and S. D. Fuller. 1987. Sorting of coronavirus from condensed secretory proteins at the exit from the trans-Golgi network of AtT20 cells. *J. Cell Biol.* 105:1215-1226.
- Tooze, J., H. F. Kern, S. D. Fuller, and K. E. Howell. 1989. Condensation-sorting events in the rough endoplasmic reticulum of exocrine pancreatic cells. *J. Cell Biol.* 109:35-50.
- Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotypic antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal. *Nature (Lond.).* 345:495-502.
- Vyas, G. N., K. R. Rao, and A. B. Ibrahim. 1972. Australia antigen (Hepatitis B antigen): a conformational antigen dependent on disulfide bonds. *Science (Wash. DC).* 178:1300-1301.
- Wagner, D. D. 1990. Cell biology of von Willebrand factor. *Annu. Rev. Cell Biol.* 6:217-246.
- Wampler, D. E., E. D. Lehman, J. Boger, W. J. McAleer, and E. M. Scolnick. 1985. Multiple chemical forms of hepatitis surface antigen produced in yeast. *Proc. Natl. Acad. Sci. USA.* 82:6830-6834.
- Warren, G. 1987. Signals and salvage sequences. *Nature (Lond.).* 327:17-18.
- Yewdell, J. W., and J. R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. *Science (Wash. DC).* 244:715-718.