Intracellular Degradation of Unassembled Asialoglycoprotein Receptor Subunits: a Pre-Golgi, Nonlysosomal Endoproteolytic Cleavage

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Abstract. The human asialoglycoprotein receptor is a heterooligomer of the two homologous subunits H1 and H2. As occurs for other oligomeric receptors, not all of the newly made subunits are assembled in the RER into oligomers and some of each chain is degraded. We studied the degradation of the unassembled H2 subunit in fibroblasts that only express H2 (45,000 mol wt) and degrade all of it. After a 30 min lag, H2 is degraded with a half-life of 30 min. We identified a 35-kD intermediate in H2 degradation; it is the COOH-terminal, exoplasmic domain of H2. After a 90-min chase, all remaining intact H2 and the 35-kD fragment were endoglycosidase H sensitive, suggesting that the cleavage generating the 35-kD intermediate occurs without translocation to the medial Golgi compartment. Treatment of cells with leupeptin, chloroquine, or NH₄Cl did not affect H2 degradation. Monensin slowed but did not block degradation. Incubation at 18–20°C slowed the degradation dramatically and caused an increase in intracellular H2, suggesting that a membrane trafficking event occurs before H2 is degraded. Immunofluorescence microscopy of cells with or without an 18°C preincubation showed a colocalization of H2 with the ER and not with the Golgi complex. We conclude that H2 is not degraded in lysosomes and never reaches the medial Golgi compartment in an intact form, but rather degradation is initiated in a pre-Golgi compartment, possibly part of the ER. The 35-kD fragment of H2 may define an initial proteolytic cleavage in the ER.

M ANY cell surface receptors and secreted proteins are composed of multiple subunits. The assembly of the individually synthesized subunits into complexes is not a perfectly efficient process; in many instances, a fraction of the subunits made is never assembled or routed to the cell surface (e.g., Dulis et al., 1982; Merlie et al., 1982; Plant and Grieninger, 1986; Corless et al., 1987; Minami et al., 1987). The human hepatic asialoglycoprotein (ASGP)[1] receptor is an attractive system in which to study this process. This receptor is composed of two strongly homologous subunits, H1 and H2. Studies using chemical cross-linking and antibody-induced degradation showed that, in the human hepatoma cell line HepG2, the receptor is a heterooligomer (Bischoff et al., 1988). The functional complex is at least a trimer, containing at least one H2 and two (or three) H1 polypeptides. H1 is synthesized as a 40-kD core-glycosylated precursor, oriented in the ER membrane with its NH₂ terminus in the cytoplasm and its COOH terminus in the ER lumen. During transit through the Golgi complex, the modification of its two N-linked oligosaccharide chains causes a size increase to ∼46 kD. Similarly, H2

is synthesized as a 43-kD transmembrane protein with three high-mannose oligosaccharide chains and is then modified in the Golgi complex to the mature form of ∼50 kD. In HepG2 cells, about one third of newly made H1 and H2 never acquires complex oligosaccharides and is degraded (Bischoff and Lodish, 1987).

The necessity for both subunits for normal receptor function was demonstrated by the expression of cDNA clones in hepatoma or fibroblast cells. For both the rat (McPhaul and Berg, 1986) and human (Shia and Lodish, 1989) ASGP receptors, expression of both subunits is needed for high-affinity binding of proteins with triantennary sugars. Moreover, the two subunits of the human receptor have different intracellular fates when expressed separately. When H1 is expressed alone in 3T3 fibroblasts (which normally do not make the receptor), most of the H1 forms oligomers containing at least three subunits, is processed, and is targeted to the cell surface. However, it cannot bind a normal ligand, asialoorosomucoid (Shia and Lodish, 1989). Cells expressing the homologous rat protein, RHL-1, can bind and endocytose a protein containing many chemically attached galactose residues but not asialoorosmucoid (Braiterman et al., 1989). In contrast, H2 expressed alone in 3T3 cells, although it is inserted normally into the RER and receives high-mannose oligosaccharides, fails to be processed to its mature form, and disappears from the cells (Shia and Lodish, 1989). The cell

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1. *Abbreviations used in this paper:* ASGP, asialoglycoprotein; endo, endoglycosidase.

line expressing H2 alone provides a convenient system to study the degradation of unassembled receptor subunits—most likely this is the same mechanism operating in fibroblasts that express both subunits but degrade part of each.

We show here that at least an initial step of H2 degradation occurs in a pre-Golgi compartment that may be part of the ER. Lysosomotropic agents do not affect the degradation of H2 nor cause accumulation of any detectable intermediates. Thus, the characteristics of degradation are similar to those described for the α and β subunits of the T cell receptor in transfected fibroblasts (Lippincott-Schwartz et al., 1988) and for the CD3-δ subunit of the T cell receptor in a T cell hybridoma lacking the β chain (Chen et al., 1988). This indicates that pre-Golgi, nonlysosomal degradation may be a general mechanism used by cells to handle improperly folded or unassembled membrane proteins. In addition, we have identified a fragment of H2, consisting of only the exoplasmic domain, that may define an initial proteolytic cleavage in the ER.

Materials and Methods

Materials

Materials were obtained from the following sources: L-[^{35}S]cysteine (specific radioactivity of >800 Ci/mmol) was from New England Nuclear (Boston, MA), Amersham Corp. (Arlington Heights, IL), or ICN Radiochemicals (Irvine, CA); ^{14}C-methylated protein standards were from Amersham Corp.; protein A-agarose was from Bethesda Research Laboratories (Gaithersburg, MD); leupeptin was from Chemicon (El Segundo, CA); chloroquine and monensin were from Sigma Chemical Co. (St. Louis, MO); endoglycosidase (endo) H was from Genzyme Corp. (Boston, MA); endo D was from Boehringer Mannheim Biochemicals (Indianapolis, IN); bicinchoninic acid protein assay reagent was from Pierce Chemical Co. (Rockford, IL); calf serum and DME were from Gibco Laboratories (Grand Island, NY); tissue culture dishes were from Costar (Cambridge, MA) and Falcon Labware (Oxnard, CA).

Antibodies

We used several antibodies specific for the ASGP receptor subunits: an antiserum specific for the H2 cytoplasmic domain, raised against a peptide corresponding to amino acids 24–33 of H2; an antiserum specific for the H2 COOH terminus, raised against a peptide corresponding to the 12 COOH-terminal residues; and an antiserum specific for H1, raised against a peptide corresponding to the 15-COOH-terminal amino acids (Bischoff et al., 1988). These were kindly provided by Drs. Joyce Bischoff and Michael Shia (Whitehead Institute for Biomedical Research, Cambridge, MA). The other antisera used were anti-RER and anti-Golgi complex, prepared against whole fractions of purified RER or Golgi membranes (Louvard et al., 1982), kindly provided by Dr. Daniel Louvard of the Pasteur Institute (Paris, France).

Cell Culture

The NIH 3T3 retrovirus-infected cell lines 1-7-1 (expressing H1 and H2) and 2-18 (expressing H2) (Shia and Lodish, 1989) were grown in DME plus 10% calf serum under an atmosphere of 5% CO_2. The cell monolayers were trypsinized and split 1:10 to 1:60 into 60-mm tissue culture dishes at least 2 d before use.

Metabolic Labeling

Subconfluent to just confluent (80–100%) monolayers were rinsed once in DME devoid of cysteine but supplemented with 10% dialyzed calf serum (cysteine-free DME), preincubated for 30 min in 1 ml cysteine-free DME, then incubated with [^{35}S]cysteine (170–250 μCi/ml) in 0.7 ml cysteine-free DME for various lengths of time at 37°C in a 5% CO_2 atmosphere. To terminate the pulse, we removed the radioactive medium and rinsed the monolayers two to three times with DME (containing cysteine) at 37°C and then returned them to the incubator with 3–5 ml DME for different chase periods. For the experiments with leupeptin, the drug was present in the medium during the preincubation, pulse, washes, and chase. For the experiments in which temperature was varied or in which monensin, chloroquine, or NH_4Cl was used, the sodium bicarbonate in the medium was substituted with 20 mM Hepes and the pH was adjusted to 7.2. In this case, incubations were done outside of the CO_2 incubator. After the given chase period, we placed the dishes on ice and rinsed them twice in PBS and then lysed the monolayers in 600 μl of 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM EDTA, 2 mM PMSF in PBS (lysis buffer). (In some experiments, the additional protease inhibitors L-1-p-tosylamino-2-phenylethyl chloromethyl ketone and N-p-tosyl-L-lysine chloromethyl ketone [100 μg/ml] were included in the lysis buffer, with no difference in the results.) Alternatively, we generated a postnuclear supernatant as described (Bischoff and Lodish, 1987), but using 50 strokes with the homogenizer (Dounce; Kontes Glass Co., Vineland, NJ); the postnuclear supernatant was adjusted to 1× PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM PMSF. After either sample preparation method, the suspension was transferred to a microfuge tube, mixed vigorously, incubated on ice for at least 1 h, remixed, and centrifuged for 15 min at 15,000 g at 4°C to remove debris. We used the bicinchoninic acid protein assay reagent (Pierce Chemical Co.), with BSA as a standard, to determine the protein concentration in the supernatant ("cell extract").

Immunoadsorption and Electrophoresis

All steps were carried out at 0–4°C unless otherwise noted. We used amounts of cell extract or postnuclear supernatant extract representing a given amount of protein, typically 200 μg, in each experiment, adjusting the total volume of the immunoadsorption with lysis buffer. We incubated samples with normal rabbit serum (used at a 1:200 dilution) for at least 1 h on ice or 30 min at room temperature and then added 12 μl of a 25% slurry of protein A-agarose in PBS, 0.02% sodium azide/μl of serum. After incubation with rotation for at least another 30 min, we centrifuged samples at 15,000 g for 30 s and transferred the supernatants to tubes containing the appropriate antiserum. Antisera were used at a 1:70 dilution, and the incubation was continued overnight, conditions we found to be sufficient to leave no immunoadsorbable material in the supernatant. Samples were then incubated with protein A-agarose as above. The protein A-agarose was washed, and the antigen–antibody complexes were eluted, denatured, and subjected to SDS-10% PAGE, fluorography, and densitometry as described (Bischoff and Lodish, 1987).

endo H Digestion

After immunoadsorption, antigen–antibody complexes were eluted from protein A-agarose by incubation at 100°C for 5 min in 20 μl 0.5% SDS, 0.1 M sodium citrate, pH 6, diluted with 80 μl 1.25 mM EDTA, 1 mM PMSF, 0.1 M sodium citrate, pH 6, and then centrifuged at 15,000 g to pellet the protein A-agarose. The supernatants were divided into halves and incubated overnight either with no addition or with 30 ng of endo H per 45-μl sample. 23 μl of 3× concentrated SDS-PAGE sample buffer was added to each sample, and samples were heated to 100°C and processed by SDS-10% PAGE as above.

Immunofluorescence Microscopy

Cells grown on coverslips were fixed and permeabilized by sequential incubation with methanol and acetone for 5 min each at −20°C. They were then rinsed with PBS containing 2% BSA (PBS-BSA) three times and subjected to the following sequential incubations: (a) 30 min with normal goat IgG; (b) 60 min with rabbit anti-H2 COOH terminus IgG affinity purified on a column of protein A-sepharose; (c) three rinses with PBS-BSA; (d) 30 min with 5 μg/ml TRITC-conjugated goat anti-rabbit IgG; (e) two rinses with PBS-BSA; (f) 60 min with 100 μg/ml unlabeled goat anti-rabbit IgG; (g) three rinses with PBS-BSA; (h) 60 min with rabbit anti-RER or rabbit anti-Golgi serum; (i) three rinses with PBS-BSA; (j) 30 min with 5 μg/ml FITC-goat anti-rabbit IgG; and (k) three rinses with PBS.

Cells were viewed on a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with FITC and TRITC filters and photographed at identical exposure times between samples to be able to compare signal intensities. Controls, in which any of the primary antibodies were omitted or replaced by preimmune IgG, showed no detectable signal with the corresponding filter.
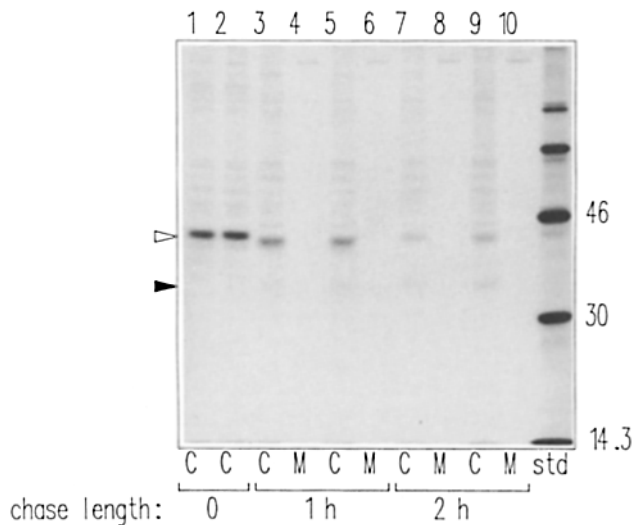
Figure 1. H2 is degraded and not secreted. 2-18 cells were incubated with [^{35}S]cysteine for 30 min then washed and incubated with nonradioactive medium for 0, 1, or 2 h. The experiment was done in duplicate. Detergent extracts of the cells (C; lanes 1-3, 5, 7, and 9) and medium (M; lanes 4, 6, 8, and 10) were immunoprecipitated with a mixture of antisera directed against the NH$_2$ and COOH termini of H2. The immunoadsorbed material was electrophoresed using SDS-PAGE, fluorographed, and subjected to autoradiography. The open and closed arrowheads at the left indicate the intact H2 polypeptide and a 35-kD putative degradation product, respectively. The protein standards at the right are in kilodaltons.

Results

H2 Is Degraded after a Small Decrease in Size

mRNAs encoding two variants of H2, H2a and H2b, are transcribed in HepG2 cells (Spiess and Lodish, 1985). Compared with H2b, H2a contains a five–amino acid insert after amino acid number 81, which is located in the exoplasmic domain just beyond the membrane-spanning region. One difference between the variants is that, when expressed alone in 3T3 fibroblasts, a portion of H2b reaches the cell surface (Lederkremer, G., and H. F. Lodish, unpublished results), while none of H2a does (Shia and Lodish, 1989). In 2-18 cells, a line of retrovirus-infected 3T3 fibroblasts expressing only H2a, the H2 polypeptide is synthesized as the usual 43-kD core-glycosylated precursor but then disappears rapidly and completely from the cells (Shia and Lodish, 1989). We used 2-18 cells to study H2 degradation in the complete absence of surface expression.

We wanted first to establish whether the disappearance of radioactively labeled H2 after a pulse labeling of 2-18 cells was due to complete intracellular proteolysis or to partial cleavage of the polypeptide followed by fragment release into the medium; the latter occurs to some viral integral membrane glycoproteins such as the vesicular stomatitis virus G protein (Chen and Huang, 1986). To test this possibility, we pulse labeled 2-18 cells, chased for 1 or 2 h, and immunoadsorbed detergent extracts of the cells and the medium with a mixture of antipeptide antisera directed against epitopes of the cytoplasmic (NH$_2$-terminal) and exoplasmic (COOH-terminal) domains of H2 (Bischoff and Lodish, 1987; Bischoff et al., 1988) (Fig. 1). Because we detected no immunoreac-

tive material in the medium, we conclude that H2 is indeed intracellularly degraded and not simply cleaved once and secreted.

The major H2 polypeptide (Fig. 1, open arrowhead) migrated slightly faster after 1 h of chase than did the pulse-labeled species. This ~1–1.3-kD decrease in size is due to modification of the oligosaccharide, not of the peptide backbone, because after endo H digestion to remove the oligosaccharides the pulse-labeled and chased samples had the same mobility (data not shown). A faint band of ~35 kD (solid arrowhead) was seen in the chase samples; as noted below, we suspect this is an intermediate in H2 degradation. This polypeptide was never detected in 3T3 cells infected with vector alone (not shown).

Time Course of H2 Degradation

The time course of degradation of newly synthesized H2 is shown in Fig. 2. In this and similar experiments, we observed that H2 was relatively stable for 30–60 min and then degraded rapidly with a half-life of 30 min, as previously reported by Shia and Lodish (1989). The 35-kD polypeptide was detected initially at 15 min of chase and was present during the time in which H2 was disappearing most rapidly; it was undetectable by 3 h of chase. The time course of the appearance and disappearance of the 35-kD species is consistent with a role as an intermediate in the degradation of H2.

The 35-kD Polypeptide Contains the COOH Terminus of H2 and Is Formed in the ER

To characterize further this putative degradation intermediate, we tested the reactivity of the 35-kD polypeptide with
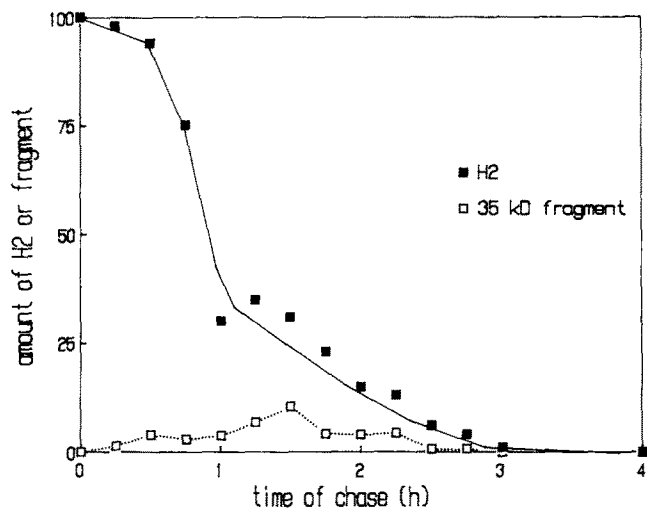

Figure 2. The time course of H2 degradation. 2-18 cells were labeled with [^{35}S]cysteine for 10 min and then washed and incubated with nonradioactive medium for the times shown. Solubilized proteins were subjected to immunoabsorption with anti–COOH terminus antiserum. The immunoadsorbed material was processed as in Fig. 1. The intensities of the radioactive species were quantitated by densitometry and normalized to the molar amount of H2 present at the beginning of the chase, taking into account that the 35-kD polypeptide contains 8 of the 11 cysteines present in intact H2 (calculated from data in Fig. 3).
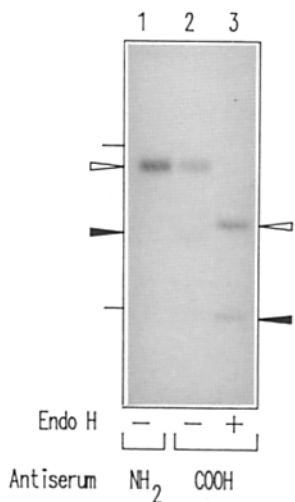
Figure 3. Immunoreactivity and endo H sensitivity of H2 and the 35-kD fragment. 2-18 cells were pulse-labeled with [^{35}S]cysteine for 15 min followed by a 90-min chase period, and a postnuclear supernatant was prepared as described in Materials and Methods. It was immunoadsorbed with antisera directed against either the NH$_2$ terminus (lane *1*) or the COOH terminus (lanes 2 and *3*) of H2. Aliquots of the sample immunoprecipitated with the anti-COOH-terminal antiserum were incubated overnight with either no addition (lane *2*) or with endo H (lane *3*). Samples were analyzed by SDS-PAGE and processed as usual. The open and closed arrowheads at the left point to intact H2 and the 35-kD fragment, respectively, in the untreated sample immunoadsorbed with the COOH terminus antiserum (lane 2); the arrowheads at right indicate their migration after endo H treatment (lane *3*). The hatch marks at left indicate the migration of the 46- and 30-kD protein standards.

antisera specific for different domains of H2 (Fig. 3). An antiserum specific for the NH$_2$ terminus of H2 immunoadsorbed only undegraded H2 (lane *1*, *open arrowhead*), whereas an antiserum specific for the COOH terminus of H2 recognized both intact H2 and the 35-kD species. Thus, we expect the 35-kD polypeptide to contain the exoplasmic domain of H2, including the three oligosaccharide chains. endo H treatment supports this notion (Fig. 3, lane *3*). All the intact H2 remaining after a 90-min pulse was endo H sensitive; endo H reduced its size by 7–8 kD. Likewise, endo H digestion completely shifted the smaller band from 35 to ∼27 kD, also a change of ∼8 kD, in agreement with the presence of three oligosaccharide chains. In addition, neither the intact H2 nor the 35-kD fragment was sensitive to endo D (data not shown); this enzyme hydrolyses high-mannose type oligosaccharides that have an unsubstituted α-mannose residue that is in an α1,3 linkage to the trisaccharide core. This structure is typically the product of Golgi α-mannosidase I, the first enzyme to act on glycoproteins that enter the *cis*-Golgi compartment. The endo H sensitivity of the two H2 species is important for two reasons: it indicates that H2 probably does not enter the Golgi complex before being degraded and that the proteolytic event generating the 35-kD polypeptide also takes place in a pre-Golgi compartment.

Because the antiserum that recognized the 35-kD species was raised against the 12 amino acids at the COOH terminus (exoplasmic domain) of H2, we can use the estimated sizes of the proteins in Fig. 3 to determine the site of cleavage that would generate the 35-kD product from H2. Using the estimated sizes of either the glycosylated or deglycosylated proteins, the predicted cleavage would be at amino acids 72–84. H2 spans the membrane between amino acids 59 and 78 (Spiess and Lodish, 1985), so the cleavage is near the junction between the membrane-spanning and exoplasmic domains. Preliminary fractionation data suggest that the 35-kD polypeptide is not tightly associated with membranes (our unpublished observation), consistent with this localization of

cleavage. We tried to find the remaining portion of H2, predicted to be ∼8–10 kD, using a gel system that can resolve proteins as small as 1 kD but we were unable to detect any smaller band immunoreactive with the anti-NH$_2$ terminus antiserum (data not shown). Either the NH$_2$-terminal fragment is degraded more rapidly than the 35-kD intermediate or, after the initial cleavage, it loses the epitope recognized by the anti-NH$_2$ terminus antiserum.

Interfering with Lysosomal Activity Does Not Affect H2 Degradation

The lag in H2 degradation suggested that H2 travels from the RER to its degradation site and that this transfer requires time. One candidate for the site of degradation is the lysosome. To find out if H2 is degraded in lysosomes in these cells, we tested the effects on H2 degradation of several compounds known to interfere with lysosomal function. Incubation of cells with high concentrations of thiol protease inhibitors inhibits degradation of proteins that are degraded in lysosomes (Ascoli, 1979; Harford et al., 1983; Schwartz et al., 1986; Lippincott-Schwartz et al., 1988); this effect is presumed to be due to fluid-phase endocytosis of the inhibitors followed by delivery to the lysosomes. We incubated cells with the protease inhibitor leupeptin (400 μg/ml) for 30 min before pulsing them with [^{35}S]cysteine and included leupeptin in the pulse, wash, and chase media (Fig. 4). The
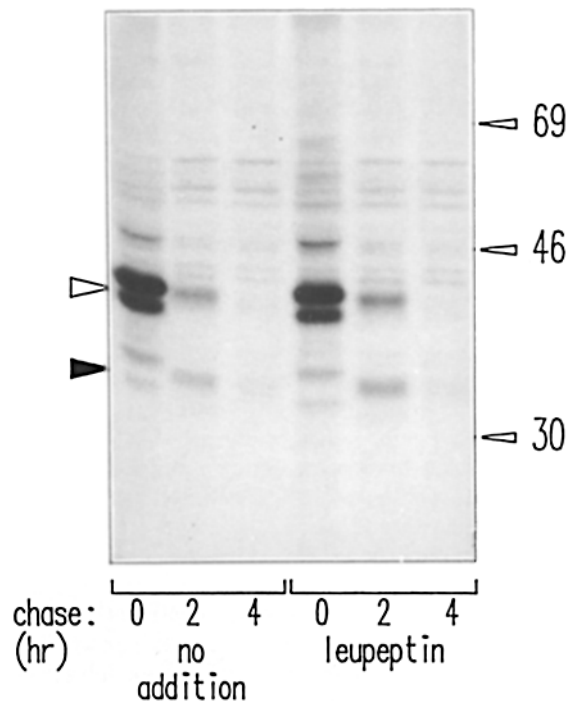

Figure 4. Effect of leupeptin incubation on H2 degradation. 2-18 cells were incubated with or without 400 μg leupeptin/ml for the 30-min period of cysteine deprivation, the 20-min pulse, and the 2- or 4-h chase period, as indicated. Cell extracts were made and processed as usual but with the inclusion of 2.5 μg leupeptin/ml lysis buffer. Anti-COOH terminus serum was used for immunoadsorption. The open and closed arrowheads at left indicate the migration of H2 and the 35-kD fragment, respectively. The arrowheads at right indicate the migration of protein standards in kilodaltons.
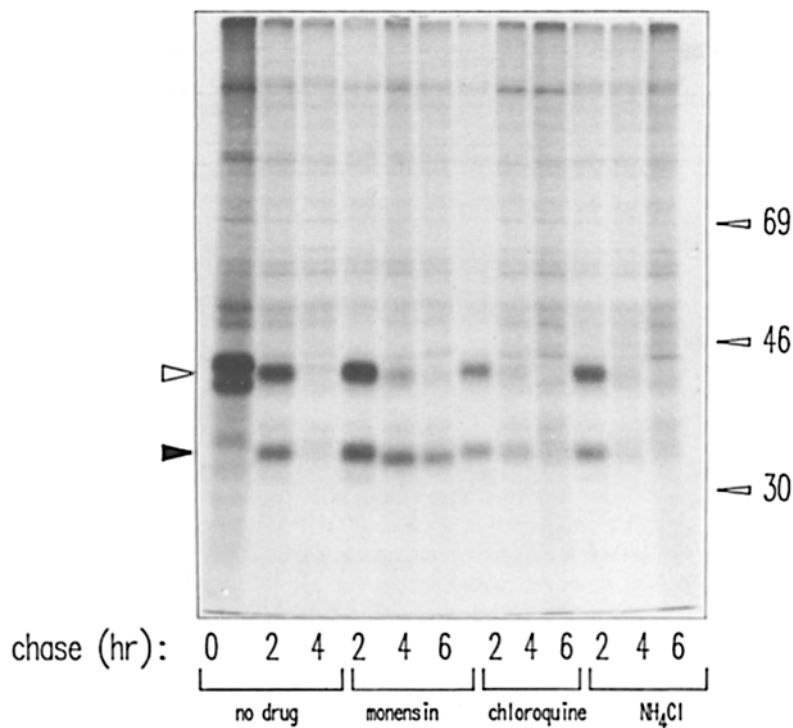
chase (hr): 0 2 4 2 4 6 2 4 6 2 4 6

no drug   monensin   chloroquine   NH₄Cl

Figure 5. Effect of monensin, chloroquine, or NH₄Cl on H2 degradation. 2-18 cells were pulse labeled with [³⁵S]cysteine for 20 min then washed and chased for 0, 2, 4, and 6 h in medium with no addition or containing monensin (100 μM), chloroquine (800 μM), or NH₄Cl (20 mM) as indicated. Solubilized proteins were immunoadsorbed with anti–COOH terminus serum and processed as usual. The arrowheads at right indicate the migration of protein standards, given in kilodaltons. Intact H2 and the 35-kD fragment are indicated on the left by open and closed arrowheads, respectively.

faster migrating polypeptides seen in the pulse-labeled samples correspond to different numbers of N-linked oligosaccharides on H2 and on the proteolytic fragment because endo H digestion reduced each pair to a single band (data not shown).

If the lysosomes were the site of H2 degradation, we would expect that degradation would be slowed by treatment with leupeptin; however, it was unaffected, as H2 decayed with approximately the same kinetics with and without leupeptin. This experiment must be considered in the light of results by Schwartz et al. (1986): although a 1-h preincubation with 500 μg leupeptin/ml inhibited the degradation of internalized ¹²⁵I-asialoorosomucoid ∼80%, the protease inhibitor had no effect on the antibody-induced degradation of the ASGP receptor. This assay measures the degradation pathway taken by cell surface receptors after they have been induced to internalize after exposure to anti–receptor antibodies. From this and other results, including evidence that the weak base primaquine could inhibit degradation of the receptor, Schwartz et al. concluded that these internalized receptors are not degraded in the lysosomes but rather in an endosomal compartment.

To determine if H2 were targeted directly to such a degradative endosomal compartment in 2-18 cells, we tested the effects of compounds that interfere with endosome and lysosome function, at concentrations greater than the concentrations that are maximally effective at neutralizing endosomes in 3T3 fibroblasts (Maxfield, 1982). Neither chloroquine nor NH₄Cl, weak bases that are thought to inhibit lysosomal and endosomal enzyme activity by raising the intralumenal pH, inhibited the breakdown of H2 nor did they affect the appearance or disappearance of the 35-kD fragment (Fig. 5). Monensin, a proton ionophore with multiple effects on cells including the neutralization of acidic compartments (Tartakoff, 1982), had very little effect on H2 degradation even

at a concentration of 100 μM, an order of magnitude higher than that used by Lippincott-Schwartz et al. (7 μM), which had no effect on degradation of T cell receptor subunits.

In a more detailed study of the time course of degradation, monensin slowed the decay of H2 by ∼30–60 min relative to untreated cells, and H2 became almost undetectable between 4 and 6 h of chase (see Fig. 5). Monensin also increased the accumulation of the 35-kD fragment (Fig. 5). Monensin at 100 μM concentration interferes not only with lysosomes; in 1-7 cells, which are 3T3 fibroblasts that synthesize H1 and express it at the cell surface (Shia and Lodish, 1989), 100 μM monensin blocked conversion of H1 and H2 oligosaccharides to the complex form (data not shown), suggesting that Golgi function or transport from the ER to the Golgi complex was affected, consistent with effects seen in other systems (Tartakoff, 1983). Taken together, the pharmacological data suggests that H2 in 2-18 cells is not degraded in lysosomes or endosomes.

H2 Degrades Slowly at 18–20°C

Incubation of cells at ∼20°C slows some processes that depend on lipid bilayer fluidity, such as membrane fission and fusion (Dunn et al., 1980; Marsh et al., 1983; Saraste et al., 1986). Lippincott-Schwartz et al. found that incubation at 18°C blocks degradation of T cell receptor α and β chains. To determine whether H2 degradation shows a similar behavior, we incubated 2-18 and 1-7-1 cells at 18–20°C for both a pulse and chase period. The 1-7-1 cells were included as a measure of how the low temperature was affecting the movement of H2 from the RER to the Golgi complex, as monitored by the modification of H2 oligosaccharides to the complex form. Some H2 was also degraded in the 1-7-1 cells, but the data are presented as a percentage of mature H2 compared with the total amount of H2 present at each time point; this may overestimate slightly the rate of maturation of H2.
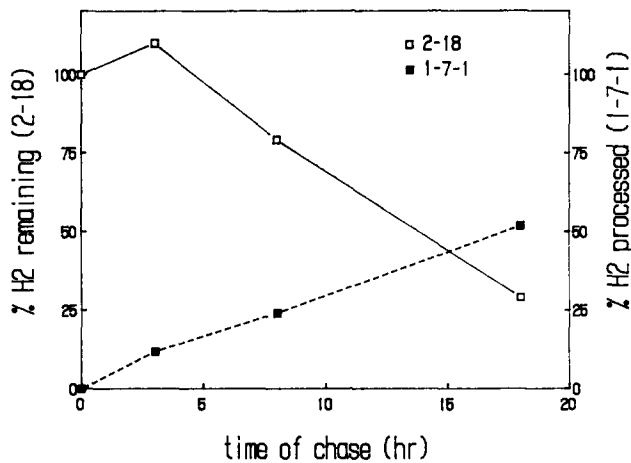
Figure 6. Slowing of H2 degradation or processing at 18–20°C. 2-18 cells and 1-7-1 cells were pulse labeled at 18–20°C with [^{35}S]-cysteine for 20 min and then washed and incubated at 18–20°C for 3, 8, or 18 h. Solubilized H2 proteins were immunoprecipitated with anti-NH$_2$ terminus antiserum, processed as usual, and then quantitated by densitometry. The data plotted for the 2-18 cells (*open squares*) are the percentage of H2 remaining at each time point compared with the pulse sample. The data for 1-7-1 cells (*solid squares*) are the amount of H2 converted to its high molecular weight form with complex sugars compared with the total amount of H2 present at each time point.

Fig. 6, *open squares*, shows that, in 2-18 cells, H2 decay was greatly slowed relative to that at 37°C (compare with Fig. 2), a half-life (after the lag period) of 11 h, relative to 30 min at 37°C. This rate was comparable with the ~18-h half-time of rate of H2 maturation in 1-7-1 cells (Fig. 6, *solid squares*), consistent with the low temperature affecting movement of protein from one vesicular compartment to another.

In 2-18 Cells H2 Is Detected in the ER and Not the Golgi Complex

To see if we could identify a particular cellular compartment containing H2 in 2-18 cells, we used immunofluorescence to label simultaneously the ER and H2 (Fig. 7, *A* and *B*) or the Golgi complex and H2 (Fig. 7, *C* and *D*). The anti-H2 COOH terminus antibodies (*A* and *C*) labeled cells coincidently with the ER-specific antibody (*B*), and this labeling pattern was easily distinguished from that of the Golgi-specific label (*D*). Because of superposition of Golgi labeling with part of the ER, we cannot exclude that some of the H2 labeling coincides with the Golgi complex, though most of it does not. Some cells that showed a stronger signal for H2, suggesting a higher level of H2 expression or a lower rate of degradation, gave an identical pattern of ER staining (Fig. 7 *A, cell at bottom*) as compared with the majority of the cells (Fig. 7 *A, two cells at top*). If H2 travels before degradation from the RER to a new compartment, (*a*) this compartment must be part of or physically adjacent to the ER and therefore not distinguished by immunofluorescence or (*b*) degradation upon reaching the new compartment must be so rapid that we cannot detect H2 in it.

Low Temperature Does Not Change the Subcellular Localization of H2

Incubation of cells at 18–20°C drastically reduced the rate

of degradation of H2 (Fig. 6). When 2-18 cells were incubated at 18°C for 5 h, we saw no change in the immunofluorescence pattern of H2 distribution, but a stronger signal was detected in all cells (Fig. 8 *A* as compared with Fig. 7 *A*). This indicates that H2 degradation is inhibited less than H2 biosynthesis. All H2 is still in a compartment that stains with the anti-ER antiserum. It is unlikely that the reduced temperature would account for such a reduction in the degradation rate if the H2 were degraded within the RER. We believe that H2 is transported for degradation to an RER-adjacent, pre-Golgi compartment such as the transitional element of the ER and that such transport is inhibited at 18–20°C.

Discussion

Here we show that the ASGP receptor subunit H2a, expressed in fibroblasts without H1, is degraded within 2–3 h of synthesis in a compartment that may be the ER itself or near the ER. Lysosomotropic agents do not affect the rate of degradation and do not cause accumulation of intermediates detectable with our antisera. Thus, we believe that degradation occurs in a pre-Golgi, nonlysosomal compartment but cannot exclude final lysosome degradation of intermediates that are not reactive with our two anti-peptide sera.

We have identified a 35-kD polypeptide that may result from the initial cleavage event in the degradation of H2. Evidence that the 35-kD protein is a fragment of H2 includes (*a*) it is not found in fibroblasts transfected with vector alone; (*b*) it has the same number of oligosaccharides as H2; (*c*) it reacts with the anti-H2 antibody specific for the COOH terminus, the part of H2 containing the sites for oligosaccharide addition; and (*d*) it is detected roughly during the period of time in which H2 is degraded. If we assume that the 35-kD protein results from a single endoproteolytic cleavage, then this site would be just COOH-terminal to the membrane-spanning region. Cell fractionation results (not shown) indicate that the 35-kD fragment is not tightly bound to the membrane, consistent with it being the exoplasmic, nonmembrane-spanning domain of H2. Our results indicate that the 35-kD H2 fragment is formed before H2 reaches the medial Golgi compartment, as the 35-kD H2 fragment bears endo H–sensitive, endo D–resistant oligosaccharides. We do not know whether this cleavage is an obligatory initial step in the degradation pathway or if it is one of several initial cleavages that can take place.

The membrane-spanning domain of the homologous protein H1 functions as an internal uncleaved signal-anchor sequence (Spiess and Lodish, 1986); we assume that the H2 membrane-spanning sequence plays the same role. The position of the cleavage we observe—just COOH-terminal to this internal signal-anchor sequence—suggests that signal peptidase may be the culpable enzyme. Interestingly, when derivatives of H1 cDNA that direct synthesis of a variant H1 protein that lacks the NH$_2$-terminal (cytoplasmic) domain were translated in vitro in the presence of microsomes, a fraction of the protein made was proteolytically cleaved at the luminal side of the membrane-spanning region, suggesting that signal peptidase recognized the internal signal sequence (Spiess and Lodish, 1986). This cleavage was not detected in normal H1, suggesting that the conformation of the protein as well as the presence of the signal-anchor sequence determines
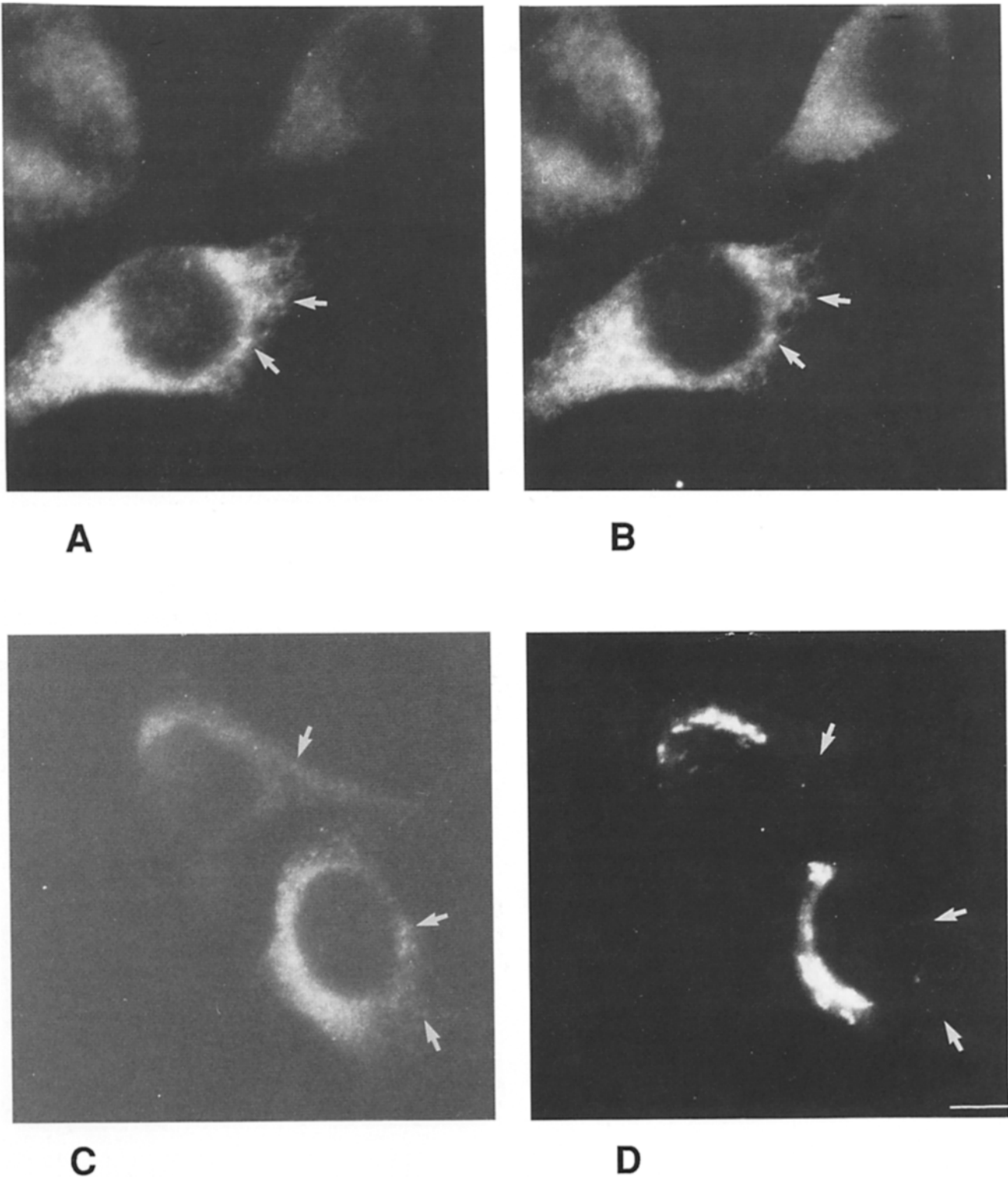
Figure 7. Double label immunolocalization of H2 and the ER or Golgi complex. Fixed, permeabilized 2-18 cells were processed as described in Materials and Methods. *A* and *B* represent double labeling with anti–H2 and anti–ER antibodies; *C* and *D* represent double labeling with anti–H2 and anti–Golgi antibodies. H2 was detected with TRITC filter (*A* and *C*), and ER (*B*) or Golgi complex (*D*) were detected with FITC filter. Identical regions are labeled by arrows in the double label pairs, pointing out similarities in staining (*A* and *B*) or differences (*C* and *D*). Bar, 10 μm.

whether signal peptidase acts. As H2 does not fold into an H1–H2 heterooligomer when synthesized in the absence of H1, we speculate that signal peptidase could recognize a c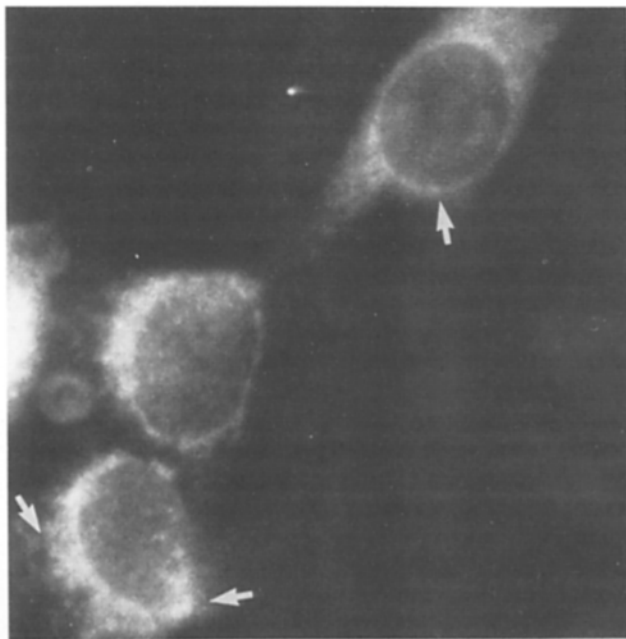ryptic cleavage site in unfolded H2. Candidate cleavage sites predicted by the method of von Heijne (1986) are present in the area of the proteolytic clip. If cleavage by signal peptidase were an obligatory initial step in H2 degradation, however, we would expect that upon incubation at 18–20°C,
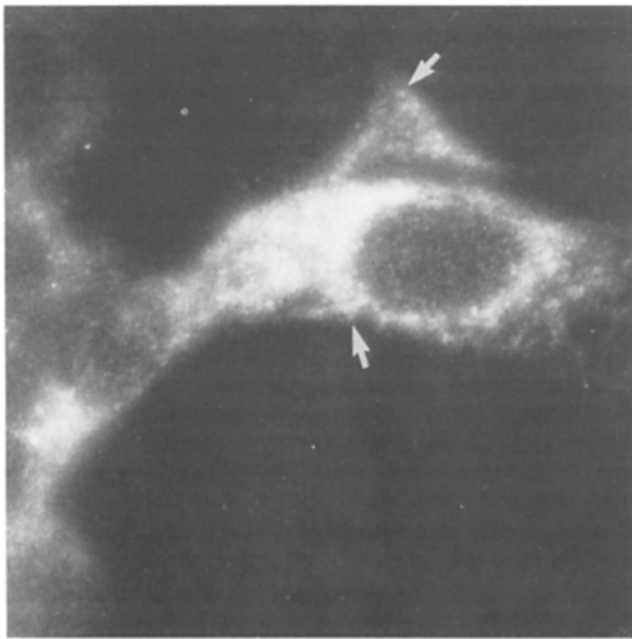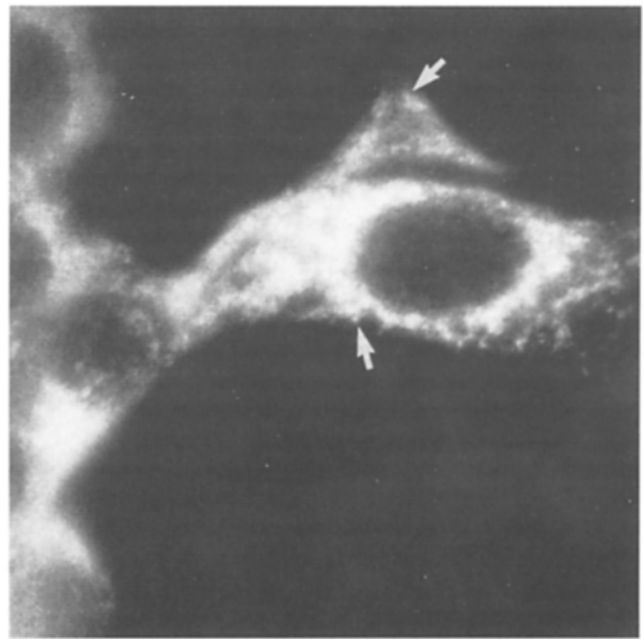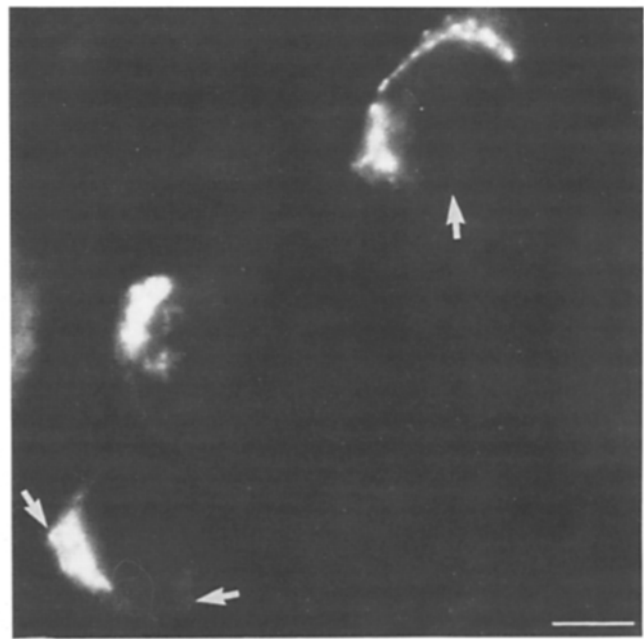
Figure 8. Effect of 18–20°C on immunolocalization of H2, the ER, and the Golgi complex. 2-18 cells were incubated at 18–20°C for 5 h before fixation, permeabilization, and immunofluorescence as in Fig. 7. (*A* and *C*) Anti–H2-TRITC; (*B*) anti–RER-FITC; (*D*) anti–Golgi-TRITC. Bar, 10 μm.

which causes H2 to accumulate in the ER, all the H2 would become cleaved; instead, we find that intact H2 is much more stable than at 37°C. Alternatively, the area of the cleavage may form a particularly protease-sensitive stalk between the membrane-spanning and more globular exoplasmic domains

and be cleaved by a protease that is not found in the RER but rather in the degradative compartment.

The proteolytic cleavage we observe with H2 is similar to the processing that occurs with many viral glycoproteins. Cells infected with viruses such as murine leukemia virus,

rabies, and mouse mammary tumor virus release soluble forms of viral glycoproteins into the extracellular fluid (Bolognesi et al., 1975; Dietzschold et al., 1983; Racevskis and Sarkar, 1978). The origin of the truncated glycoprotein has been well characterized for vesicular stomatitis virus, which directs the synthesis of a membrane bound glycoprotein (G protein) in the RER. A truncated, soluble form of G protein, G$_s$, lacks the membrane-spanning and cytoplasmic portions of the protein. G$_s$ is formed intracellularly and released by infected cells (Garreis-Wabnitz and Kruppa, 1984). Use of the temperature-sensitive ts045 mutant of vesicular stomatitis virus G protein, which fails to fold into a trimer and leave the ER (Knipe et al., 1977a,b; Bergman et al., 1981; Kreis and Lodish, 1986), was instrumental in proving that G$_s$ is generated in the ER by cleavage of G protein at a site near the luminal surface of the membrane-spanning domain (Chen and Huang, 1986). Perhaps this is another example of a virus exploiting a normal cellular function, in this case quality control for multisubunit protein assembly, for its own purposes.

The characteristics of H2 degradation are essentially the same as those described by Lippincott-Schwartz and coworkers (1988) for the α and β subunits of the T cell receptor, which are also degraded in a pre-Golgi compartment in 3T3 fibroblasts. The ASGP receptor bears no striking sequence homology with the T cell receptor subunits and yet it is a substrate for a similar pre-Golgi degradation pathway. Since not all of the T cell receptor subunits have yet been coexpressed in fibroblasts, we do not know if 3T3 cells are capable of assembling the subunits and transporting them to the surface. 2B4 T cell hybridoma cells, which express all seven subunits of the T cell receptor complex, degrade the unassembled α and β subunits in the lysosomes (Lippincott-Schwartz et al., 1988). That leaves open the possibility that the fibroblasts are incapable of synthesizing the α and β subunits into a normal conformation, and the grossly abnormal conformation triggers the use of the pre-Golgi pathway. In contrast, we know that 3T3 cells are capable of making a functional ASGP receptor and yet degrade a portion of both H1 and H2 in the process (Shia and Lodish, 1989). We therefore suspect that the degradation process we describe here is the same as that occurring during cosynthesis of the two subunits in fibroblasts. It remains to be tested whether ER degradation is the mechanism operating in hepatocytes to allow only properly assembled H1–H2 complexes to mature. In addition, we would like to find out whether H2 is completely degraded in 2-18 cells because it cannot self-associate as compared with H1 which forms dimers and trimers in fibroblasts that express it alone (Shia and Lodish, 1989). A similar example would be vesicular stomatitis virus ts045 G protein, which cannot trimerize and becomes stuck in the ER (Kreis and Lodish, 1986). The generation and expression of mutant H2 proteins may help us resolve this question.

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