

Two Pathways for the Degradation of the H2 Subunit of the Asialoglycoprotein Receptor in the Endoplasmic Reticulum

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Abstract. An intermediate of 35 kD accumulates transiently during ER degradation of the H2 subunit of the asialoglycoprotein receptor; it is derived by an endoproteolytic cleavage in the exoplasmic domain near the transmembrane region. In the presence of cycloheximide all of the precursor H2 is converted to this intermediate, which is degraded only after cycloheximide is removed (Wikström, L., and H. F. Lodish. 1991. *J. Cell Biol.* 113:997-1007). Here we have generated mutants of H2 that do not form the 35-kD fragment, either in transfected cells or during in vitro translation reactions in the presence of pancreatic microsomes. In transfected cells the kinetics of ER degradation of these mutant proteins are indistinguishable from that of wild-type H2, indicating the existence of a second pathway of ER degradation which does not involve formation of the 35-kD fragment. Degradation of H2 in the ER by this alternative pathway is inhibited by TLCK or TPCK, but neither formation nor degradation of the 35-kD fragment is blocked by these reagents. As determined by NH₂-terminal sequencing of the 35-kD fragment, formed either in transfected cells or during in vitro translation reactions in the presence of pancreatic microsomes, the putative cleavage sites are between small polar, un-

charged amino acid residues. Substitution of the residues NH₂- or COOH-terminal to the cleavage site by large hydrophobic or charged ones decreased the amount of 35-kD fragment formed and in some cases changed the putative cleavage site. Cleavage can also be affected by amino acid substitutions (e.g., to proline or glycine) which change protein conformation. Therefore, the endoprotease that generates the 35-kD fragment has specificity similar to that of signal peptidase.

H2a and H2b are isoforms that differ only by a pentapeptide insertion in the exoplasmic juxtamembrane region of H2a. 100% of H2a is degraded in the ER, but up to 30% of H2b folds properly and matures to the cell surface. The sites of cleavage to form the 35-kD fragment are slightly different in H2a and H2b. Two mutant H2b proteins, with either a glycine or proline substitution at the position of insertion of the pentapeptide in H2a, have metabolic fates similar to that of H2a. These mutations are likely to change the protein conformation in this region. Thus the conformation of the juxtamembrane domain of the H2 protein is important in determining its metabolic fate within the ER.

CELLULAR homeostasis involves continuous turnover of proteins in all cellular compartments. The endoplasmic reticulum (ER), the site of synthesis of secretory, membrane, lysosomal, and vacuolar proteins, is a site for protein degradation. Proper folding and oligomerization of proteins in the ER are prerequisites for further routing into the Golgi complex (Lodish, 1988; Hurlley and Helenius, 1989; Pelham, 1989). Most misfolded polypeptides and unassembled subunits of oligomeric proteins are eventually degraded without exiting the ER. The rapid degradation of

unassembled subunits of several oligomeric membrane proteins within the ER (reviewed by Klausner and Sitia, 1990; Bonifacino and Lippincott, 1991) is of interest as it represents a pathway for protein degradation distinct from the well studied lysosomal protein breakdown, and it is part of an important, but still largely uncharacterized, regulatory step in the cellular secretory pathway.

There are many examples of selective ER degradation. Expression of the T cell receptor, α , β , or δ subunits alone, in fibroblasts, or in mutant T cells lead to their rapid degradation within the ER, whereas under similar circumstances the ϵ and ζ chains are metabolically stable (Chen et al., 1988; Lippincott et al., 1988; Bonifacino et al., 1989; Wileman et

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al., 1990). Monomeric 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)¹ reductase, a transmembrane protein, also exhibits regulated degradation in the ER (Chun et al., 1990; Inoue and Simoni, 1992). Another system which exhibits rapid ER degradation is that of the human asialoglycoprotein receptor H2 subunit, the focus of this study.

The human asialoglycoprotein (ASGP) receptor is a type 2 transmembrane glycoprotein that is normally expressed only on the sinusoidal (basolateral) surface of hepatocytes. This Ca²⁺-dependent lectin binds and removes by receptor-mediated endocytosis glycoproteins with carbohydrate side chains bearing terminal galactose residues (asialoglycoproteins) (Lodish, 1991; Spiess, 1990). The functional ASGP receptor is a hetero-oligomer consisting of two types of subunits, H1 and H2, with a minimum stoichiometry of (H1)₃(H2)₁ (Henis et al., 1990). H1 and H2 are 60% homologous in amino acid sequence (Spiess et al., 1985; Spiess and Lodish, 1985; Bischoff and Lodish, 1987; Bischoff et al., 1988). The polypeptide chain of each subunit consists of four main domains: a short cytosolic NH₂-terminal segment, a single transmembrane segment that also functions as an uncleaved signal anchor sequence, a stalk domain, and, at the very COOH terminus, the Ca²⁺-dependent galactose binding domain. There are two subtypes of H2: H2a and H2b which differ only by the presence of five extra amino acids in H2a near the transmembrane region on the exoplasmic side which results from alternative splicing of the mRNA. In Hep G2 hepatoma cells, of the H2 expressed 90% is H2b and 10% H2a (Lederkremer and Lodish, 1991).

Shia and Lodish (1989) showed that more than 50% of newly synthesized H1 subunits, expressed without H2 in NIH 3T3 fibroblasts, mature through the Golgi complex to the cell surface. In contrast, all newly made H2a, synthesized in fibroblasts without H1, is rapidly degraded in a non-lysosomal, pre-Golgi compartment (Amara et al., 1989; Shia and Lodish, 1989). H2a is synthesized, inserted into the ER, and core-glycosylated normally. However, H2a remains within the ER and its carbohydrate chains do not get processed by medial Golgi enzymes. Instead, it is degraded in the ER after a lag of ~30 min by a process not affected by agents that inhibit lysosomal degradation (Wikström and Lodish, 1991). When H2b is expressed in fibroblasts, ~30% of newly synthesized protein becomes folded normally, exits the ER, and reaches the cell surface while 70% remains unfolded and is degraded in the ER (Lederkremer and Lodish, 1991; Wikström and Lodish, 1993). During ER degradation of both the H2a and H2b subunits, a 35-kD fragment accumulates transiently; it is formed by proteolytic cleavage in the exoplasmic domain near the transmembrane region (Wikström and Lodish, 1991, 1992). In the presence of cycloheximide all of the precursor H2 is converted to this intermediate, which is degraded completely only after cycloheximide is removed. Thus, we suggested that proteolytic cleavage to generate this 35-kD fragment is an obligatory step in ER degradation of H2 (Wikström and Lodish, 1991).

Here we analyze further two aspects of ER degradation of

the H2 subunit. First, we find that the amino termini of the 35-kD fragment generated from H2a and H2b are slightly different, suggesting a different site of cleavage in each protein. The same proteolytic cleavages are observed in an *in vitro* translation system in the presence of pancreatic microsomes. We examined by mutational analyses of the amino acid residues NH₂- and COOH-terminal to the cleavage sites the sequence specificity of the cleavage. We find that the protease responsible for the formation of the 35-kD fragment prefers to cleave between small neutral or polar residues. Furthermore, the selection of cleavage site and the extent of cleavage is affected by mutations that are thought to alter the local conformation of the protein. Thus, the specificity of this protease is similar to signal peptidase.

Second, we show that the overall rates and extents of ER degradation of H2 mutants that do not generate the 35-kD fragment are similar to those of the wild-type protein and to H2 mutants which do generate the 35-kD fragment. Thus, the cleavage of the H2 protein to form the 35-kD intermediate is not obligatory for ER degradation, despite other evidence that all H2 can be converted into the 35-kD fragment which is then degraded (Wikström and Lodish, 1991). Therefore, there are at least two pathways for the ER degradation of the H2 protein, one not dependent on the proteolytic cleavage that generates the 35-kD intermediate. This second ER degradation process can be inhibited by *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), or iodoacetamide, but these compounds do not block the cleavage process that forms the 35-kD fragment nor its ultimate degradation. The TLCK/TPCK-sensitive ER degradation pathway is probably the principal one that recognizes and degrades unfolded forms of H2b in the ER, while properly folded forms of the protein mature to the cell surface (Wikström and Lodish, 1993).

Materials and Methods

Materials

Materials were purchased from sources previously listed (Amara et al., 1989; Wikström and Lodish, 1991, 1992). In addition, reagents and enzymes for *in vitro* transcription and translation were obtained from Promega Corp. (Madison, WI). Reagents and enzymes for PCR reactions were from Perkin-Elmer Cetus Instrs. (Norwalk, CT). Dog pancreatic microsomes, prepared by a standard protocol (Walter and Blobel, 1983), were a kind gift from Dr. C. Hwang (Genzyme Corp., Framingham, MA). Restriction enzymes were from New England Biolabs (Beverly, MA). Immobilon-P paper was from Millipore Corp. (Bedford, MA). The Sequenase 2.0 kit for DNA sequencing was from United States Biochemical Corp. (Cleveland, OH).

In Vitro Transcription and *In Vitro* Translation

In vitro transcription/translation reactions were done as previously described (Bischoff and Lodish, 1987). Briefly, mRNAs were transcribed from cDNAs subcloned in pSP64 vectors, using SP6 RNA polymerase following the manufacturer's protocol. mRNAs were translated in nuclease-treated rabbit reticulocyte lysates using [³⁵S]Cys or [³H]Leu as radiolabel, in a total reaction volume of 12.5 μ l. At the end of the reaction, microsomes were isolated by centrifugation at 165,000 *g* for 15 min through a cushion of 0.5 M sucrose, 10 mM Tris, pH 7.4, and 150 mM NaCl. The pelleted microsomes were solubilized using lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, and 10 mM EDTA in phosphate buffered saline, pH 7.4, with 2 mM PMSF) or SDS-gel sample buffer (125 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue) for further analysis.

1. *Abbreviations used in this paper:* ASGP, asialoglycoprotein; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; Endo H, endoglycosidase H; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PTH, phenylthiohydantoin; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

Mutagenesis of H2 cDNA, Subcloning, and DNA Sequencing

Substitution mutations of H2 were introduced by overlap extension PCR (Landt et al., 1990). The concentrations of reagents and enzymes used in the PCR reactions were according to the manufacturer's recommendations. Four primers were used to generate each mutation. As an example, the H2 S79W mutant was generated with the following four primers:

primer 1: 5'-CCTCAGACAACTCAG-3',

corresponding to bp -63 to -47 of the H2 cDNA sequence (Spiess and Lodish, 1985) (with the starting ATG codon designated as bp 1-3);

primer 2: 3'-CACCAGTAGACACTGA-5',

corresponding to bp 213-230 (antisense);

primer 3: 5'-GTGGTCATCTGTGTGACTGGGTGC~~CA~~AAGTGAG-3',

corresponding to bp 225-257 (mutant bases are underlined);

primer 4: 3'-ATGTTGTGTGCTTGGGG-5',

corresponding to bp 673-689 (antisense).

The 5'-most 18 bp of primer 3 is complementary to the 5'-most 18 bp of primer 2. PCR reactions using primers 1 and 2 and templated by the wild-type cDNA produced the expected 350-bp fragment. PCR using primer 3 (which contains the mutated sequence) and primer 4, templated by the wild-type cDNA, produced the expected 460-bp product, containing the mutated sequence. The two reaction products were then purified by agarose gel electrophoresis, mixed, and used as templates for the third PCR reaction containing primers 1 and 4. The expected 800-bp fragment, containing the desired mutant sequence, was digested with XmaI and DraIII, which have unique sites within the H2 sequence (bp -12 and 632, respectively), to generate a 640-bp fragment. This was then gel purified and subcloned into a pSP64 vector containing the wild-type cDNA sequence that had been digested with the same enzymes, thereby replacing the wild type with the desired mutant sequence. All PCRs were done under the following conditions: 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C for 30 cycles. Primers 1, 2, and 4 were used to generate all of the H2 mutants; primer 3 was changed at the appropriate positions to generate desired mutations at different positions. All mutations were verified by double-stranded dideoxy sequencing using the Sequenase 2.0 kit from United States Biochemical Corp.

Cell Culture

NIH 3T3 cells expressing wild-type H2a (2-18 cells) and H2b (2C cells) were kind gifts of Drs. L. Wikström (Ludwig Institute for Cancer Research, Ludwig, Sweden) and G. Lederkremer (Tel Aviv University, Tel Aviv, Israel) (Shia and Lodish, 1989; Lederkremer and Lodish, 1991). NIH 3T3 cells expressing H2 mutants were generated by using a calcium phosphate transfection protocol (Chen and Okayama, 1988), using the pMEX-neo mammalian expression vector (Martin-Zanca et al., 1989) containing mutant H2 cDNAs subcloned into the BamHI and EcoRI sites in the multicloning site of the vector. Colonies resistant to G418 were subcloned and tested for expression of H2 protein by metabolic labeling. All 3T3 cells were cultured in DME supplemented with 10% heat inactivated calf serum.

Metabolic Labeling, Immunoprecipitation, and Enzyme Digestions

Confluent or near confluent (80%) cells in 100- or 60-mm diameter tissue culture dishes were labeled with [³⁵S]cysteine or [³H]leucine using techniques previously described (Amara et al., 1989; Wikström and Lodish, 1991).

Antisera against the carboxyl and amino termini of the ASGP receptor H2 subunit (Bischoff et al., 1988) were kind gifts of Drs. L. Wikström and G. Lederkremer. Immunoprecipitation and Endo H digestions of cell or microsome lysates were done as previously described (Amara et al., 1989; Wikström and Lodish, 1991).

Gel Electrophoresis, Fluorography and Scanning Densitometry

Immunoprecipitates or in vitro translation products were subjected to SDS-PAGE using 0.75 mm thick 10% Laemmli gels and analyzed by fluorography

using 20% 2,5-diphenyloxazole as previously described (Bischoff and Lodish, 1987). Autoradiograms were quantitated with a Molecular Dynamics (Sunnyvale, CA) laser microdensitometer as previously described (Lodish and Kong, 1991).

Radiosequencing Analysis of the NH₂ Termini of the 35-kD Fragment

Limited NH₂-terminal protein sequencing was performed essentially as previously described (Matsudaira, 1990). Tritium-labeled immunoprecipitates or in vitro translation products were subjected to SDS-PAGE on 0.75 mm thick 10% Laemmli gels and then electroblotted onto prewet Immobilon-P paper at 0.5 A for 30 min in 10 mM CAPS, pH 11.0, 10% methanol. The paper was then rinsed in water and air-dried. Protein bands of interest were located on the paper by autoradiography and then excised for automated Edman degradation with an Applied Biosystem Inc. (Foster City, CA) model 470A protein sequencer. The PTH (phenylthiohydantoin)-amino acid derivatives from each reaction cycle were then subjected to liquid scintillation counting.

Results

H2a and H2b Are Cleaved at Different Sites Near the Transmembrane Region to Form the 35-kD Fragment

Metabolic labeling of the H2 subunit of the ASGP receptor expressed in NIH 3T3 cells demonstrated that the core-glycosylated ER forms of these proteins undergo rapid degradation in the ER (Wikström and Lodish, 1991, 1992). A 35-kD proteolytic fragment, observed in the course of degradation of the ER precursor forms, is formed in the ER, and is thought to be the first step in the ER degradation of the protein (Amara et al., 1989; Lederkremer and Lodish, 1991; Wikström and Lodish, 1991). The 35-kD fragment can be immunoprecipitated by an antiserum specific for the H2 carboxyl terminus but not an antiserum specific for the amino terminus, and its molecular weight suggests that it is generated by a proteolytic cleavage on the exoplasmic side of the protein near the transmembrane region (Amara et al., 1989). We used a radiosequencing strategy to determine the NH₂-terminal amino acid sequence of the 35-kD fragment. 3T3 fibroblasts were pulse labeled with [³H]leucine and chased for 3 h in medium containing 0.5 mM cycloheximide. Under these conditions all newly made H2 is converted into the 35-kD fragment (Wikström and Lodish, 1991). Because the protein contains several leucine residues in the region where cleavage occurs, [³H]leucine was used as the radioactive label. The fragments were subjected to NH₂-terminal sequencing by automated Edman degradation.

Fig. 1 *a* shows that the 35-kD cleavage fragment formed by H2b in fibroblasts generates three radioactive peaks (arrows) at cycles 3, 7, and 10 of the Edman degradation. These three peaks correlate precisely (and only) to leucines 84, 88, and 91 of the H2b amino acid sequence. Therefore, the amino terminus of the H2b 35-kD fragment is Ala82. Fig. 1 *b* shows the results of a similar radiosequencing of the cleavage fragment formed by H2a. Radioactive peaks are detected at cycles 11, 12, 15, 16, 18, and 19. This pattern indicates that there must be at least two species of 35-kD fragment which differ by only one amino acid residue at the amino terminus. One would generate [³H]leucine peaks at cycles 11, 15, and 18, corresponding positions 89, 93, and 96 of the H2a amino acid sequence, and thus its amino terminus would be Ser79. The other would generate [³H]leucine

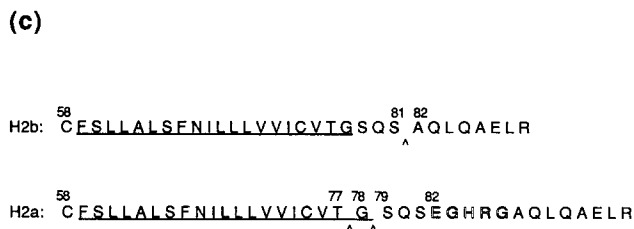
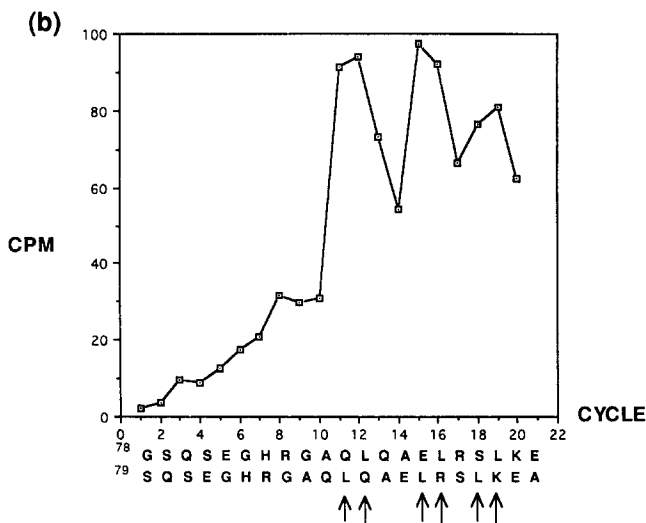
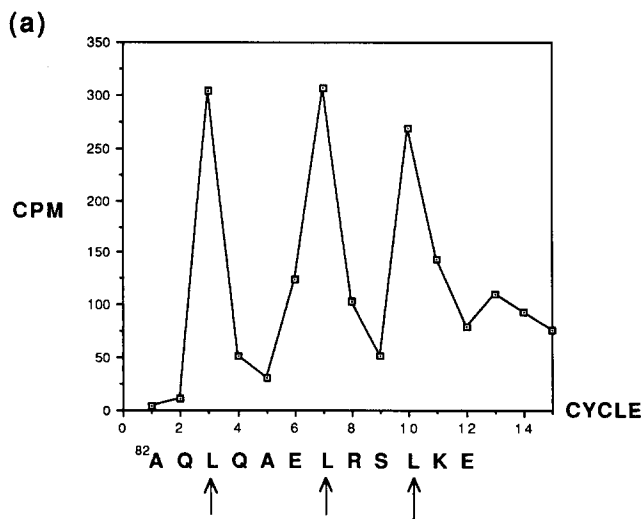


Figure 1. Determination of the cleavage site of H2b and H2a that generates the 35-kD fragment in NIH 3T3 fibroblasts. 10 million 3T3 cells expressing either H2b (a) or H2a (b) were pulse labeled with 0.3 mCi/ml of [³H]Leu for 30 min and chased in unlabeled medium containing 0.5 mM cycloheximide for 3 h. Cells were lysed in lysis buffer, immunoprecipitated with the anti-carboxyl-terminal H2 antiserum, and subjected to SDS-PAGE. Proteins were electrophoretically transferred onto Immobilon-P paper and the 35-kD cleavage fragment located by autoradiography. Bands on the paper were then cut out for NH₂-terminal protein sequencing and the radioactivity of each cycle of the Edman degradation was determined by liquid scintillation counting. Arrows indicate positions where radioactive leucines were detected. From the known amino acid sequence of this region, the position of these radioactive peaks were extrapolated to determine the cleavage site. (c) Results of radiosequencing of intracellular 35-kD fragment: A indicates a deduced cleavage site. The underlined section is the putative transmembrane region. The residues in H2a represented in shadow are not found in H2b.

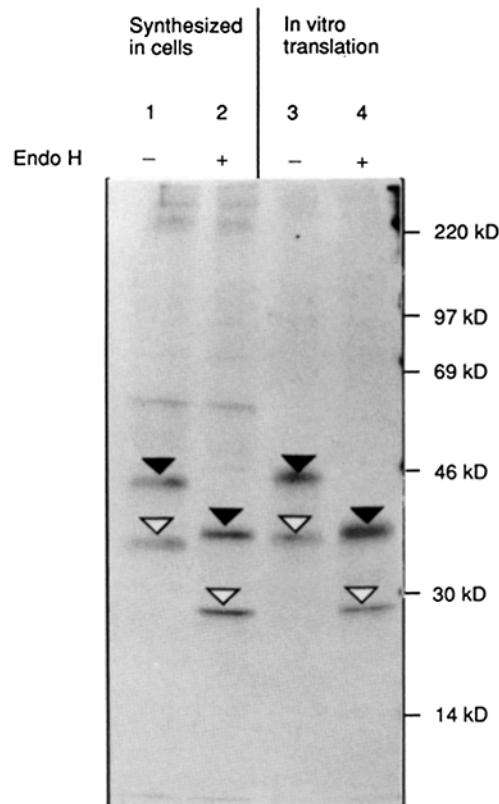


Figure 2. Formation of the 35-kD fragment during in vitro translation of H2 mRNA and in transfected cells. Lanes 1 and 2: 3T3 cells expressing H2a were pulse labeled with [³⁵S]-Cys for 30 min, chased for 2 h with 1 mM 2,4-dinitrophenol, 5 mM 2-deoxyglucose, and 0.5 mM cycloheximide, and then lysed in lysis buffer. Lanes 3 and 4: H2a mRNA was translated with [³⁵S]-Cys in a rabbit reticulocyte lysate with dog pancreatic microsomes for 2 h. At the end of reaction, the microsomes were pelleted and then solubilized in lysis buffer. All samples were immunoprecipitated with antisera specific for the H2 carboxy terminus, treated with (lanes 2 and 4) or without Endo H (lanes 1 and 3), and analyzed by SDS-PAGE and fluorography. Solid arrows indicate the intact protein. Open arrows indicate the cleavage fragment.

peaks at cycles 12, 16, and 19, corresponding leucines 89, 93, and 96; its deduced amino terminus would be Gly78.

If we assume that the 35-kD fragments are formed by a single endoproteolytic cleavage with no further trimming of the amino terminus, the amino termini of the 35-kD fragments would correspond to the cleavage sites (Fig. 1 c). H2b exhibits a single cleavage site between Ser81 and Ala82, and H2a exhibits two cleavage sites, one between Thr77 and Gly78, and the other between Gly78 and Ser79. Therefore, H2a and H2b utilize different cleavage sites for formation of the 35-kD fragment. For both proteins the cleavage sites are between small uncharged or small polar residues and the cleavage site in the H2b is at the position where the five extra amino acids are inserted in H2a.

The 35-kD Fragment Is Produced by In Vitro Translation of H2a or H2b mRNA in the Presence of Microsomes

Lane 3 of Fig. 2 shows that when H2a mRNA (synthesized by in vitro transcription of the corresponding cDNA) is sub-

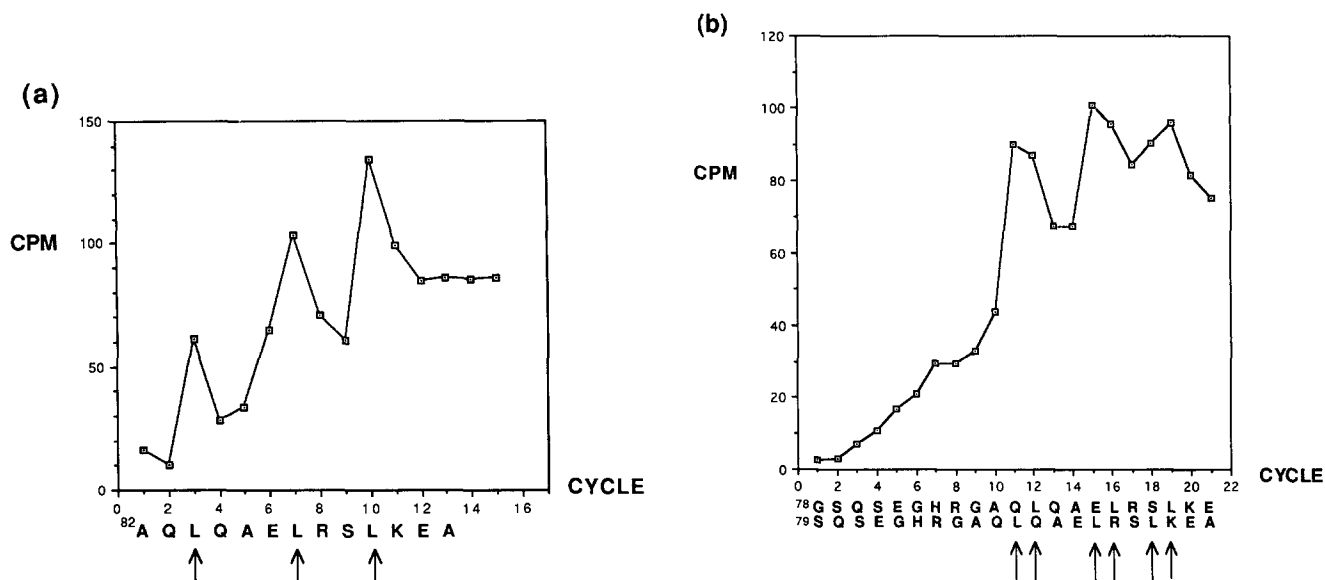


Figure 3. Radiosequencing of 35-kD fragment of H2b (a) and H2a (b) produced by in vitro translation. H2b (a) or H2a (b) mRNA was translated for 2 h with [³H]Leu in a rabbit reticulocyte lysate with dog pancreatic microsomes. At the end of the reaction, the microsomes were pelleted, solubilized in lysis buffer and then subjected to SDS-PAGE. Proteins were electroblotted onto Immobilon-P paper and the 35-kD cleavage fragment located by autoradiography. Bands on the paper were then cut out for NH₂-terminal protein sequencing and radioactivity of each cycle of the Edman degradation was determined by liquid scintillation counting. Arrows indicate positions where radioactive leucines were detected. From the known amino acid sequence of this region, the position of these radioactive peaks were extrapolated to determine the cleavage site.

jected to in vitro translation in a rabbit reticulocyte lysate in the presence of dog pancreatic microsomes, a 35-kD fragment (*open arrow*) is observed in addition to the expected full-length 43-kD core-glycosylated H2a (*solid arrow*). Lane 4 shows that both of these species are sensitive to Endo H (endoglycosidase H) digestion. Lane 1 shows that the full-length H2a and the 35-kD H2a fragment formed in transfected fibroblasts exhibit the same gel mobility as the corresponding species synthesized in vitro (lane 3). Lane 2 shows that the full-length and 35-kD fragments formed in fibroblasts are sensitive to Endo H digestion and that they also have the same gel mobility as the in vitro products (lane 4).

The 35-kD fragment is formed only if microsomes are present during the in vitro translation. It is immunoprecipitated only by antisera specific for the carboxyl terminus but not the amino terminus of H2, and it is present inside the microsomes as it is totally protected from digestion by proteinase K unless detergents are added to permeabilize the microsomes (data not shown). Identical results (data not shown) were observed by in vitro translation of H2b mRNA in the presence of microsomes. To confirm that these 35-kD fragments formed in vitro are identical to those formed in fibroblasts, they were subjected to radiosequencing analyses. Fig. 3, a and b show that the 35-kD fragments formed in vitro from both H2b and H2a mRNA have the NH₂-terminal sequences, and thus the same cleavage sites (H2b between Ser81 and Ala82, H2a between Thr77 and Gly78, and between Gly78 and Ser79), respectively, as those generated in intact cells (compare to Fig. 1). This result also confirms that the 35-kD fragment is formed in the ER without the need for vesicular transport (Wikström and Lodish, 1992). The full-length H2a and H2b proteins and fragments produced during the in vitro translation reaction are stable and do not undergo

further degradation, even after incubations of over 24 h after the termination of protein synthesis (data not shown). In contrast, both the core-glycosylated precursor and the 35-kD fragment formed in fibroblasts are quickly degraded (Amara et al., 1989).

Substitution of Residues Around the Cleavage Sites with Large Hydrophobic or Charged Amino Acids Inhibit Formation of the 35-kD Fragment During In Vitro Translation

Analysis of the sequence specificity of the cleavage process can provide important information regarding the proteolytic enzyme involved. Therefore, we constructed a number of substitution mutants around the putative cleavage sites of H2a and H2b, and analyzed the amount and NH₂ termini of the 35-kD fragment formed by these mutant proteins. As the residues surrounding the cleavage sites are small, polar, and noncharged, we suspected that signal peptidase may be responsible for this proteolytic cleavage (von Heijne, 1986). If this were true, substituting these residues with large hydrophobic or charged ones should cause a decrease in amount of cleavage (Folz et al., 1988; Shaw et al., 1988). The in vitro translation system was used initially to screen mutants for changes in the formation of the 35-kD fragment.

Since H2b exhibits only a single putative cleavage site, we first constructed two sets of H2b mutants, changing either the residue NH₂-terminal to the cleavage site (Ser81) or the COOH-terminal residue (Ala82). mRNAs encoding these mutant proteins were synthesized by in vitro transcription and then translated in vitro, and the amount of intact H2 protein and 35-kD fragment formed quantified by scanning densitometry. All mutant proteins studied were inserted nor-

Table I. Formation of 35-kD Fragment by H2 Mutants During In Vitro Translation and In Transfected Fibroblasts

Species	Sequence	Amount of 35-kD fragment formed in vitro (wild type = 1)	Formation of 35-kD fragment in fibroblasts
H2b wild type	<u>VICVTGSQS</u> ^{81 82} AQLQAE [^] LR	1	+
a. H2b Ser 81 Mutants:			
Ser81Ile	<u>VICVTGSQI</u> ^{81 82} AQLQAE [^] LR	0.50	-
Ser81Trp	<u>VICVTGSQW</u> ^{81 82} AQLQAE [^] LR	0.54	-
Ser81Arg	<u>VICVTGSQR</u> ^{81 82} AQLQAE [^] LR	0.47	-
Ser81Lys	<u>VICVTGSQK</u> ^{81 82} AQLQAE [^] LR	0.55	-
Ser81Asn	<u>VICVTGSQN</u> ^{81 82} AQLQAE [^] LR	0.80	+
Ser81Pro	<u>VICVTGSQP</u> ^{81 82} AQLQAE [^] LR	1.06	+
Ser81Gly	<u>VICVTGSQG</u> ^{81 82} AQLQAE [^] LR	2.60	+
Ser81Ala	<u>VICVTGSQA</u> ^{81 82} AQLQAE [^] LR	2.68	+
b. H2b Ala 82 Mutants:			
Ala82Pro	<u>VICVTGSQS</u> ^{81 82} PQLQAE [^] LR	1.37	+
Ala82Gly	<u>VICVTGSQS</u> ^{81 82} GQLQAE [^] LR	0.97	+
Ala82Arg	<u>VICVTGSQS</u> ^{81 82} RQLQAE [^] LR	0.91	+
Ala82Thr	<u>VICVTGSQS</u> ^{81 82} TQLQAE [^] LR	1.26	+
Ala82Glu	<u>VICVTGSQS</u> ^{81 82} EQLQAE [^] LR	1.44	+
Ala82His	<u>VICVTGSQS</u> ^{81 82} HQLQAE [^] LR	1.14	ND
c. H2a wild type	<u>VICVT</u> ^{77 78 79 82} <u>G</u> SQSEGH [^] RG [^] AQLQAE [^] LR	1	+
H2a Mutants:			
Ser79Pro	<u>VICVT</u> ^{77 78 79} <u>G</u> PQSEGH [^] RG [^] AQLQAE [^] LR	0.29	-
Ser79Trp	<u>VICVT</u> ^{77 78 79} <u>G</u> WQSEGH [^] RG [^] AQLQAE [^] LR	0.35	-
Gly78Trp	<u>VICVT</u> ^{77 78 79} <u>W</u> SQSEGH [^] RG [^] AQLQAE [^] LR	0.23	-
Gly78Arg	<u>VICVT</u> ^{77 78 79} <u>R</u> SQSEGH [^] RG [^] AQLQAE [^] LR	0.27	-
Thr77Trp	<u>VICVW</u> ^{77 78 79} <u>G</u> SQSEGH [^] RG [^] AQLQAE [^] LR	0.22	-
Gly78Pro	<u>VICVT</u> ^{77 78 79} <u>P</u> SQSEGH [^] RG [^] AQLQAE [^] LR	0.82	+
Glu82Ala	<u>VICVT</u> ^{77 78 79} <u>G</u> SQSAGH [^] RG [^] AQLQAE [^] LR	0.77	+

Boldface indicates the mutant residue. ^ indicates deduced cleavage site(s) in the wild type proteins. The underlined section is part of the putative transmembrane region. Residues in italics are the five extra amino acids in H2a. In vitro translations: mRNA encoding H2 wild-type or mutant protein was translated with [³⁵S]Cys in rabbit reticulocyte lysates in the presence of dog pancreatic microsomes for 2 h. Pelleted microsomes were immunoprecipitated, treated with Endo H, and then subjected to SDS-PAGE. Intact H2 protein and the 35-kD cleavage fragment were detected by fluorography, quantitated by scanning densitometry, and normalized to the number of cysteine residues. The extent of endoproteolytic cleavage of each sample was calculated as the fraction of the fragment relative to total (intact plus fragment) H2. (The average extent of cleavage of the wild type H2a was 0.3 and that of the H2b was 0.2). The extent of cleavage of each mutant was normalized to the value of the wild-type protein from the same experiment, set at 1.0. Intact fibroblasts: 3T3 cells expressing H2 wild-type or mutant proteins were pulse labeled with [³⁵S]Cys for 15 min, chased with 0.5 mM cycloheximide in unlabeled medium for 3 h, and then lysed in lysis buffer. The cell lysates were immunoprecipitated with anti-carboxyl-terminal H2 antisera and analyzed by SDS-PAGE and fluorography. + indicates that 35-kD fragment is detected. - indicates that the fragment is not detected. ND, not determined.

H2b Wild type:	VICVTG	⁸¹ SQS	⁸² AQLQAE [^] LR
H2b Mutants:			
S81I:	VICVTG	SQI	AQLQAE [^] LR
S81R:	VICVTG	SQR	AQLQAE [^] LR
S81G:	VICVTG	SQG	AQLQAE [^] LR
S81A:	VICVTG	SQA	AQLQAE [^] LR
A82R:	VICVTG	SQS	RQLQAE [^] LR
A82P:	VICVTG	SQS	PQLQAE [^] LR
A82G:	VICVTG	SQS	GQLQAE [^] LR
A82T:	VICVTG	SQS	TQLQAE [^] LR
A82E:	VICVTG	SQS	EQLQAE [^] LR

Figure 4. Determination of cleavage site of H2b mutants by radiosequencing of 35-kD fragment produced during in vitro translation. H2b mutant mRNAs were translated with [³H]Leu in rabbit reticulocyte lysates with dog pancreatic microsomes for 2 h. At the end of the reaction, the microsomes were pelleted, solubilized in lysis buffer, and then subjected to SDS-PAGE. Proteins were electroblotted onto Immobilon-P paper and the 35-kD cleavage fragment located by autoradiography. The band on Immobilon-P paper was then excised for NH₂-terminal protein sequencing and radioactivity of each cycle of the Edman degradation was determined by liquid scintillation counting. From plots of radioactivity against reaction cycles, peaks corresponding to [³H]Leucines were determined and data extrapolated to determine the cleavage site. Boldface indicates the mutant residue. A indicates a deduced cleavage site. The underlined section is part of the putative transmembrane region.

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mally into microsomal membranes and were N-glycosylated (data not shown). For each mutant protein, the fraction of the amount of 35-kD fragment relative to the total (intact and fragment) was calculated and then normalized to that of the wild-type protein which is given a reference value of 1.0. Table I a, column 3, summarizes the data from H2b mutants in which Ser81 residue was mutated. Mutation of Ser81 to a large hydrophobic (Ile, Trp) or positively charged residue (Arg, Lys) causes a twofold decrease in the amount of 35-kD fragment formed during in vitro translation. Two mutants (S81P and S81N) generate amounts of 35-kD fragment similar to that of the wild type while two mutants (S81A and S81G) generated twofold increased amounts of the 35-kD fragment.

These results are consistent with the notion that signal peptidase may be responsible for the cleavage, as this protease normally does not cleave COOH-terminal to large hydrophobic or positively charged residues. However, the S81I, S81W, S81R, and S81K mutants do generate some 35-kD fragment formation, and this might be due to a change in the position of the cleavage site. Therefore, the amino termini of the H2b S81R and S81I 35 kD fragments produced in vitro were determined by radiosequencing. Fig. 4 shows that the NH₂ termini of the H2b S81R and S81I 35-kD fragments, defining the putative cleavage site, have been shifted from that of the H2b wild-type site (S81/A82) to G78/S79, one of the cleavage sites used by H2a. In contrast, radiosequencing of the 35-kD fragments formed during in vitro translation of the H2b S81G and S81A mutants, which exhibit increased amounts of 35-kD fragments, showed that the putative cleavage sites are the same as wild type (Fig. 4).

Six mutants of Ala82 in H2b were also analyzed (Table I b). No mutant showed any significant change in the amount of 35-kD fragment formed during in vitro translation (column 3). However, analyses of the NH₂ termini of these 35-kD fragments showed that the cleavage sites of the A82P and A82G mutants are shifted from that of the H2b wild-type site (S81/A82) to G78/S79, one of the cleavage sites found in

H2a. The A82E and A82T mutants generated two species of 35-kD fragments, with NH₂ termini corresponding to that of the H2b wild-type S81/A82 and to cleavage at the G78/S79 site. The A82R mutant exhibited only cleavage at the wild-type S81/A82 site.

A less extensive mutational analysis was done with H2a (Table I c). The putative cleavage sites of the wild-type H2a that generate the 35-kD fragment are T77/G78 and G78/S79. Mutants of T77, G78, or S79 were constructed and analyzed for amount of 35-kD fragment formation during in vitro translation in the presence of microsomes. The T77W, G78W, G78R, and S79W mutants showed significantly (70–80%) decreased formation of the 35-kD fragment. All of these mutants either have a large hydrophobic amino acid (Trp) or a positively charged residue (Arg) adjacent to the putative cleavage site. Interestingly, the S79P mutant, but not the G78P mutant, also exhibited a large decrease in formation of the 35-kD fragment.

Mutations That Cause a Decrease in Formation of the 35-kD Fragment during In Vitro Translation Also Cause a Decrease in the Amount of 35-kD Fragment Formed in Intact Fibroblasts

The cDNAs encoding the H2a and H2b mutants were subcloned into a mammalian expression vector and then transfected into NIH 3T3 cells to form stable cell lines. These were used to study the effect of the mutations on the formation of the 35-kD fragment in intact cells. All the mutant proteins studied were properly inserted into the ER and became N-glycosylated. The extent of formation of the 35-kD fragment was determined by experiments in which cells expressing the H2 mutants were pulse labeled and then chased in unlabeled medium containing cycloheximide. In cells expressing the wild-type H2 proteins the presence of cycloheximide in the chase medium results in conversion of all newly synthesized H2 into the 35-kD fragment (Wikström and Lodish, 1991). The presence or absence of the 35-kD fragment in cells expressing the mutants was determined, as shown for two typical mutants in Fig. 5. Both the H2b S81G and the H2b S81W mutants are synthesized as 43-kD core-glycosylated precursors (Fig. 5, lanes 1 and 3, respectively, *solid arrow*). During a chase of 3 h in the presence of cycloheximide the S81G mutant clearly produces much 35-kD fragment (lane 2, *open arrow*) but the S81W mutant produces no 35-kD fragment (lane 4). Column 4 of Table I summarizes the results obtained in similar studies for all our H2a and H2b mutants. When the H2b S81I, S81W, S81R, and S81K mutants are expressed in fibroblasts, no detectable amount of 35-kD fragment is observed. However the H2b S81P, S81G, S81A, and S81N mutants form wild-type levels of the 35-kD fragment. Similarly, the H2b A82G, A82P, A82R, A82E, and A82T mutants produce approximately normal amounts of the 35-kD cleavage fragment. No detectable amount of the 35-kD fragment (or any other immunoprecipitable fragment) was detected in cells expressing the H2a T77W, G78W, G78R, S79W, and S79P mutants, but wild-type amounts of the 35-kD fragment were formed by the G78P and E82A mutants.

Therefore, all H2a and H2b mutants which exhibit a significant decrease in the amount of 35-kD fragment formed during in vitro translations generate no detectable amount of

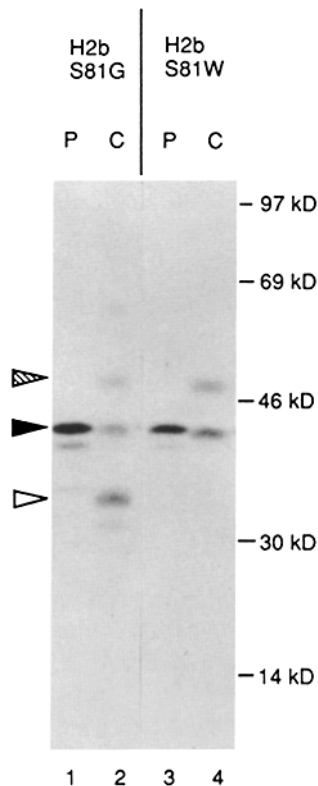


Figure 5. Formation of the 35-kD fragment in fibroblasts expressing H2b mutant proteins S81G and S81W. 3T3 cells expressing H2b S81G or H2b S81W were pulse labeled with 0.3 mCi/ml of [³⁵S]Cys for 15 min, chased with 0.5 mM cycloheximide in unlabeled medium for 3 h, and then lysed in lysis buffer. The cell lysate was immunoprecipitated with antisera specific for the H2 carboxyl terminus and analyzed by SDS-PAGE and fluorography. The solid arrow indicates the position of the 43-kD core-glycosylated protein and the open arrow indicates the position of the 35-kD fragment. The striped arrow indicates the complex glycosylated form. *P*, sample that is pulse labeled only; *C*, sample that is pulse labeled and then chased with 0.5 mM cycloheximide in unlabeled medium for 3 h.

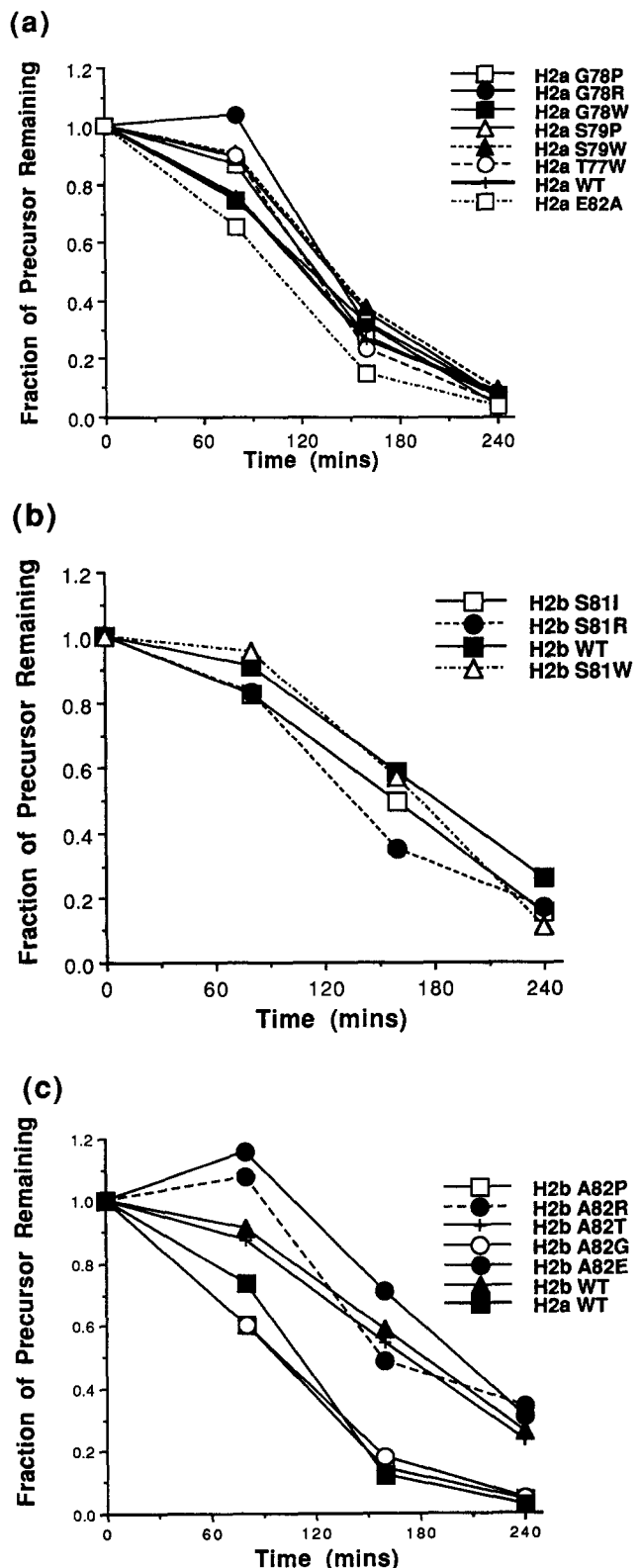
35-kD fragment in transfected fibroblasts. All mutants which generated a normal or above-normal amount of 35-kD fragments during *in vitro* translations formed, in transfected cells, approximately normal amounts of a 35-kD fragment. We did not determine the NH₂ termini of the 35-kD fragments generated in cells expressing the mutant proteins, and we assume that they are identical to those generated during *in vitro* translations in the presence of microsomes (summarized in Fig. 4).

These experiments demonstrate that the protease responsible for formation of the 35-kD cleavage fragment prefers to cleave between small neutral or small polar residues. Large hydrophobic or charged amino acids at either side of the cleavage site are inhibitory. Mutations which may affect the conformation of the protein around the putative cleavage site (e.g., H2b A82G or A82P mutations) appear to affect the position of the cleavage. We therefore suspect that signal peptidase, which has such properties (Nothwehr and Gordon, 1989), or a protease in the ER with similar sequence specificity, is responsible for generating the 35-kD fragment. However, we do not have direct evidence for this hypothesis.

Mutant H2a and H2b Proteins Which Do Not Generate the 35-kD Cleavage Fragment Exhibit Normal Kinetics of ER Degradation in Transfected Fibroblasts

If ER cleavage of the H2 protein to form the 35-kD fragment

Figure 6. Kinetics of ER degradation of mutant H2a and H2b proteins in transfected fibroblasts. 3T3 cells expressing wild-type or mutant H2 proteins were pulse labeled with 0.3 mCi/ml of [³⁵S]-Cys for 15 min and then chased in unlabeled medium for up to 4 h.



Anti-H2 immunoprecipitates of cell lysates made after various times of chase were subjected to SDS-PAGE. The 43-kD high mannose precursor was detected by fluorography and quantitated by scanning densitometry. (a) Pulse chase of H2a wild type and mutants. (b) Pulse chase of H2b wild type and mutants at Ser81 that do not generate a 35-kD fragment. (c) Pulse chase of H2a wild type, H2b wild type, and H2b mutants at Ala82.

is an obligatory step in ER degradation, we would expect the rate of ER degradation of H2 mutant proteins that do not generate the 35-kD fragment to be significantly slower than that of the H2 wild type. To study the kinetics of the ER degradation of various H2 mutant proteins, fibroblasts expressing H2 proteins were pulse labeled and then chased in unlabeled complete medium for up to 4 h. The amount of the 43-kD core-glycosylated ER precursor was monitored at intervals (Fig. 6).

Fig. 6 *a* shows a comparison of the H2a wild type with seven different mutants. After a lag of 30–60 min, the core-glycosylated precursor of the wild-type H2a protein is degraded rapidly (half-life of ~ 1 h) with kinetics similar to those previously described (Amara et al., 1989). After 4 h of chase, less than 10% of the pulse labeled precursor remains. The core-glycosylated precursors of the five mutants that do not generate a 35-kD fragment (G78W, G78R, S79P, S79W, and T77W) and the two which do (G78P and E82A) are degraded at rates similar to that of the H2a wild type. For the mutants G78W, G78R, S79P, S79W, and T77W, no 35-kD (or other) fragment was seen at any time during the pulse or chase (data not shown). Among the different mutant cell lines there are differences in the extent of the lag period prior to degradation. However, these variations are not reproducible and may be due to slightly different metabolic states of the cells at the time of the experiment. Similar to the wild type, none of the H2a mutants studied generated any detectable amount of the 50-kD complex-glycosylated Endo H-resistant form (data not shown). Therefore, H2a mutant proteins that are not cleaved to form the 35-kD fragment do not escape ER degradation and do not reach the medial Golgi where complex oligosaccharides are attached.

Fig. 6 *b* shows that the rate of ER degradation of the core-glycosylated precursors of several mutants H2b proteins that do not form a 35-kD fragment (S81I, S81R, S81W) is the same as that of the H2b wild type (*solid squares*). For these mutants no 35-kD (or other) fragment was seen at any time during the pulse or chase (data not shown). The rate of degradation of the ER precursors of the S81P, S81A, S81G, and S81N mutants that do generate the 35-kD fragment is similar to that of the H2b wild type (data not shown).

About 30% of newly made H2b wild-type protein folds properly in the ER and matures to the cell surface (Lederkremer and Lodish, 1991). The other 70% is degraded in the ER. Table II shows that, for all of the S81 mutant proteins studied, ~ 25 –40% of the pulse labeled protein acquires complex oligosaccharides by 4 h of chase. Thus, transport of these mutant proteins from the ER to the Golgi and eventually to the cell surface is normal. Thus, H2b mutants which do not generate a 35-kD fragment in the ER do not persist for longer periods in that organelle nor do a significantly greater fraction acquire complex oligosaccharides. All the mutant S81 proteins exhibit normal rates of ER degradation of the ER core-glycosylated precursor, regardless of the ability to form the 35-kD fragment. Therefore, the cleavage event that generates the 35-kD fragment is not obligatory for ER degradation of H2b proteins.

H2b Ala82Pro and Ala82Gly Mutants Have Metabolic Fates Similar to That of the Wild-type H2a

H2a and H2b have very different metabolic fates in fibro-

Table II. Fraction of Pulse Labeled H2b Protein Acquiring Complex Oligosaccharides After a 4 h Chase

Species	Fraction complex glycosylated at 4 h
H2b wt	0.31
H2b S81I	0.39
H2b S81R	0.33
H2b S81W	0.32
H2b S81N	0.40
H2b S81P	0.28
H2b S81G	0.26
H2b S81A	0.33
H2b A82P	0
H2b A82G	0
H2b A82R	0.41
H2b A82T	0.30
H2b A82E	0.42

3T3 cells expressing H2b wild type or mutants were pulse labeled with 0.3 mCi/ml of [35 S]Cys for 15 min and then chased in unlabeled medium for 4 h. Immunoprecipitates of cell lysates were subjected to SDS-PAGE. The 50-kD complex-glycosylated forms of H2 were detected by fluorography and quantitated by scanning densitometry, relative to the total amount of H2 precursor after a pulse label.

blasts although H2a differs from H2b only by having five extra amino acids inserted between Ser81 and Ala82. All ER core-glycosylated H2a is degraded in the ER while, as noted, $\sim 30\%$ of H2b matures to the cell surface (Lederkremer and Lodish, 1991; Wikström and Lodish, 1993). As described above, H2a and H2b are cleaved at different sites to generate the 35-kD fragments. In vitro translation studies (Fig. 4) showed that the putative cleavage sites that generate the 35-kD fragment from the precursor of the mutant H2b A82P and A82G proteins are not the one used by the wild-type H2b, but are one of the two cleavage sites used by wild-type H2a.

Fig. 6 *c* compares the kinetics, in transfected fibroblasts, of ER degradation of the core-glycosylated precursors of five H2b mutants, A82P, A82R, A82T, A82G, and A82E, with those of the H2a and H2b wild types. The H2a wild-type protein (*solid squares*) exhibits a faster rate of degradation than the H2b wild-type protein (*solid triangles*). The ER precursors of three of the H2b mutants, A82R, A82T, and A82E, exhibit a rate of degradation similar to the H2b wild type. The fractions of A82R, A82T, and A82E protein that acquires complex oligosaccharides are also similar to that of the H2b wild type (Table II). In contrast, the ER precursors of the H2b A82P (Fig. 6 *c*, *open squares*) and A82G (*open circles*) mutants exhibit a faster rate of degradation than the H2b wild-type (*solid triangles*). The rates of ER degradation of these two mutant proteins are similar to that of wild-type H2a rather than H2b. Equally significant, none of the pulse labeled precursors of these two mutant proteins acquires complex-glycosylated (Golgi-processed) oligosaccharides (Table II), another characteristic of H2a.

Thus, the H2b A82G and A82P mutants have a metabolic fate similar to that of H2a; they are degraded very rapidly and no detectable fraction matures from the ER to the Golgi. However, with respect to the rate of ER degradation and the ability of a fraction of the protein to mature to the Golgi, the H2b A82E mutant has the same metabolic fate as H2b, not H2a (Fig. 6 *c*; Table II), although this mutant has a glutamate residue at position 82, as does wild-type H2a (Fig. 1 *c*).

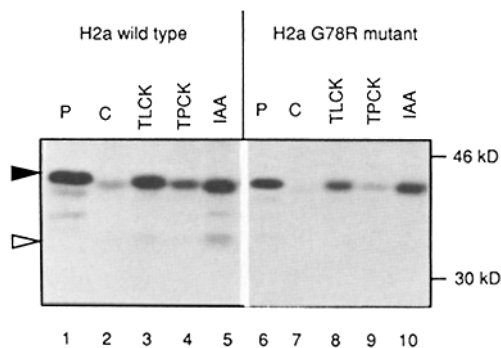


Figure 7. Inhibition of TLCK, TPCK, and iodoacetamide of ER degradation in transfected fibroblasts of the core-glycosylated precursors of the H2a wild type and H2a G78R mutant proteins. 3T3 cells expressing the H2a wild type or G78R mutant proteins were pulse labeled with 0.3 mCi/ml of [³⁵S]Cys for 15 min and then chased in unlabeled complete medium containing various inhibitors for 4 h. Immunoprecipitates of cell lysates were analyzed by SDS-PAGE and fluorography. Lanes 1 and 6: Pulse labeled only. Lanes 2 and 7: Chased in medium without any inhibitors. Lanes 3 and 8: Chased in medium with 100 μ M TLCK. Lanes 4 and 9: Chased in medium with 20 μ M TPCK. Lanes 5 and 10: Chased with 30 μ M iodoacetamide (IAA). The G78R mutant does not form the 35-kD cleavage product. The solid arrow indicates the position of the coreglycosylated ER precursor and the open arrow the position of the 35-kD fragment. P, samples that are only pulse labeled; C, samples that are pulse labeled and then chased for 4 h without any inhibitor.

Therefore, the difference in metabolic fates between wild-type H2a and H2b is not caused by the presence of glutamate (the most NH₂-terminal of the five amino acid insert) at position 82 in H2a. Furthermore, the H2a E82A mutant has an Ala residue at position 82, the same residue found at this position in H2b (Fig. 1 c). This mutant has the same metabolic fate as wild-type H2a (Fig. 6 a); no complex-glycosylated forms are generated. Thus, the nature of the amino acid at residue 82 is unlikely to be a determinant of the different metabolic fates of the two H2 isoforms. More likely, the pentapeptide present in H2a introduces a conformational change in this region of the protein which blocks folding of the exoplasmic domain and/or labilizes the protein to the ER degradation machinery, and this change can be mimicked by introduction of proline or glycine residues at residue 82 of H2b.

TLCK, TPCK, and Iodoacetamide Block ER Degradation of Both Wild-type H2 and Mutants That Do Not Form the 35-kD Fragment

The ER degradation of H2 can be inhibited by TLCK or TPCK (Wikström and Lodish, 1991, 1992). Fig. 7 shows that TLCK, TPCK, or iodoacetamide inhibit ER degradation in fibroblasts of both the core-glycosylated precursors of H2a wild type (lanes 3–5) and of the H2a G78R mutant which does not generate any 35-kD fragment (lanes 6–10). Over 90% of the 43-kD core-glycosylated protein (solid arrow) labeled during the pulse (lanes 1 and 6) is degraded after 4 h of chase in the absence of any inhibitors (lanes 2 and 7). The presence of 100 μ M TLCK (lanes 3 and 8) or 20 μ M TPCK (lanes 4 and 9) in the chase medium inhibits ER

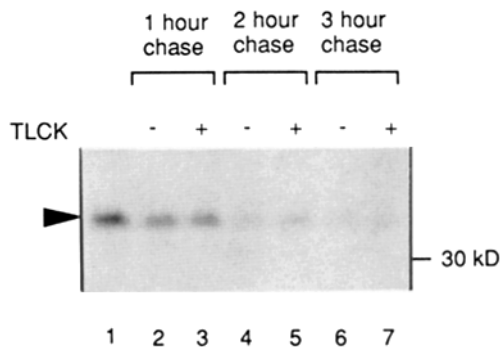


Figure 8. TLCK does not inhibit degradation of the 35-kD fragment in transfected cells. 3T3 cells expressing wild-type H2a were pulse labeled with 0.3 mCi/ml of [³⁵S]Cys for 15 min and then chased in unlabeled complete medium containing 0.5 mM cycloheximide for 5 h (lane 1). Cycloheximide was then removed and medium without any inhibitor (lanes 2, 4, and 6) or medium with 100 μ M TLCK (lanes 3, 5, and 7) was added and the chase continued for 1 (lanes 2 and 3), 2 (lanes 4 and 5), or 3 (lanes 6 and 7) more h. Immunoprecipitates of cell lysates at various time points after removal of cycloheximide were analyzed by SDS-PAGE and fluorography. Arrow indicates position of the 35-kD fragment. Time points on figure refer to time after the removal of cycloheximide.

degradation, as shown by the accumulation of the 43-kD core-glycosylated protein. The presence of 30 μ M iodoacetamide in the chase medium (lanes 5 and 10) also inhibits ER degradation. Like TPCK and TLCK (Wikström and Lodish, 1991), this concentration of iodoacetamide does not affect maturation of ASGP receptor H1 subunit proteins to the Golgi (data not shown), indicating that it does not generally inhibit cellular functions. In experiments not shown here, TLCK, TPCK, and iodoacetamide blocked ER degradation of H2a mutants that do not generate any 35-kD fragment (T77W, G78W, S79W, S79P) as well as H2a mutants that do (G78P, E82A).

TLCK and TPCK Do Not Inhibit Formation or Degradation of the 35-kD Fragment

Fig. 8 shows that TLCK does not inhibit the degradation of the 35-kD fragment in transfected fibroblasts. 3T3 cells expressing wild-type H2a were pulsed labeled and then chased in the presence of 0.5 mM cycloheximide which causes all precursor H2a to be converted to a 35-kD fragment which is not further metabolized (Fig. 8, lane 1, solid arrow) (see Wikström and Lodish, 1991). The cycloheximide was then removed and the chase continued for up to 3 h without any inhibitor (lanes 2, 4, and 6) or in the presence of 100 μ M TLCK (lanes 3, 5, and 7). The 35-kD fragment is degraded rapidly both in the absence of inhibitors and in the presence of 100 μ M TLCK. The same result is observed when TPCK is used instead of TLCK (data not shown). Therefore, degradation of the 35-kD fragment is not blocked by TLCK or TPCK.

Fig. 9 a shows that in transfected fibroblasts TLCK does not block the formation of 35-kD fragment from wild-type H2a. The overall degradation of the pulse labeled core-glycosylated H2a precursor (Fig. 9, lane 1, solid arrow) is inhibited by 100 μ M TLCK (lanes 5–7) compared to lanes 2–4 (chase without any inhibitor). When the chase medium

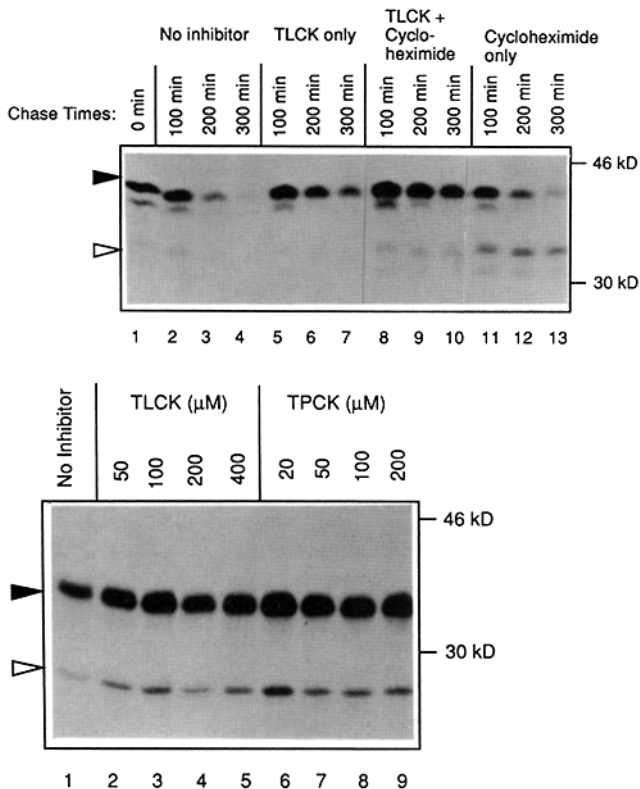


Figure 9. TLCK and TPCK do not inhibit formation of the 35-kD fragment. (a) Transfected cells. 3T3 cells expressing wild-type H2a were pulse labeled with 0.3 mCi/ml of [³⁵S]Cys for 15 min and then chased in unlabeled complete medium containing various inhibitors for up to 5 h. Immunoprecipitates of cell lysates at 0, 100, 200, and 300 min of chase were analyzed by SDS-PAGE and fluorography. Lane 1: Pulse labeled only. Lanes 2-4: Chased in medium without any inhibitor. Lanes 5-7: Chased in the presence of 100 μ M TLCK. Lanes 8-10: Chased in the presence of 100 μ M TLCK and 0.5 mM cycloheximide. Lanes 11-13: Chased in the presence of 0.5 mM cycloheximide. The solid arrow indicates the position of the core-glycosylated ER precursor and the open arrow indicates the position of the 35-kD fragment. (b) Cell free translation reactions. H2a mRNA was translated with [³⁵S]Cys in a rabbit reticulocyte lysate with dog pancreatic microsomes for 2 h in the absence of inhibitor (lane 1) or in the presence of TLCK (lanes 2-5) or TPCK (lanes 6-9). Inhibitors were added to the translation mixture and the reactions were incubated for 30 min at 30°C before addition of RNA. At the end of the reaction, microsomes were pelleted and then solubilized in lysis buffer. Samples were immunoprecipitated with antisera specific for the H2 carboxy terminus, treated with Endo H, and analyzed by SDS-PAGE and fluorography. The solid arrow indicates the position of the intact protein and the open arrow the position of the cleavage fragment.

contained both 100 μ M TLCK and 0.5 mM cycloheximide (lanes 8-10), 40% as much 35-kD fragment accumulated during the chase period as in samples (lanes 11-13) in which only cycloheximide is present. Thus TLCK does not block formation of the 35-kD fragment.

Fig. 9 b shows that up to 400 μ M of TLCK or 200 μ M of TPCK have no effect on formation of the 35-kD fragment during in vitro translation of the H2a protein in the presence of microsomes (open arrow). In this experiment the inhibitors were preincubated in the translation mixture for 30 min

before addition of mRNA and the concentrations of TLCK and TPCK used are well above those required for inhibition of H2 degradation in transfected fibroblasts.

Therefore, TLCK or TPCK blocks the ER degradation pathway of H2a that does not involve formation of the 35-kD fragment, but does not block either the formation or further degradation of the 35-kD fragment. This indicates that the cleavage event that forms the 35-kD fragment is only one of at least two pathways for the ER degradation of H2. Since iodoacetamide has a similar effect as TLCK and TPCK, it is likely that these compounds are inhibiting the same protease, one that requires an essential sulfhydryl group for activity.

Discussion

There Are at Least Two Pathways for the ER Degradation of the H2 Subunit of the Asialoglycoprotein Receptor

During ER degradation of the core-glycosylated precursors of both the H2a and H2b subunits, a 35-kD fragment accumulates transiently and is ultimately degraded; it is formed by proteolytic cleavage in the exoplasmic domain near the transmembrane region (Wikström and Lodish, 1991, 1992). In the presence of cycloheximide all precursor H2 is converted to this intermediate, which is degraded completely only after cycloheximide is removed. Thus, the proteolytic cleavage generating this 35-kD fragment was thought to be an obligatory step in ER degradation of H2 (Wikström and Lodish, 1991). Here, we show that this is not the only pathway for ER degradation.

If the initial cleavage process that forms the 35-kD fragment is obligatory for ER degradation of H2, mutants which show a decrease in formation of the 35-kD fragment would be expected to have a slower rate or extent of degradation. However, in transfected fibroblasts the ER precursors of mutant H2 proteins that do not generate a 35-kD fragment have similar rates of ER degradation to that of the H2 wild type and H2 mutants that do generate a 35-kD fragment (Fig. 6). It is unlikely these observations are due to very rapid degradation of a 35-kD fragment generated by precursors of some of the mutants, so as to make them undetectable, because they are not detected even in cells that have been treated with cycloheximide, which causes all pulse labeled wild-type H2 precursor to be converted to a stable 35-kD fragment. This suggests that there is an alternate degradation pathway for the H2 protein in the ER that is not dependent on the cleavage process that generates the 35-kD fragment.

In both Hep G2 cells and transfected 3T3 cells, TLCK and TPCK inhibit the degradation of both H2a and H2b precursors in the ER (Wikström and Lodish, 1991, 1992). It was then thought that formation of the 35-kD fragment is obligatory for the degradation of H2 in the ER and that the inhibitors work by blocking the formation of the 35-kD fragment. The main reason for thinking so was that little 35-kD fragment could be detected in pulse chase experiments when cells were treated with TLCK or TPCK. Also, cycloheximide blocks further degradation of the 35-kD fragment; in its presence all precursor H2 is converted to a 35-kD fragment. However, we showed here that mutant H2 proteins that do not generate the 35-kD fragment in transfected fibroblasts

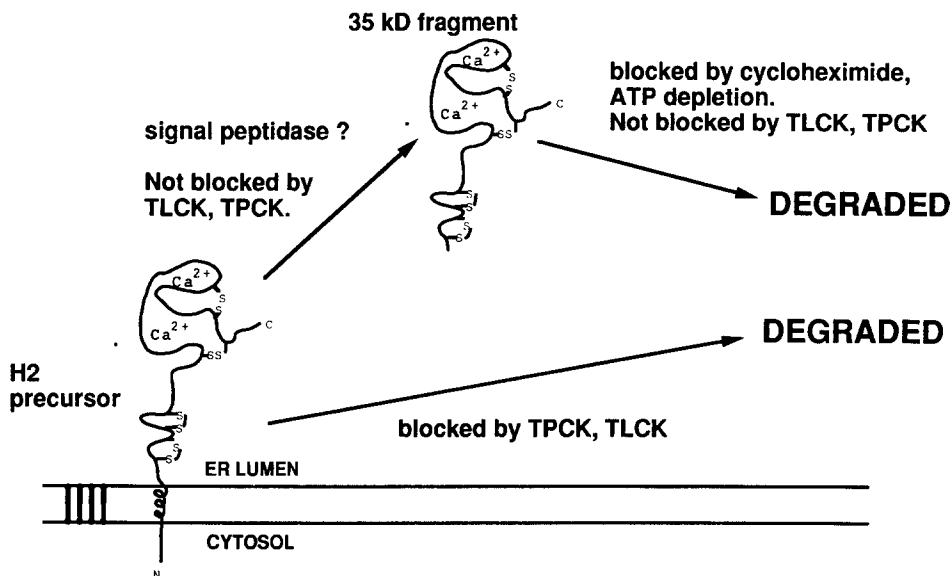


Figure 10. Model illustrating the two pathways for ER degradation of H2.

are degraded in the ER at the same rate as those that do form the fragment. Furthermore, TLCK and TPCK are able to inhibit the degradation of the mutants which do not form the fragment (Fig. 7). These results led us to postulate that two pathways exist for the degradation of the H2 protein and to reassess the role of TLCK and TPCK in the two degradation pathways. We showed directly that the degradation of the 35-kD fragment is not inhibited by TLCK or TPCK (Fig. 8). We also found that the 35-kD fragment does accumulate in cells when both TLCK and cycloheximide are present (Fig. 9 a). Most likely, the 35-kD fragment is formed in the presence of TLCK and absence of cycloheximide but is rapidly degraded and does not accumulate. Furthermore, formation of the 35-kD fragment during *in vitro* translation is not inhibited by TLCK or TPCK (Fig. 9 b). Therefore, TLCK and TPCK neither block the formation nor the degradation of the 35-kD fragment. Thus, both the enzyme(s) that generate the fragment and those that degrade it are unlikely to be involved in the second ER degradative pathway, that not involving the 35-kD fragment. As TLCK or TPCK can inhibit the ER degradation of the vast majority of pulse labeled H2, we think that the pathway not involving the 35-kD intermediate is normally the major one and this is the pathway that is sensitive to TLCK and TPCK. Fig. 10 shows a model for the ER degradation of H2 that illustrates these two pathways of degradation. Inoue and Simoni (1992) showed that the degradation of the T cell receptor α subunit and the HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase in the ER exhibit different sensitivities to several protease inhibitors. This suggests that multiple mechanisms of protein degradation exist in the ER which affect different proteins. The present study indicates that the same protein may be subjected to more than one degradation pathway.

Wikström and Lodish (1993) showed that, for the H2b protein, ~30% of the newly synthesized protein becomes properly folded and is transported to the Golgi while the ~70% that is degraded in the ER is not properly folded. They also showed that inhibition of the ER degradation with TLCK or TPCK causes accumulation of unfolded forms of the protein within the ER. Therefore, the ER degradation

process that is inhibited by TLCK or TPCK recognizes the fraction of the protein that remains unfolded in the ER and targets them for degradation. Here we showed that the same fraction of ER precursors of mutant H2b proteins that do not generate 35-kD fragment (e.g., S81I, S81W, and S81R) acquires complex oligosaccharides as does wild-type H2b or those mutants that do generate the 35-kD fragment. This indicates that the fraction of H2b precursor protein that folds properly in the ER, moves to the Golgi complex, and presumably is transported to the cell surface is not increased when formation of the 35-kD fragment in the ER is inhibited by mutation.

Iodoacetamide has similar effect to those of TLCK and TPCK in inhibiting one of the pathways of ER degradation of H2. This suggests that this degradation system utilizes active sulfhydryl group(s) or cysteine protease(s). Proteases of this class have been suggested to be involved in the ER degradation of the T cell receptor subunits (Wileman et al., 1991) and that of the HMG-CoA reductase (Inoue et al., 1991).

The Endoprotease That Cleaves H2 to Generate the 35-kD Fragment Has Properties Similar to Signal Peptidase

By determining the NH₂ termini of the 35-kD fragments, we have localized the most likely proteolytic cleavage sites in both H2a and H2b (Fig. 1). The cleavage sites are slightly different in H2a and H2b but they are all near the putative transmembrane region on the exoplasmic side of the protein. H2b has only one putative cleavage site and H2a has two. The occurrence of endoproteolytic cleavage within the lumen of the ER and the proximity of the cleavage sites to the transmembrane region suggests that the proteolytic enzyme involved in the cleavage may be a membrane protein. Although different sites are used for H2a and H2b, they are only a few amino acid residues away from each other and in both cases the residues NH₂- and COOH-terminal to the cleavage sites are all small neutral or small polar amino acids (Thr, Gly, Ser, and Ala). One proteolytic enzyme in the ER membrane that is known to have preferences for such amino

acids NH₂- and COOH-terminal to the cleavage site on its substrate is signal peptidase (Dalbey and von Heijne, 1992).

Applying von Heijne's algorithm for the prediction of signal peptidase cleavage sites (von Heijne, 1986) to the region of the H2b protein just exoplasmic to the transmembrane sequence, the optimal site for signal peptidase action is between Ser81 and Ala82, precisely the cleavage site deduced from our experiments. However, a similar calculation for H2a indicates that the optimal signal peptidase cleavage site should be between Ser81 and Glu82, but the experimentally deduced sites are T77/G78 and G78/S79.

H2 is a type 2 transmembrane protein with a single transmembrane region that functions during ER insertion as an uncleaved signal anchor (Spiess and Lodish, 1985). There are examples of type 2 signal-anchor sequences which are cleaved by signal peptidase, but only after removal of the amino-terminal cytosolic domain. One is the invariant chain of the Class II histocompatibility antigens (Lipp and Dobberstein, 1986). Another is the ASGP receptor H1 subunit. This is also a type 2 transmembrane protein that is 60% homologous to H2 but it is normally not subjected to proteolytic cleavage when expressed in fibroblasts or synthesized by *in vitro* translation in the presence of pancreatic microsomes. However, when the NH₂-terminal cytosolic segment of the protein is deleted, the transmembrane region is cleaved from the exoplasmic domain, most probably by signal peptidase (Schmid and Spiess, 1988). The NH₂-terminal cytosolic domain of H1 may modulate the accessibility of the potential cleavage site in the transmembrane region. When the NH₂-terminal cytosolic domain is removed, the site becomes accessible and the protein is cleaved. The site of cleavage of the mutant H1 lacking the NH₂-terminal domain is between amino acids Gly60 and Ser61 of H1, numbered according to wild-type H1 (Schmid and Spiess, 1988). The homologous site, G78/S79 in H2a is also one of the deduced sites of endoproteolytic cleavage on precursor H2a. Thus, it is possible that H2 also has a potential site for signal peptidase cleavage at the COOH terminus of the transmembrane region and perhaps the cytosolic domain of the H2 protein is not as effective as that of H1 in preventing access of signal peptidase to this site.

To determine the specificity of the endoproteolytic cleavage that generates the 35-kD fragment, we mutated amino acid residues NH₂- and COOH-terminal to the deduced cleavage sites. All the mutants were analyzed both *in vitro* translation microsome-insertion systems and in transfected fibroblasts, and showed proper membrane insertion and core asparagine-linked glycosylation. Therefore any decrease in formation of the 35-kD fragment is not due to incompetence of the mutant protein for insertion in the ER membrane. *In vitro* translation studies of mRNA encoding mutant H2 proteins showed that the extent of formation of the 35-kD fragment was decreased (but not abolished) when the residue NH₂-terminal to the cleavage site (Ser81) was mutated to a large hydrophobic (Trp, Ile) or charged residue (Arg, Lys). Conversely, mutation of Ser81 to perhaps even more favorable residues for signal peptidase cleavage (H2b S81A and S81G) increased the amount of 35-kD fragment formed. The same qualitative results were seen when these mutant proteins were synthesized in transfected fibroblasts: H2b mutants S81W, S81I, and S81R did not generate detectable amounts of the 35-kD fragment, while normal amounts of

the fragment were produced in cells expressing mutants H2b S81A and S81G. Mutant H2 proteins that generated low amounts of the 35-kD fragment during *in vitro* translation generated none in transfected cells. There are several possible explanations, including tissue and species differences, dog pancreatic microsomes versus mouse fibroblasts, and differences in ionic and redox conditions in the experimental systems. However, there is an excellent qualitative correlation between the two systems on the effect of mutations on formation of the 35-kD fragment.

The 35-kD fragment produced in low amounts during the *in vitro* translation of the H2b S81I and S81R mutants had a different NH₂ terminus from that of the wild type, suggesting that a different cleavage site was used to generate the 35-kD fragments. The presumed cleavage site, G78/S79, is one of the two cleavage sites utilized to form the 35-kD fragment from H2a. This change in cleavage site may be due to the selection of the next most favorable site for cleavage by signal peptidase once the original site has been mutated to become unfavorable. This property of signal peptidase has been previously described (Folz et al., 1988).

When Ala82 in H2b, the residue COOH-terminal to the normal cleavage site, was mutated, there was no significant decrease in the amount of 35-kD fragment formed, either during *in vitro* translations or in transfected fibroblasts. However, during *in vitro* translations the H2b A82G and A82P mutants were cleaved at G78/S79, rather than at the wild-type cleavage site of S81/A82, as judged from the NH₂ termini of the 35-kD fragments. As mentioned above, the G78/S79 site is one of the cleavage sites utilized in H2a for generation of the 35-kD fragment. The H2b A82E and A82T mutants were cleaved at two sites, G78/S79 as well as the normal H2b site S81/A82, whereas the H2b A82R mutant was only cleaved at S81/A82. Thus cleavage site selection may also be affected by changes in protein conformation that may be introduced by these mutations. Proline and glycine residues are most likely to affect the protein conformation by inducing β -turns or breaking α helices (Chou and Fasman, 1978). The conformational changes introduced by these amino acids may in turn affect the recognition of cleavage sites by the protease and result in proteolytic cleavage at an alternative site.

Single amino acid substitutions in H2a can inhibit formation of the 35-kD fragment (see Table Ic) even though there are two putative cleavage sites. Similar to H2b, large hydrophobic or positively charged residues NH₂- or COOH-terminal to the putative cleavage sites are inhibitory to formation of the 35-kD fragment. Interestingly, the H2a S79P mutant exhibits a marked decrease in extent of formation of the 35-kD fragment. This may be due to conformational changes introduced by the insertion of five extra amino acids in H2a which in turn affect the specificity of the proteolytic cleavage. It is also possible that different enzymes cleave H2a and H2b to generate the 35-kD fragments.

Folz et al. (1988) showed that the amino acid occupying the -1 position of the signal cleavage site is important in determining the efficiency and site selection of cleavage by eukaryotic signal peptidase. They showed that small residues at the -1 position are preferred by signal peptidase. Nothwehr and Gordon (1989) and Nothwehr et al. (1990) further demonstrated that conformational features of the signal sequence influence the efficiency and position of cleavage. The

amino acid specificity of the presumed endoproteolytic cleavage that generates the H2 35-kD fragment, and the effects of introduction of amino acids that tend to change protein conformation on the selection of the cleavage site, are similar to those exhibited by eukaryotic signal peptidase (reviewed by von Heijne, 1990; Dalbey and von Heijne, 1992). Therefore, the protease that cleaves H2 to produce the 35-kD fragment may be signal peptidase or another protease that has similar properties. As the putative cleavage sites of the H2 wild type and mutants have been determined by NH₂-terminal protein sequencing of the 35-kD fragments, we cannot rule out the possibility that all H2a and H2b mutants are initially cleaved by an endoprotease at the same site, and that differences of the NH₂ termini of the various fragments analyzed are due to differential exoprotease activity at the NH₂ terminus.

Conformational Differences in the Juxtamembrane Region May Explain the Difference in Metabolic Fate between H2a and H2b

H2a and H2b have different metabolic fates when expressed in fibroblasts. When H2b is expressed in fibroblasts in the absence of H1, ~30% of newly synthesized protein becomes folded normally, exits the ER, and reaches the cell surface while 70% remains unfolded and is degraded in the ER. In contrast, all H2a, expressed in the absence of H1, is degraded within the ER (Lederkremer and Lodish, 1991; Wikström and Lodish, 1991). H2b mutant proteins A82G and A82P, synthesized in transfected cells, have metabolic fates similar to that of wild-type H2a, not H2b. No newly made A82G and A82P mutant proteins acquire complex oligosaccharides, evidence that none exits the ER. The ER precursor proteins are degraded at the faster rate characteristic of H2a (Fig. 6 c). Most interestingly, the cleavage site used for generation of the A82G and A82P 35-kD fragments is one of those utilized by wild-type H2a, rather than the Ser81/Ala82 site used by wild-type H2b. Proline and glycine are residues commonly found in β -turns of proteins and the pentapeptide (Glu-Gly-His-Arg-Gly) inserted in H2a perhaps could form this type of secondary structure (Lederkremer and Lodish, 1991). Therefore, the H2b A82P and A82G mutants and wild-type H2a may have similar metabolic fates because they have similar conformations in the juxtamembrane region. The introduction of a β turn in this region of the protein, either by the pentapeptide or by introduction of proline or glycine, may cause a change in the cleavage site that generates the 35-kD fragment. This conformational change can also make the protein incapable of exiting the ER and thus, it becomes totally degraded within this organelle. It is possible that the juxtamembrane region of H2 is critical for correct folding of the entire exoplasmic domain because unfolded forms of the protein do not exit the ER and are quickly degraded. Alternatively, this region of the protein may not affect folding of the rest of the molecule, but certain conformations in this region (which would be disrupted in the H2b A82P and A82PG mutants and in wild-type H2a) may be essential for recognition by some factors that regulate ER-to-Golgi transport of plasma membrane proteins. It is interesting that only the H2b A82P and A82G mutants have metabolic fates similar to that of H2a; the H2b S81P and S81G mutants have metabolic fates similar to that of the wild-type H2b, suggesting that introduction of proline or glycine resi-

dues only at certain specific positions can alter the metabolic fate of the H2 protein.

The transmembrane domain of T cell receptor subunits is thought to be critical for targeting the proteins for ER degradation (Bonifacino et al., 1990, 1991; Wileman et al., 1991). In contrast, our evidence suggests that the domain of the ASGP receptor H2 subunit that determines the rate and extent of ER degradation is probably not within the transmembrane domain but is in the juxtamembrane exoplasmic region. This region modulates the fate of the H2 protein in the ER as shown by the difference in metabolic fate between H2a and H2b and the effect of introduction of proline and glycine residues in this region of H2b. However, the proteolytic cleavage in this region is not critical in determining the overall rate of ER degradation, since mutant H2 proteins that do not generate the 35-kD fragment are degraded at the normal rate, presumably by the second pathway that does not involve formation of the 35-kD fragment (Fig. 10).

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