Nonlysosomal, pre-Golgi Degradation of Unassembled Asialoglycoprotein Receptor Subunits: A TLCK- and TPCK-sensitive Cleavage within the ER

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Abstract. The human asialoglycoprotein receptor subunit H2a is cotranslationally inserted into the ER membrane. When expressed together with subunit H1 in mouse fibroblasts part forms a hetero-oligomer that is transported to the cell surface, but when expressed alone it is all rapidly degraded. Degradation is insensitive to lysosomotropic agents and the undegraded precursor is last detected in the ER region of the cell. Small amounts of an intermediate 35-kD degradation product can be detected (Amara, J. F., G. Lederkremer, and H. F. Lodish. 1989. *J. Cell Biol.* 109:3315). We show here that the oligosaccharides on both precursor H2a and the 35-kD fragment are $Man_{6-9}GlcNAc_2$, structures typically found in pre-Golgi compartments. Subcellular fractionation shows that the intermediate degradation product does not cofractionate with the lysosomal enzyme β-galactosidase, but is found in a part of the ER that contains ribosomes. Thus the intermediate degradation product is localized in the ER, indicating that the initial degradation event does take place in the ER.

All degradation of H2a, including the initial endoproteolytic cleavage generating the 35-kD intermediate, is blocked by the protease inhibitors *N*-tosyl-L-lysine chloromethyl ketone and *N*-tosyl-L-phenylalanine chloromethyl ketone. These drugs do not inhibit ER-to-Golgi transport of H1. Depleting the cells of ATP or inhibiting protein synthesis allows the initial endoproteolytic cleavage to occur, but blocks further degradation of the 35-kD intermediate; thus we can convert all cellular H2 into the 35-kD intermediate. Approximately 50% of H2b, a splicing variant differing from H2a by a five amino acid deletion, can be transported to the cell surface, and the rest appears to be degraded by the same pathway as H2a, both when expressed alone in fibroblasts and together with H1 in HepG2 cells. Addition of *N*-tosyl-L-lysine chloromethyl ketone or *N*-tosyl-L-phenylalanine chloromethyl ketone blocks degradation of the ~50% that is not transported, but does not affect the fraction of H2b that moves to the Golgi region. Thus, a protein destined for degradation will not be transported to the Golgi region if degradation is inhibited.

CELL surface molecules often exist as oligo- or multimeric complexes. The assembly of individual subunits into complexes occurs in the ER and is in many cases a prerequisite for transport out of the ER (reviewed by Hurtley and Helenius, 1989). The oligomerization process can be viewed as a quality control step where aberrant or unassembled polypeptides and incompletely assembled complexes are prevented from leaving the ER. The degradation of single subunits or incompletely assembled complexes has been described for the T cell receptor (TCR)[1] α and β subunits (Lippincott-Schwartz et al., 1988) and the asialoglycoprotein receptor H2 subunit (Amara et al., 1989). In both instances the degradation was rapid, independent of lysosomal function, and insensitive to inhibitors of lysosomal proteases. Based on the findings that the intact precursor protein could last be detected in the ER by a variety of methods, it was suggested that degradation took place in or in close association to the ER, and the term "ER-degradation" was coined.

Here we show that indeed the ER is the site for initiation of degradation of unassembled subunits, using the asialoglycoprotein receptor as a model system. The functional human asialoglycoprotein receptor is composed of two homologous subunits, H1 and H2, forming at least a trimer $(H1)_2(H2)$ (Bischoff et al., 1988). H1 and H2 are N-glycosylated proteins oriented in the ER membrane with their COOH-terminal domains in the ER lumen and the NH_2 termini in the cytoplasm. Both chains are needed to form a high-affinity receptor (Shia and Lodish, 1989). Lederkremer and Lodish (1990) showed that a human hepatoma cell line (HepG2) expresses two forms of H2, H2a and H2b, arising by alterna-

1. *Abbreviations used in this paper*: Endo D, endoglycosidase D; Endo H, endoglycosidase H; E 64, *N*-(*N*-[L-3-*trans*-carboxyoxiran-2-carbonyl]-L-leucyl)-agmatine; GlcNAc, *N*-acetyl-glucosamine; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; man, mannose; TCR, T cell receptor.

tive splicing of a 15-bp mini-exon encoding five amino acids next to the transmembrane region in the exoplasmic domain. H2b, which lacks the five amino acids, accounts for >90% of H2 RNA. In fibroblasts expressing either H2 subunit alone, 20–40% of H2b, but no H2a, reaches the cell surface. The remainder is degraded. Here we demonstrate a *N*-tosyl-L-lysine chloromethyl ketone (TLCK)- and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-sensitive proteolytic step initiating the degradation. No degradation of precursor H2a or H2b occurs in the presence of these protease inhibitors, but also there is no increase in the amount of H2b that reaches the Golgi region, as compared to untreated cells, indicating an early, irreversible differential tagging or sorting of proteins destined for degradation and proteins destined for transport.

Importantly, Amara et al. (1989) found that, concomitant with the disappearance of precursor H2a, small amounts of a soluble 35-kD polypeptide could be detected. The fragment contains the COOH terminus and is a product of an endoproteolytic cleavage near the five amino acid insertion. This fragment could not be detected in the medium. By finding ways to block the degradation of the fragment without affecting the initial cleavage, we were able to accumulate sufficient amounts of the intermediate degradation product to use it as a marker for studying the initial cleavage in the degradation process. By localizing the 35-kD intermediate to the rough ER, where the intact precursor H2b is also found, we established that the TLCK- and TPCK-sensitive endoproteolytic cleavage must take place in that organelle.

Materials and Methods

Materials

Materials were obtained from the following sources: L-[^{35}S]cysteine (specific radioactivity of >1,000 Ci/mmole) from ICN Radiochemicals (Irvine, CA); D-[2-^{3}H(N)]-mannose (specific radioactivity 30 Ci/mmole) from New England Nuclear Du Pont Products (Boston, MA); ^{14}C-methylated protein standards from Amersham Corp. (Arlington Heights, IL); Affigel-protein A from Bio-Rad (Richmond, CA); cycloheximide, anisomycin, emetine, 2,4-dinitrophenol, aprotinin, chymostatin, PMSF, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-glucoside, and 3-methyl adenine from Sigma Chemical Co. (St. Louis, MO); α2-macroglobulin, bestatin, *N*-(*N*-[L-3-*trans*-carboxyoxiran-2-carbonyl]-L-leucyl)-agmatine (E-64), leupeptin, pepstatin, TLCK and TPCK from Boehringer-Mannheim Biochemicals (Indianapolis, IN); Pronase from Calbiochem-Behring Corp. (La Jolla, CA); bicinchoninic acid protein assay reagent from Pierce Chemical Co. (Rockford, IL); calf serum, FCS, MEM, DME, glutamine, and penicillin/streptomycin from Gibco Laboratories (Grand Island, NY); cysteine-free media from Hazelton Products (Denver, PA); tissue culture dishes from Costar (Cambridge, MA) and Falcon Labware (Oxnard, CA).

Cell Culture

NIH 3T3 fibroblasts cell lines 1-7-1, expressing H1 and H2b; 2-18, expressing H2a (Shia and Lodish, 1989); and 2C, expressing H2b (Lederkremer and Lodish, 1990) were grown in DME supplemented with glutamine, penicillin, streptomycin, and 10% calf serum under an atmosphere of 5% CO_2. HepG2 cells were grown in MEM with glutamine, penicillin, streptomycin, and 10% FCS. The cell monolayers were trypsinized and split into 35-, 60-, or 100-mm tissue culture dishes at least 2 d before use. The medium for cells incubated at 18°C outside the incubator was supplemented with 20 mM Hepes, pH 7.2.

Metabolic Labeling

Subconfluent cell monolayers were rinsed twice in medium devoid of cysteine but supplemented with 10% dialyzed serum, glutamine, penicillin, and streptomycin and preincubated for 30 min in this medium. The cells were labeled with [^{35}S]cysteine (200–600 μCi/ml) for various lengths of time at 37°C. After the pulse, the plates were washed three times with cold PBS and transferred to ice, or with 37°C medium containing cysteine and returned to the incubator for different chase periods in cysteine-containing medium.

Immunoprecipitation and Electrophoresis

Cells were lysed in PBS containing 1% Triton X 100/0.5% sodium deoxycholate/10 mM EDTA/2 mM PMSF (lysis buffer) for 1 h on ice, and debris and nuclei were pelleted in a microfuge for 20 min at 4°C. An aliquot of the supernatant, referred to as the cell extract, was spotted on Whatman filter paper circles for TCA precipitation, and another aliquot was used for determining the protein concentration, using the bicinchoninic acid kit with BSA as standard. For experiments involving only a short chase (<3 h) the same amount of acid precipitable radioactivity, found to be equivalent to the amount of total protein, was immunoprecipitated. For immunoprecipitation of samples that had been subjected to longer chase periods, the same amount of protein was used for each sample, typically 150 μg, and the volume was adjusted to 150 μl with lysis buffer. The samples were precleared by incubating them on ice for 1 h with 2 μl normal rabbit serum and then adding 12 μl of a 25% Affigel-protein A slurry per μl of serum, rotating the samples for 30 min in 4°C, and pelleting the beads 20 s in a microfuge. The supernatant was incubated with 3 μl antiserum per 100 μg of protein at 0°C overnight. The samples were then incubated with Affigel-Protein A as above, and the beads were washed and processed for SDS-PAGE analysis on a 10% gel under reducing conditions and fluorography as described in Bischoff and Lodish (1987). The antisera used were: H2 COOH antibodies, raised against a peptide of the 12 most COOH-terminal amino acids of H2, and H1 COOH antibodies, raised against a peptide of the 15 most COOH-terminal amino acids of H1 (Bischoff et al., 1988) and were kindly provided by Drs. Bischoff and Shia (Whitehead Institute, Cambridge, MA).

Subcellular Fractionation on Sucrose/D_2O Gradients

HepG2 cells grown on 100-mm plates were metabolically labeled as described, rinsed with PBS, and scraped off in 10 mM Hepes, pH 7.2, 0.25 M sucrose. After 10 min on ice, the cells were homogenized with 10 strokes of a tight-fitting Dounce homogenizer. Intact cells and nuclei were spun down (centrifuge model RC-3B; Sorvall Instruments, Newton, CT) for 10 min at 1,000 rpm and the supernatant was loaded on a preformed sucrose gradient consisting of 1 ml each of 10, 15, 20, 25, 30, 34, 38, 42, 46, and 50% sucrose in D_2O with 10 mM Hepes, pH 7.2. The gradient was centrifugated for 3 h (rotor model SW 41; Beckman Instruments, Palo Alto, CA) at 36,000 rpm and 4°C, and fractions were collected from the top. An aliquot from each fraction was mixed with an equal volume of two-times concentrated lysis buffer and subjected to immunoprecipitation with anti-H2 COOH antibodies. Another aliquot was used for determining α-glucosidase activity.

Separation of Lysosomes from ER/Golgi Vesicles on Percoll Gradients

HepG2 cells grown on 100-mm plates were metabolically labeled as described, rinsed with PBS, and scraped off the plate in 10 mM triethanolamine/1 mM EDTA/0.25 mM sucrose. After 20 min on ice, the cells were homogenized with a ball bearing stainless steel homogenizer (1.85×10^{-3} in clearance) (Balch and Rothman, 1985). Intact cells and nuclei were pelleted (centrifuge model RC-3B; Sorvall Instruments Div., Newton, CT) for 10 min at 2,000 rpm and the supernatant was loaded on a preformed Percoll gradient consisting of 1 ml each of 5, 10, 15, 20, 25, 30, 35, 40, and 45% of a Percoll stock solution (90% Percoll in 10 mM triethanolamine/1 mM EDTA/0.25 mM sucrose). On the bottom of the gradient was a 1-ml cushion of 2.5 M sucrose. The gradient was centrifuged for 1 h in a Beckman SW 41 rotor (model SW41; Beckman Instruments Inc., Palo Alto, CA) at 24,500 rpm and 4°C and fractions were collected from the bottom. The fractions were subjected to immunoprecipitation as described above, and aliquots were taken for determination of β-galactosidase activity.

Enzyme Assays

To determine β-galactosidase activity, 50 μl of the gradient fractions were mixed with 10 μl of 20% Triton X 100, 20 μl of 0.5 M citrate buffer, pH 3.5, and 2 μl of 0.2 M *p*-nitrophenol-β-D-galactopyranoside. The reaction

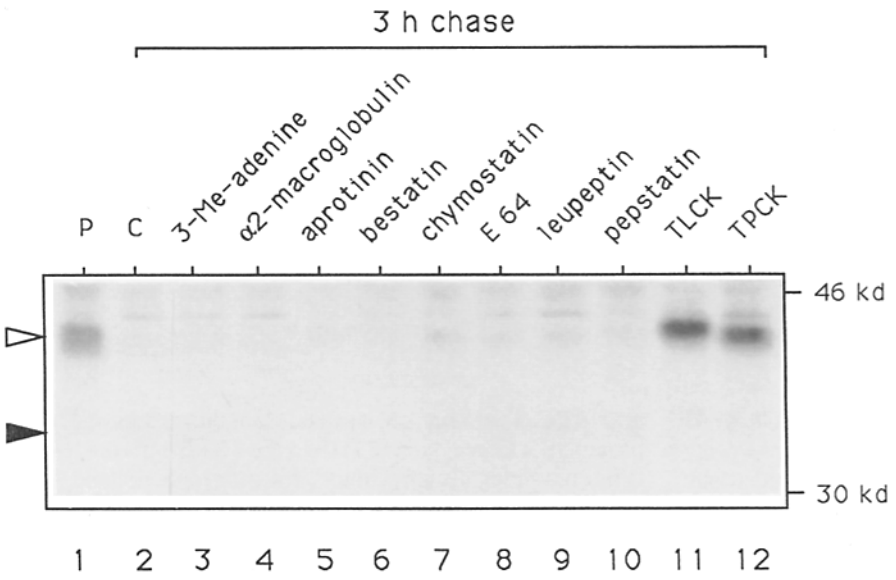

Figure 1. Protease inhibitors TLCK and TPCK block degradation of H2a. 2-18 cells were pulse labeled with ^{35}S-cysteine for 30 min, washed, and chased for 3 h in nonradioactive medium containing protease inhibitors. (Lane *1*) pulse only; (lane 2) chase without inhibitor; (lane *3*) chase with 1 mg/ml 3-methyl adenine; (lane *4*) chase with 0.2 mg/ml α2-macroglobulin; (lane *5*) chase with 0.2 inh. U/ml aprotinin; (lane *6*) chase with 50 μg/ml bestatin; (lane *7*) chase with 0.2 mg/ml chymostatin; (lane *8*) chase with 1 μg/ml E 64; (lane *9*) chase with 0.4 mg/ml leupeptin; (lane *10*) chase with 1 μg/ml pepstatin; (lane *11*) chase with 100 μM TLCK; (lane *12*) chase with 20 μM TPCK. Cell extracts were immunoprecipitated with H2 COOH antibodies and subjected to SDS-PAGE and fluorography. The open arrow indicates the precursor form of H2a and the solid arrow the position of the 35-kD intermediate degradation product.

mixture was incubated for 5 h at 37°C, and terminated by adding 1 ml of 0.2 M Na_2CO_3; the OD_{410} was read.

α-glucosidase assay was performed essentially according to Michael and Kornfeld (1980) by mixing 50 μl of the gradient fraction with 10 μl of 10% Triton X 100, 10 μl of 25 mM of *p*-nitrophenol-α-D-glucoside, 10 μl of 1 M phosphate buffer, pH 6.9, and 20 μl of H_2O. The reaction mixture was incubated for 5 h at 37°C, and terminated by adding 1 ml of 0.2 M Na_2CO_3; the OD_{410} was read. This assay will detect the activities of both α-glucosidase I and II, and the activity is completely inhibited by 1-deoxynojirimycin (Lodish and Kong, unpublished observation), a specific inhibitor of these enzymes (Saunier et al., 1982).

Oligosaccharide Analysis

2-18 cells were labeled with ^{3}H-mannose (200 μCi/ml) for 3 h in low glucose (100 mg/l) medium, solubilized, and immunoprecipitated with H2 COOH antibodies, and run on an SDS-PAGE, as described. The bands corresponding to intact H2a and the 35-kD fragment were excised from the gel. The gel pieces were incubated with Pronase and the glycopeptides were processed as described by Bischoff et al. (1986). The free oligosaccharides were size fractionated by HPLC on a Micropax AX-5 column by the method of Mellis and Baenziger (1981). Starting at acetonitrile/water 60:40 and increasing the proportion of water, the oligosaccharides were eluted and fractions collected. Aliquots from each fraction were taken for determining the radioactivity by liquid scintillation counting. Standards were prepared from CHO cells labeled with ^{3}H-mannose for 3 h, as described by Bischoff et al. (1986).

Results

TLCK and TPCK Block Degradation of H2a

Fig. 1, lane *1* shows that in 2-18 cells synthesizing H2a, a pulse label reveals a 43-kD precursor with Endo H–sensitive oligosaccharides (Amara et al., 1989). After a 3-h chase all of it has been degraded (Fig. 1, lane 2). Furthermore, of all protease inhibitors tested, only TLCK and TPCK block degradation of the H2a precursor (Fig. 1, lanes *11* and *12*). TLCK acts on trypsin and related proteases, and can also inhibit endoproteolytic cleavage by some serine and cysteine proteases, whereas TPCK inhibits chymotrypsin and some endoproteases, including cysteine proteases. The difference in size of H2a accumulating in TLCK- and TPCK-treated cells is not always seen, and we do not know the significance of the observation. Preincubation of cells with inhibitors before labeling gave similar results but were more difficult to interpret, since some of the inhibitors also reduced the level of protein biosynthesis. TLCK- or TPCK-treated cells, however, showed the same incorporation of ^{35}S-cysteine into H2a as did control cells (data not shown). 3-Methyl adenine, a specific inhibitor of autophagy (Seglen and Gordon, 1982) did not interfere with H2 degradation (Fig. 1, lane *3*).

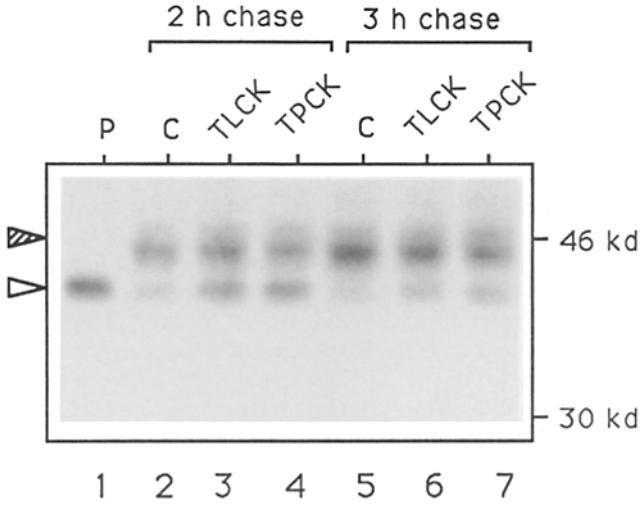

Figure 2. TLCK and TPCK do not affect intracellular transport and maturation of H1. 1-7-1 cells were pulse labeled with ^{35}S-cysteine for 30 min, washed, and chased for 2 or 3 h in the absence (lanes *2* and *5*) or presence of 100 μM TLCK (lanes *3* and *6*) or 20 μM TPCK (lanes *4* and *7*). Cell extracts were immunoprecipitated with H1 COOH-terminal antibodies and subjected to SDS-PAGE and fluorography. The open arrow indicates the H1 precursor and the striped arrow the mature form of H1.

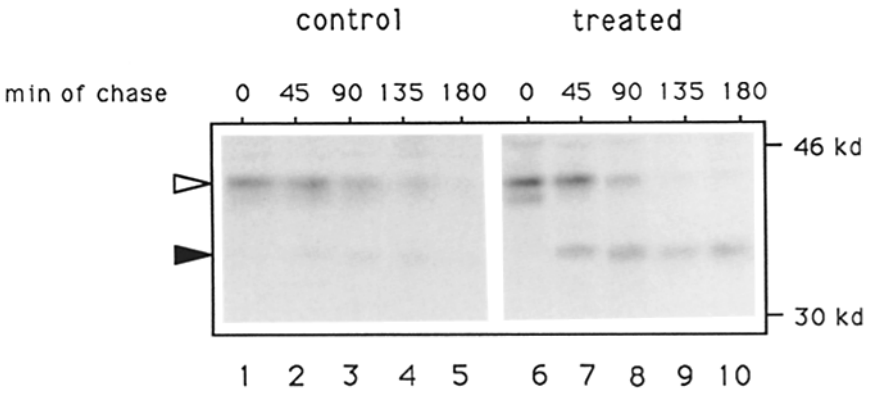

Figure 3. Effects of 2,4-dinitrophenol and cycloheximide on degradation of H2a. 2-18 cells were pulse labeled with ^{35}S-cysteine for 15 min, washed, and chased in nonradioactive medium for the times shown in the absence (lanes *1–5*) or presence (lanes *6–10*) of 0.3 mM cycloheximide, 1 mM 2,4-dinitrophenol, and 5 mM 2-deoxy-D-glucose. Cell extracts were immunoprecipitated with H2 COOH antibodies and subjected to SDS-PAGE analysis and fluorography. The open arrow indicates the H2a precursor and the solid arrow the 35-kD intermediate degradation product. Molecular weight standards are shown on the right.

To establish whether the actions of TPCK and TLCK were specific or because of toxic effects, we carried out an experiment in which we followed the intracellular transport and glycosylation of H1 in drug-treated cells (Fig. 2). In 1-7-1 cells, which express both H1 and H2b, H1 is synthesized as a 40-kD precursor (lane *1*) and is converted after a 2- or 3-h chase to a 46-kD species with Endo H–resistant oligosaccharides (Shia and Lodish, 1989) (lanes *2* and *5*). TLCK and TPCK did not block conversion of precursor H1 to the 46-kD species (lanes *3, 4, 6,* and *7*). In control 1-7-1 cells, conversion of precursor H1 to the mature species is not quantitative; about half of the precursor is degraded. In TLCK- or TPCK-treated cells, the H1 that would have been degraded is recovered as the 40-kD precursor (lanes *3, 4, 6,* and *7*). Thus, in 1-7-1 cells TLCK and TPCK do not block maturation of the normal amount of H1 through the Golgi but do block degradation of the ~50% of the H1 precursor that would normally be destroyed.

Inhibitors of Protein Biosynthesis or ATP Production Block Further Degradation of the 35-kD Degradation Intermediate

Small amounts of a 35-kD putative degradation intermediate can be observed during degradation of H2a in 2-18 cells. The fragment is recognized by H2 COOH-terminal antibodies but not by antibodies recognizing a peptide in the NH_2-terminal cytoplasmic domain (Amara et al., 1989). The size of the fragment corresponds to that of the exoplasmic domain of H2a, which suggests that it is the product of an endoproteolytic cleavage near the five amino acids that differentiate H2a from H2b. Since a degradation intermediate would be an important tool for localizing the degradation process, we set out to block selectively subsequent steps of degradation. Disappearance of the 35-kD fragment was followed as the loss of reactivity in the 35-kD fragment, using the H2 COOH antibody.

Lanes *1–5* in Fig. 3 show the time course of degradation of H2a precursor in untreated 2-18 cells. Small amounts of a 35-kD intermediate could be detected after a 45-min chase period (lane *2*), and by 180 min of chase (lane *5*), all precursor H2a and 35-kD fragment had disappeared. If cycloheximide, 2,4-dinitrophenol, and 2-deoxy-D-glucose are added to the chase medium, all pulse-labeled H2a precursor is converted to a 35-kD fragment (lanes *7–10*). As before, the 35-kD fragment is immunoprecipitable with the H2 COOH antibody, but not with an NH_2-terminal antibody (Amara et al., 1989). Therefore, we conclude that the cleavage is endoproteolytic. Conversion of H2a to the 35-kD intermediate in cells containing cycloheximide, 2,4-dinitrophenol and 2-deoxy-D-glucose is quantitative, considering the loss of three cysteines because of endoproteolysis: the H2a precursor has 11 cysteine residues (Spiess and Lodish, 1985), and the 35-kD fragment probably eight.

Fig. 4 shows that conversion of precursor H2a (lane *1*) to the 35-kD fragment during a 3-h chase is less complete when 2,4-dinitrophenol and 2-deoxy-D-glucose (lane *3*) and cycloheximide (lane *4*) are added separately than when they are

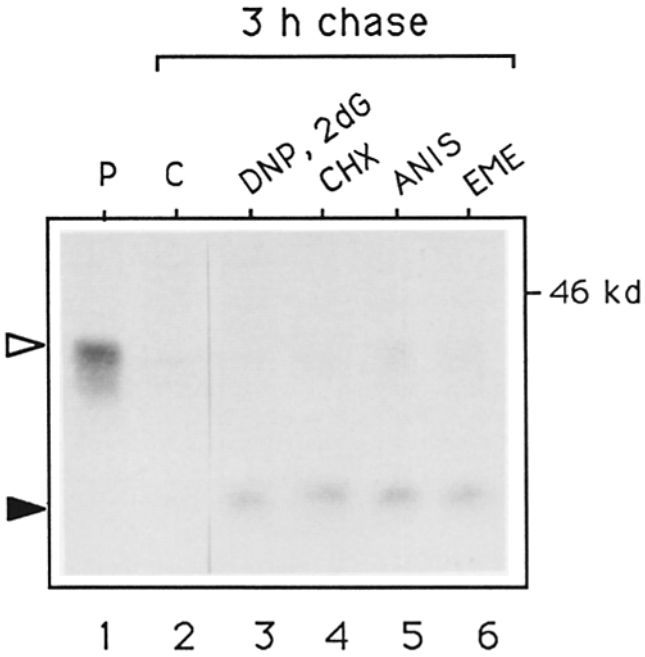

Figure 4. Inhibitors of ATP production or protein synthesis block degradation of the 35-kD intermediate fragment. 2-18 cells were pulse labeled with ^{35}S-cysteine for 30 min (lane *1*); washed and chased for 3 h in nonradioactive medium without drugs (lane *2*); or containing 1 mM 2,4-dinitrophenol and 5 mM 2-deoxy-D-glucose (lane *3*); 0.3 mM cycloheximide (lane *4*); 0.5 μM anisomycin (lane *5*); or 0.5 μM emetine (lane *6*). The cell extracts were immunoprecipitated with H2 COOH antibodies and subjected to SDS-PAGE and fluorography. The open arrow indicates the H2a precursor and the solid arrow the 35-kD intermediate degradation product.

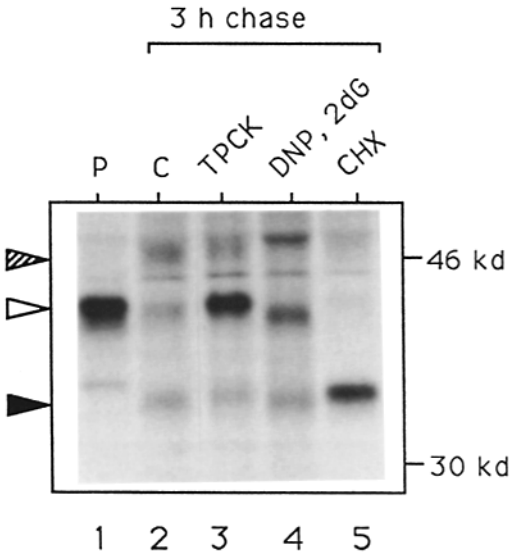

Figure 5. Effects of cycloheximide, 2,4-dinitrophenol, and 2-deoxy-D-glucose and TPCK on the intracellular fate of H2b. 2C cells were pulse labeled with ^{35}S-cysteine for 30 min, washed, and chased in nonradioactive medium for 3 h under the following conditions: (lane 2) without drugs; (lane *3*) in the presence of 20 μM TPCK; (lane *4*) in the presence of 1 mM 2,4-dinitrophenol and 5 mM 2-deoxy-D-glucose; and (lane *5*) in the presence of 0.3 mM cycloheximide. Lane *1* represents the pulse-labeled sample. The cell extracts were immunoprecipitated with H2 COOH antibodies, and subjected to SDS-PAGE and fluorography. The open arrow indicates precursor H2b, the solid arrow the 35-kD intermediate degradation product, and the striped arrow the mature form of H2b.

added together (compare with Fig. 3). Anisomycin and emetine (lanes *5* and *6*) have the same effect as cycloheximide. The concentrations of these drugs are sufficient to inhibit completely protein biosynthesis in 3T3 fibroblasts (data not shown).

Precursor H2b Is Degraded by the Same Pathway as H2a When Expressed Alone in Fibroblasts or Together with H1 in HepG2 Cells

Lederkremer and Lodish (1990) showed that between 20 and 40% of H2b reaches the cell surface when expressed alone in fibroblasts (2C cells). What happens to the remaining H2b? Is it degraded by the same pathway as H2a, or are the extra five amino acids in H2a a target sequence for an endoprotease or signal for sorting to a putative degradation compartment? In an attempt to approach these questions we conducted a pulse-chase experiment in 2C cells (Fig. 5) using the inhibitors we found useful in studying degradation of H2a. A pulse label (lane *1*) reveals the 43-kD H2b precursor. After a 3-h chase in the absence of drugs (lane *2*), roughly equal amounts of the precursor, a mature 50-kD form and the 35-kD intermediate are seen. If TPCK is present during the chase (lane *3*), the amount of mature H2b roughly equals that of the control cells (lane *2*), indicating that TPCK does not interfere with transport and maturation of H2b. Importantly, more precursor is found in TPCK-treated cells, consistent with TPCK inhibition of the initial endoproteolysis of H2a (Fig. 1). For unknown reasons, some of the 35-kD fragment is also formed. Addition of 2,4-dinitrophenol and 2-deoxy-D-glucose to the chase medium (Fig. 5, lane *4*), partially blocks degradation of the 35-kD fragment; ER-to-Golgi transport of H2b is also reduced, resulting in an increased level of H2b precursor, and a decrease in the level of mature protein. In the presence of cycloheximide (Fig. 5, lane *5*), the level of mature H2b is comparable to that of the control, indicating no effect of cycloheximide on ER-to-Golgi transport. Importantly, no precursor can be detected; instead virtually all precursor H2b has been converted to the 35-kD fragment. Thus, in the presence of cycloheximide, the remaining portion of H2b that would be degraded during a 3-h chase (compare lanes *1* and *2*) is converted to the 35-kD intermediate. We conclude that 20–40% of precursor H2b in 2C cells will move from the ER to the Golgi in the absence (Fig. 5, lane *2*) or presence of drugs (lanes *3* and *5*). Apparently some segregation occurs early after biosynthesis between H2b molecules destined for Golgi transport and those that will be degraded; the fraction destined for degradation does not mature to the Golgi if degradation is blocked by TPCK.

The reason for the slight difference in mobility between the fragment generated in cycloheximide-treated cells (lane *4*) and in 2,4-dinitrophenol–treated cells (lane *5*) is probably because of differences in carbohydrate processing. The 35-kD fragment is in both cases resistant to cleavage by Endo D, but after digestion with Endo H the size of both fragments appears to be the same (data not shown). A similar size difference of the 35-kD fragments is seen in HepG2 cells (see Fig. 7, lanes *B3* and *B4*). The 35-kD fragment derived from H2b is recognized by H2 COOH antibodies, but not by NH_2-terminal antibodies (data not shown), suggesting a similar origin for the 35-kD H2b fragment as for the 35-kD H2a fragment. Thus the extra five amino acids in H2a do not constitute an obligatory degradation signal; rather H2b can

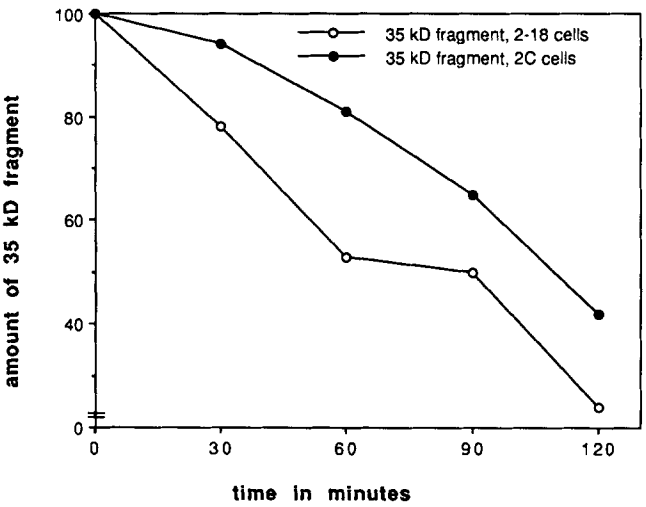

Figure 6. Degradation of the 35-kD intermediate forms of H2a and H2b. 2-18 (○) and 2C (●) cells were pulse labeled with ^{35}S-cysteine for 30 min and chased in nonradioactive medium for 3 h in the presence of 0.3 mM cycloheximide. The cycloheximide was removed and the incubation continued in the absence of drugs for the indicated periods of time. The cell extracts were immunoprecipitated with H2 COOH antibodies and the samples subjected to SDS-PAGE analysis and fluorography. The bands on the autoradiogram were quantitated by densitometric scanning and normalized to the amount present at 0 min.

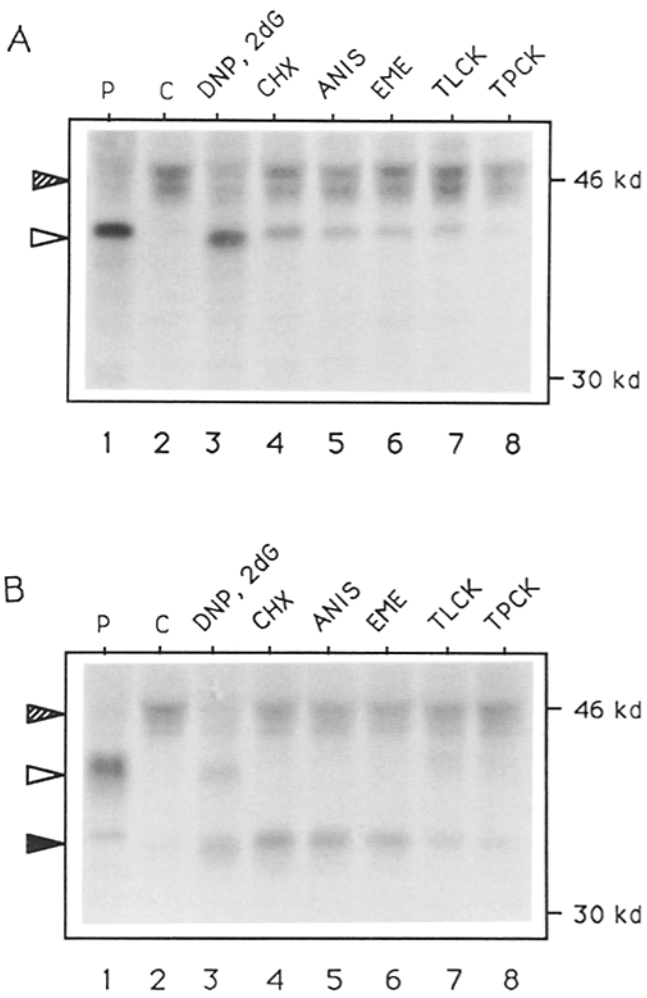

Figure 7. The fates of H1 and H2 in HepG2 cells treated with 2,4-dinitrophenol, 2-deoxy-D-glucose, cycloheximide, anisomycin, emetine, TPCK, and TLCK. HepG2 cells were pulse labeled with ^{35}S-cysteine for 30 min (lanes *A1* and *B1*) and chased in nonradioactive medium for 4 h in the absence (lanes *A2* and *B2*) or presence of 1 mM 2,4-dinitrophenol and 5 mM 2-deoxy-D-glucose (lanes *A3* and *B3*); 0.3 mM cycloheximide (lanes *A4* and *B4*); 0.5 μM anisomycin (lanes *A5* and *B5*); 0.5 μM emetine (lanes *A6* and *B6*); 100 μM TLCK (lanes *A7* and *B7*); or 20 μM TPCK (lanes *A8* and *B8*). One half of each sample was immunoprecipitated with H1 COOH antibodies (*A*) and the other half with H2 COOH antibodies (*B*) and then the samples were subjected to SDS-PAGE analysis and fluorography. The open arrow indicates precursor H1 or H2, the solid arrow the 35-kD intermediate degradation product of H2, and the striped arrow indicates the mature forms of H1 or H2.

be degraded by a mechanism that shows the same inhibitor sensitivity as does H2a.

To obtain information on the kinetics of degradation of the 35-kD fragment, we designed the following experiment (Fig. 6): 2-18 (expressing H2a) and 2C (expressing H2b) cells were pulse labeled for 30 min and chased for 3 h in the presence of cycloheximide to accumulate the 35-kD fragment. At zero minutes on the figure, the drug was removed and the incubation continued. The relative amounts of H2a and H2b were quantified by densitometric scanning of the autoradiogram. Disappearance of the 35-kD fragment generated from H2a (*open circles*) and H2b (*closed circles*) occur with roughly similar kinetics. The half-life of H2a is ~90 min and the H2b 35-kD fragment is degraded slower than that from H2a.

Studies by Bischoff and Lodish (1987) show that in HepG2 cells only about two thirds of newly synthesized H1 and H2 subunits reach the cell surface; a third is degraded. (As noted, >90% of H2 in HepG2 cells is H2b, as judged by RNA quantification). We wanted to study H2 degradation in this more normal system, where H2 is expressed together with H1. To this end, HepG2 cells were pulse labeled and then chased in the presence or absence of drugs (Fig. 7). Lanes *A1* and *B1* show the pulse-labeled precursor forms of H1 and H2, respectively, and the mature forms, generated in untreated cells after a 4-h chase are seen in lanes *A2* and *B2*. Cells treated with inhibitors of protein biosynthesis (lanes *A4, A5,* and *A6*) or TLCK and TPCK (lanes *A7* and *A8*) show the same level of mature H1 as control (lane *A2*), again showing that these inhibitors have no effect on intracellular maturation of H1. In cells treated with cycloheximide (lane *A4*), anisomycin (lane *A5*), emetine (lane *A6*), and to some extent TLCK (lane *A7*), some precursor protein (*open arrow*) can still be detected. The effect of 2,4-dinitrophenol and 2-deoxy-D-glucose (Fig. 7, lane *A3*), a lower level of mature protein and an increased level of precursor H1, can be attributed mainly to inhibited ER-to-Golgi transport.

Similarly, the level of Golgi-processed mature H2 is not affected by inhibitors of protein biosynthesis (lanes *B4, B5,* and *B6*) or TLCK or TPCK (lanes *B7* and *B8*). 2,4-Dinitrophenol and 2-deoxy-D-glucose (Fig. 7, lane *B3*), cycloheximide (lane *B4*), anisomycin (lane *B5*), and emetine (lane *B6*) all cause accumulation of the 35-kD intermediate (*solid arrow*). That is, the fraction of precursor H2 that is degraded in control cells (lane *B2*) is converted to a 35-kD intermediate by the same treatments that in 2C cells cause all precursor H2b to be converted into the 35-kD intermediate fragment (Fig. 5). Cells treated with 2,4-dinitrophenol and 2-deoxy-D-glucose (Fig. 7, lane *3*) do accumulate some H2 precursor protein and show reduced levels of mature H2, probably because of inhibition of intracellular transport of H2. The effects of TLCK and TPCK on degradation of H1 and especially H2 in HepG2 cells are not as strong as in 2-18 or 2C fibroblasts. We do not know why. One possibility is that the drugs cannot enter into HepG2 cells as easily.

Oligosaccharide Analyses of Precursor H2a and the 35-kD Fragment Indicate a Pre-Golgi Localization

The subcellular localization of the 35-kD intermediate in H2 degradation need not be the same as the site of the initial TLCK- and TPCK-sensitive endoproteolytic degradation that generates it, nor the site of its ultimate degradation. However, since we can show, by several criteria, that the 35-kD fragment is in the ER, it would appear that at least the initial stage of degradation occurs in that organelle.

Determining the composition of oligosaccharides on glycoproteins is a widely used method to obtain information about the compartment in which a protein destined for transport is residing (for review see Kornfeld and Kornfeld, 1985). We know that the three N-linked glycans on both precursor H2a and the 35-kD fragment are resistant to cleavage by Endo D, but sensitive to Endo H (Amara et al., 1989). To obtain more detailed information about them, HPLC analysis was performed on the glycans from H2a and the 35-

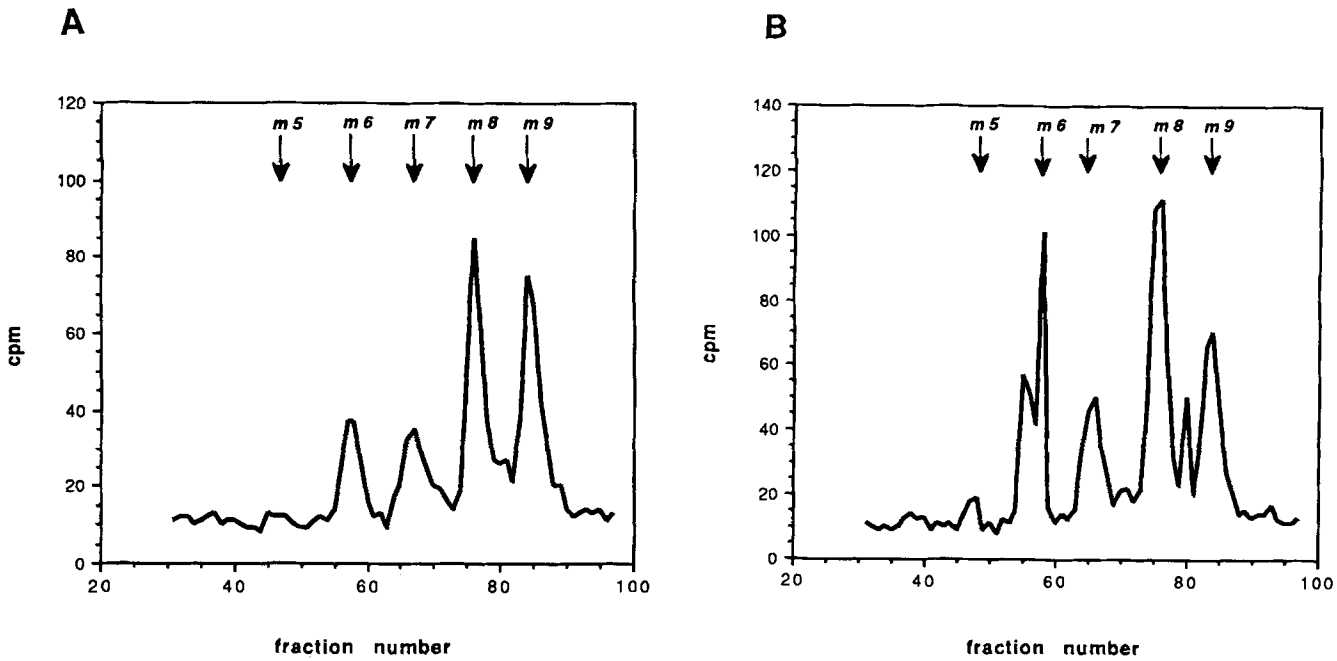

Figure 8. Only high mannose oligosaccharides are seen on precursor H2a and the 35-kD intermediate degradation product. 2-18 cells were labeled with D-[2-^{3}H(N)]-mannose for 3 h in low glucose medium, immunoprecipitated with H2 COOH antibodies, and analyzed by SDS-PAGE. The bands corresponding to H2a and the 35-kD fragment were recovered from the gel. The oligosaccharides from H2a (*A*) and the 35-kD fragment (*B*) were released, purified, and size fractionated by HPLC on a Micropax AX-5 column. The collected fractions were analyzed for radioactivity by liquid scintillation counting. Standards were prepared according to Bischoff et al. (1986). Positions of $Man_5GlcNAc$ to $Man_9GlcNAc$ are designated by m_5 to m_9.

kD fragment (Fig. 8). Fig. 8 *A* shows that the H2a precursor has mainly $Man_9GlcNAc_2$ and $Man_8GlcNac_2$ oligosaccharides, and so does the 35-kD fragment (Fig. 8 *B*), which also contains substantial amounts of $Man_6GlcNAc_2$. No significant amounts of $Man_5GlcNAc_2$ can be detected on either H2a or the 35-kD fragment. The enzyme responsible for catalyzing the formation of $Man_5GlcNAc_2$, Golgi-α-mannosidase 1, is thought to reside in the *cis*-Golgi (Kornfeld and Kornfeld, 1985), and the lack of $Man_5GlcNAc_2$ on H2a and the 35-kD intermediate indicates that neither of the molecules has reached the Golgi complex. We do not know the identity of the single-fraction peaks seen between $Man_8GlcNAc$ and $Man_9GlcNAc$ and between $Man_5GlcNAc$ and $Man_6GlcNac$. They do not arise by readdition of glucose residues as has been seen for some ER-localized proteins (Suh et al., 1989), since they are completely digested by Jack Bean α-mannosidase (J. Bischoff, personal communication).

The 35-kD Fragment is Absent from Lysosomes and Localized to the Rough ER

Previous evidence against participation of lysosomes in degradation from the ER comes from experiments showing absence of inhibition by lysosomotropic agents or inhibitors of lysosomal proteases, and also showing absence of any intact precursor protein in lysosomes. By showing (Fig. 9) that the 35-kD fragment is not in lysosomes, we can eliminate this organelle as the site of the initial endoproteolytic cleavage. In this experiment we separate lysosomes from ER/Golgi vesicles on Percoll gradients. These studies, as well as separations using D_2O/sucrose gradients (below), were done on HepG2 cells, not on transfected fibroblasts, since preliminary fractionation of fibroblasts did not cleanly resolve lysosomes, rough ER, and Golgi (not shown).

HepG2 cells were pulse labeled with radioactive cysteine for 15 min (Fig. 9 *A*), or 30 min followed by a 4-h chase in the presence of cycloheximide, to convert about one third of the H2 precursor to the 35-kD fragment (Fig. 9 *B*). We used labeled transferrin as a reference ER protein, as it exits the ER slowly; after a pulse or chase virtually all labeled transferrin in the cell is in the ER (Lodish et al., 1983). In both gradients between 55 and 65% of the lysosomal enzyme β-galactosidase was detected in the three bottom fractions, whereas virtually all 43-kD precursor H2 and transferrin (Fig. 9 *A*) and all mature H2 and intermediate 35-kD fragment (Fig. 9 *B*) was localized to the middle fractions of the gradient. We conclude that the initial endoproteolytic degradation step does not occur in lysosomes.

In an attempt to localize further the compartment in which the intermediate degradation product accumulates, we turned to subcellular fractionation on D_2O/sucrose gradients (Lodish et al., 1983, 1987). HepG2 cells were pulse labeled (Fig. 10 *A*) or chased for 4 h in the presence of cycloheximide (Fig. 10, *B* and *C*). Following the protocol of Lodish and Kong (1990) the postnuclear supernatant loaded on the gradient shown in Fig. 10 *C* was pretreated with EDTA and RNase to strip off ribosomes and decrease the density of the rough ER. In this study, α-glucosidase activity was used as a marker for the ER, since α-glucosidases I and II reside in the ER and act on ER-localized substrates (Hubbard and Robbins, 1979; Kornfeld and Kornfeld, 1985; Lucocq et al., 1986). Similar results were obtained using Endo H-sensitive transferrin precursor as an ER marker (not shown). After a pulse (Fig. 10 *A*), α-glucosidase activity and precursor H2 are

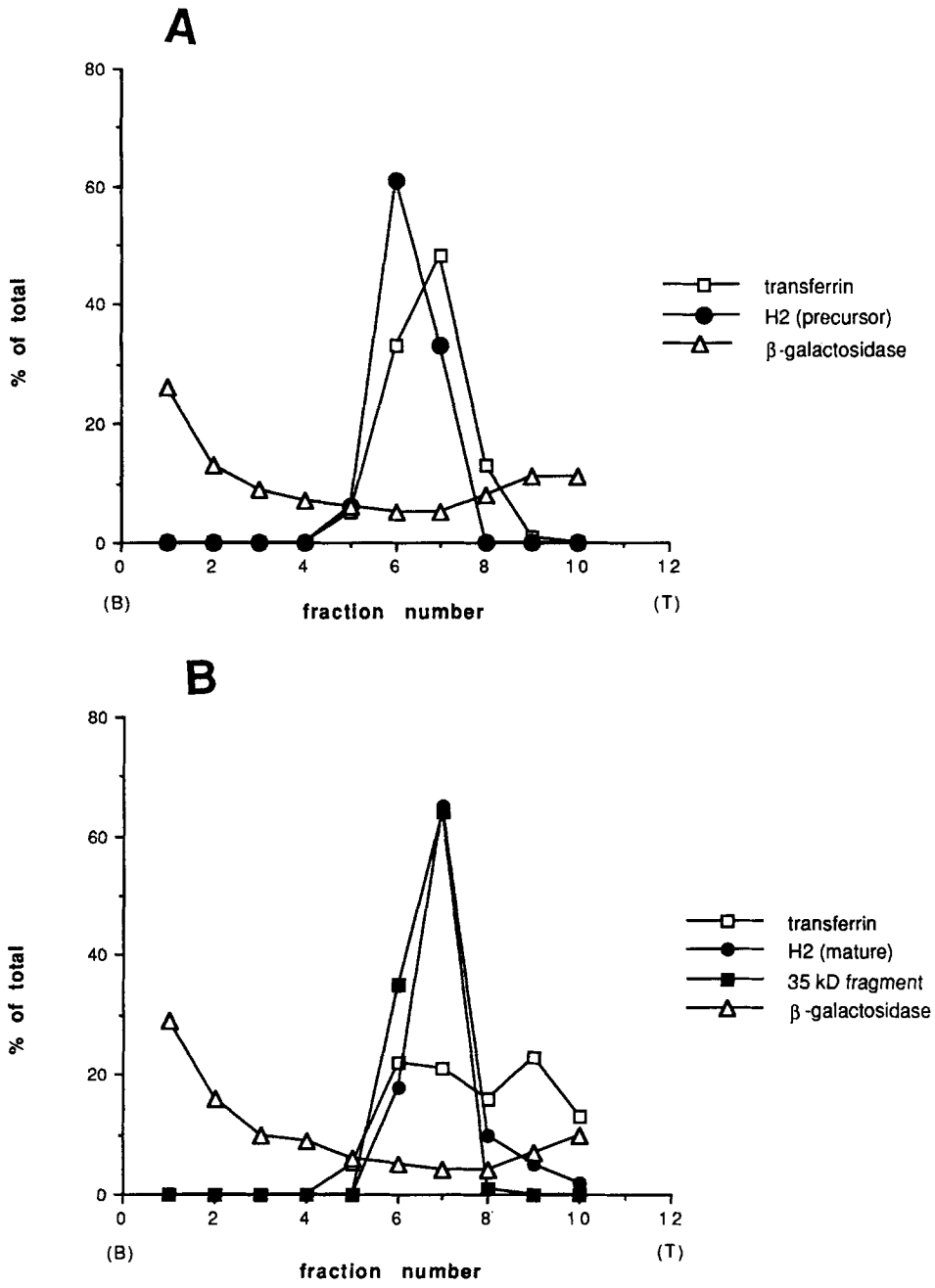

Figure 9. H2 and the 35-kD intermediate degradation product do not cofractionate with lysosomes. HepG2 cells were labeled with ^{35}S-cysteine for 15 min (*A*) or 30 min followed by a 4-h chase in the presence of 0.3 mM cycloheximide (*B*). The cells were homogenized, and the postnuclear supernatants were loaded on 5–45% preformed Percoll gradients with cushions of 2.5 M sucrose. After 1 h of centrifugation (rotor model SW41; Beckman Instruments) at 24,500 rpm, 10 fractions were collected, assayed for β-galactosidase activity, and immunoprecipitated with antibodies against H2 COOH and transferrin. The immunoprecipitated material was separated by SDS-PAGE and subjected to fluorography. The quantification of precursor and mature H2, the 35-kD H2 fragment, and precursor and mature transferrin was done by densitometric scanning of the autoradiograms, and normalized to the total amount recovered in all fractions.

found mainly in fractions 7–10 (21–31% sucrose), defining the ER. After a 4-h chase in the presence of cycloheximide (Fig. 10 *B*), a portion of H2 has reached the Golgi and acquired complex glycans. It bands in a less dense region, fractions 3–5 (Fig. 10 *B*, indicated by arrows), that correspond to the density of Golgi and plasma membranes. Importantly, a portion of H2 has been converted to the 35-kD intermediate, and virtually all bands in fractions 7–10 coincident with ER α-glucosidase. Upon EDTA and RNAse treatment (Fig. 10 *C*), vesicles containing both the 35-kD intermediate and α-glucosidase activity band at a slightly lighter density (mainly fractions 5–7 [16–23% sucrose]). The mature H2 in the Golgi still bands in fractions 3–5. We conclude that the density of vesicles containing the 35-kD degradation intermediate is indistinguishable from that of an authentic ER protein (α-glucosidase) as well as that of transferrin precursors, both with and without removal of ribosomes by RNAse and EDTA. Thus, the 35-kD intermediate resides in a part of the ER that contains ribosomes, and the initial endoproteolytic cleavage that generates it must also be localized to that organelle.

Discussion

Our data suggests that degradation of H2a occurs in at least two distinct steps. First, the intact H2a molecule is clipped by an endoprotease, generating a 35-kD fragment. Judging from the size of the fragment and its reactivity with antibodies, the clip occurs on the exoplasmic side of the transmembrane domain. The 35-kD fragment is probably soluble, and

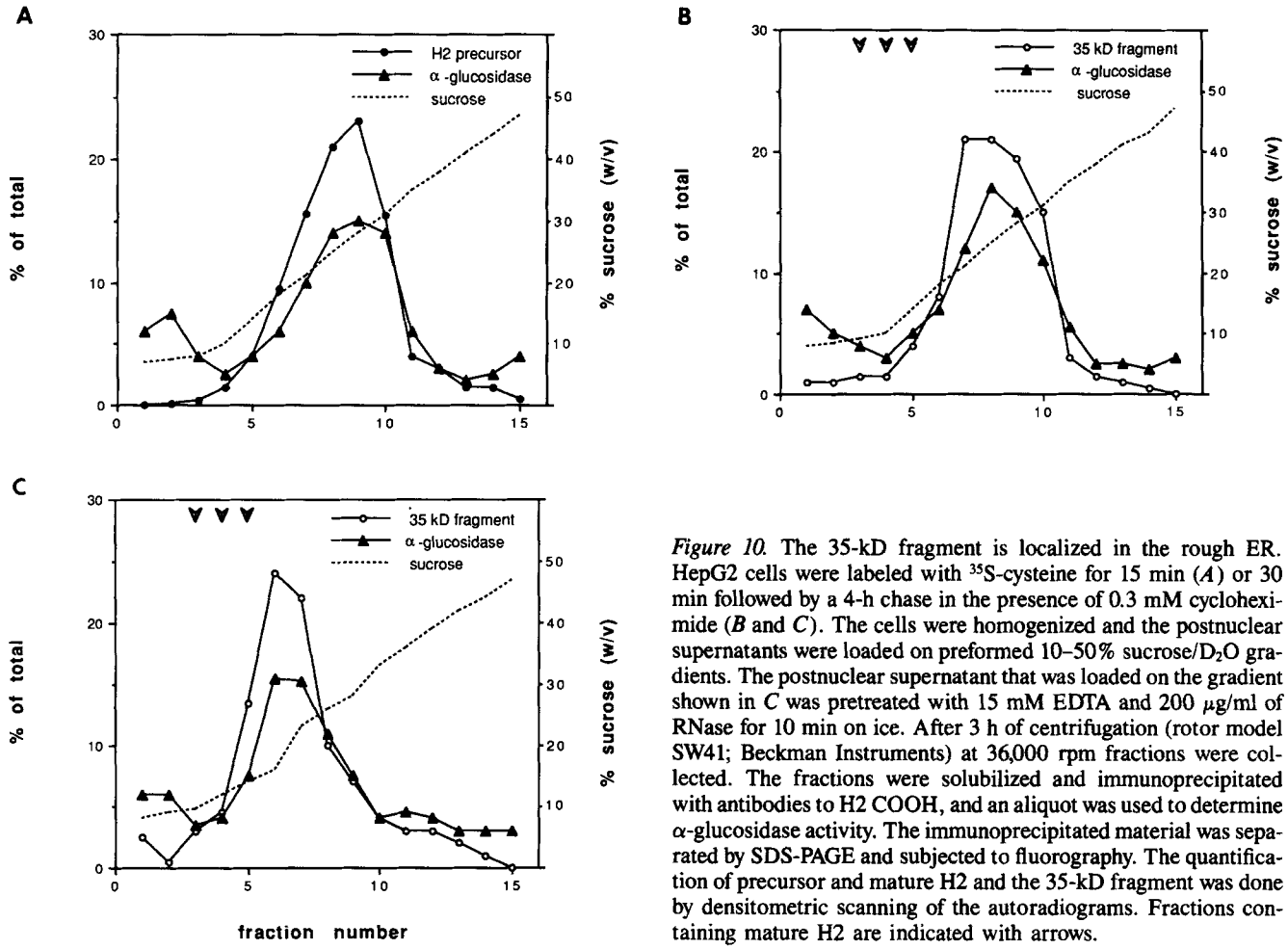

Figure 10. The 35-kD fragment is localized in the rough ER. HepG2 cells were labeled with ^{35}S-cysteine for 15 min (*A*) or 30 min followed by a 4-h chase in the presence of 0.3 mM cycloheximide (*B* and *C*). The cells were homogenized and the postnuclear supernatants were loaded on preformed 10–50% sucrose/D_2O gradients. The postnuclear supernatant that was loaded on the gradient shown in *C* was pretreated with 15 mM EDTA and 200 μg/ml of RNase for 10 min on ice. After 3 h of centrifugation (rotor model SW41; Beckman Instruments) at 36,000 rpm fractions were collected. The fractions were solubilized and immunoprecipitated with antibodies to H2 COOH, and an aliquot was used to determine α-glucosidase activity. The immunoprecipitated material was separated by SDS-PAGE and subjected to fluorography. The quantification of precursor and mature H2 and the 35-kD fragment was done by densitometric scanning of the autoradiograms. Fractions containing mature H2 are indicated with arrows.

since the size of the fragment is slightly different for H2a and H2b (Fig. 6), we believe that the clip occurs NH_2-terminal to the extra five amino acids in H2a. The initial endoproteolytic cleavage is inhibited by protease inhibitors TLCK and TPCK (Figs. *1*, *2*, and *5*). These drugs probably stabilize precursor H2a and H2b by inhibiting the endoprotease that would otherwise initiate the degradation process. Since neither TLCK nor TPCK inhibit protein biosynthesis, ER-to-Golgi transport, or processing of glycans (Figs. *2*, *5*, and *7*), their effect on H2 degradation is specific and indicates participation of a TLCK- and TPCK-sensitive protease. This initial endoproteolytic clip is not sensitive to inhibitors of protein biosynthesis or to ATP depletion, but these substances inhibit degradation of the 35-kD intermediate fragment (Figs. *3–5*, and *7*).

The 35-kD fragment does not accumulate in lysosomes, as judged by subcellular fractionation (Fig. 9), eliminating a role for lysosomes in at least the initial endoproteolytic, TLCK- and TPCK-sensitive step of H2 degradation. Further, subcellular fractionation shows that the 35-kD fragment accumulates in a membrane compartment which has the density of the rough ER, and contains ribosomes (Fig. 10). Oligosaccharide analysis confirms the localization in the ER by revealing a composition of the N-linked oligosaccharides on the 35-kD fragment that is typical of proteins with a pre-Golgi localization (Fig. 8). The 35-kD fragment is not localized to an ER-to-Golgi transport vesicle, since in HepG2 cells the density of these is lighter than that of the Golgi (Lodish et al., 1987) while the compartment containing the 35-kD fragment has the much heavier density of the rough ER (Fig. 10). We conclude that the initial TPCK and TLCK-sensitive cleavage of H2 occurs in the rough ER. However, our data is also consistent with the notion that the fragment is formed in some ER-to-Golgi intermediate compartment (Pelham, 1988) and recycled back to the ER.

Proteolytic clipping of several viral glycoproteins have been reported, of which the VSV G protein is perhaps the most extensively characterized. The precursor ts045 G protein accumulates in the rough ER membrane and a soluble fragment, G_s, is generated in the ER or other pre-Golgi compartment and is subsequently released into the medium (Garreis-Wabnitz and Kruppa, 1984; Chen and Huang, 1986). It would be of interest to see whether TPCK or TLCK block generation of G_s.

Since the transmembrane domain in H2 serves as a noncleavable signal sequence, Amara et al. (1989) proposed that the ER protease signal peptidase might generate the 35-kD fragment. We tested this hypothesis by metabolically labeling fibroblasts expressing recombinant human albumin (a nonglycosylated protein) in the presence of TLCK or TPCK. There was no difference in the size of albumin compared to untreated cells, indicating that signal sequence cleavage oc-

curred in the presence of these inhibitors (data not shown). Thus, the participation of signal peptidase in H2 degradation is unlikely.

The finding that both H2a and H2b are degraded by very similar pathways is interesting, but the role of the five amino acids (the sole difference between the two forms of H2) in the degradation remains an open question; they probably do not act as signal for targeting H2 to a putative degradation compartment or function as a target sequence for an endoprotease.

For two other proteins which are subject to ER degradation, the transmembrane domain is the determinant for rapid and selective degradation. The basal turn-over of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, a monomeric ER-resident protein with a highly regulated degradation pattern (reviewed by Goldstein and Brown, 1990), is inhibitable by lysosomotropic agents, whereas the rapid, inducible degradation is not (Tanaka et al., 1986). A fusion protein consisting of the membrane spanning, noncatalytic part of HMG CoA reductase and β-galactosidase is localized to the ER (Skalnik et al., 1988). The β-galactosidase activity is as sensitive to rapid degradation induced by sterols as is intact HMG CoA reductase. Also, deletions of single amino acids in the membrane-spanning domain of the chimeric molecule results in abolition of sterol-inducible degradation of β-galactosidase, while the localization of the fusion protein is unaffected. Bonifacino et al. (1990) identified the transmembrane domain of TCR α chain as a determinant for the ER degradation; a normally stable cell surface protein, the Tac antigen, becomes a target for ER degradation when its transmembrane domain is substituted with that of the TCR α chain.

Considering our data on the degradation of H2 in light of these results, we believe that precursor H2 folding or oligomerization prevents ER degradation. H2a in 2-18 cells might be unable to oligomerize, and as a result all is degraded; when coexpressed with H1, some H2a is stabilized by forming oligomers and is transported to the cell surface (Lederkremer and Lodish, 1990). Some H2b in 2C cells and HepG2 cells do form oligomers and mature to the Golgi and are protected from degradation. Wileman et al. (1990) and Bonifacino et al. (1989) showed that rapidly degraded TCR subunits can be stabilized by coexpressing them with degradation-resistant chains.

Oligomerization is a prerequisite for transport of many proteins out of the ER (Kreis and Lodish, 1986; Copeland et al., 1986; Gething et al., 1986). Our results show that even if degradation was prevented by TLCK or TPCK, the amount of H1 and H2b transported out of the ER was not increased (Figs. 2, 5, and 7) leading us to believe that the rest of the H2 molecules cannot leave the ER even if they are not degraded. In the presence of TLCK or TPCK H2a in 2-18 cells does not acquire complex carbohydrate side chains (Fig. 1), again suggesting that none is transported to the Golgi. These results suggest that there is an early sorting or tagging of proteins destined for degradation and proteins destined for transport to the Golgi, and that once a protein has been selected for degradation the decision is irreversible, even if degradation is blocked. Evidence suggesting sorting within the ER of proteins destined for degradation was obtained by Chen et al. (1988), who studied the fate of the TCR ϵ, γ, and δ subunits (the CD3 complex) in a T cell hybridoma deficient in expression of the β subunit. The δ chain is degraded by the ER pathway and the ϵ and γ chains are stable. Failure of the ϵ-γ complex to be co-immunoprecipitated with antibodies to the δ chain led them to conclude that degradation of δ occurs in a compartment different from that of receptor synthesis and assembly (presumably the rough ER), otherwise the ϵ-γ complex would have access to newly made δ chain and coprecipitate.

Inhibitors of protein biosynthesis and energy production block degradation of the 35-kD H2 degradation intermediate. Reportedly, they have no effect on degradation of the TCR α chain (Lippincott-Schwartz et al., 1988). However, no degradation intermediate has been detected for the TCR α chain.

Cycloheximide is often used as an inhibitor of lysosomal degradation. However, Amara et al. (1989) showed that lysosomotropic agents do not affect degradation of H2a, and our results reveal that the 35-kD fragment is not in lysosomes, indicating no role for lysosomes in at least the initial endoproteolytic step of H2 degradation. Inhibition of degradation of the 35-kD intermediate by cycloheximide and other inhibitors of protein biosynthesis could indicate that this second degradation step involves an unstable protease, a point that remains to be investigated. Energy inhibitors could block the same step as does cycloheximide by inhibiting protein synthesis. Other possibilities are that an ATP-dependent protease is involved, or that a membrane transport step is required. Dunn (1990) showed that autophagosomes are formed from ribosome-free regions of the ER, but there are no indications that autophagy would account for the ER degradation of H2. In particular, 3-methyl adenine, an inhibitor of autophagy (Seglen and Gordon, 1982), has no effect on the degradation of H2 (Fig. 1).

Thus, while we can state that the initial endoproteolytic cleavage in H2 degradation occurs in the rough ER, we do not know where the subsequent degradation of the 35-kD intermediate takes place, except that it is probably not in lysosomes. It could also occur in the ER or an ER-related compartment, near the site of accumulation of the 35-kD intermediate.

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