Degradation and Folding of the Asialoglycoprotein Receptor in the Endoplasmic Reticulum

by

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B.Sc.(Hons) National University of Singapore (1988)

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at the Massachusetts Institute of Technology
July 1995

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Abstract

There are two pathways for the degradation of the asialoglycoprotein (ASGP) receptor H2 subunit in the endoplasmic reticulum (ER). One pathway involves the formation of an intermediate of 35 kD derived by an endoproteolytic cleavage in the exoplasmic domain near the transmembrane region. As determined by amino-terminal sequencing of the 35 kD fragment, the putative cleavage sites are between small, uncharged amino acid residues. Substitution of the residues amino- or carboxy-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. Therefore, the endoprotease that generates the 35 kD fragment has specificity similar to that of signal peptidase. Mutants of H2 which do not form the 35 kD fragment show the same kinetics of ER degradation in transfected cells as the wild-type. Therefore, there is a second pathway of ER degradation of the H2 protein which does not involve formation of the 35 kD fragment. This alternative pathway is inhibited by two protease inhibitors, TLCK or TPCK, but neither formation nor degradation of the 35 kD fragment is blocked by these reagents.

H2a and H2b are subunit isoforms of the ASGP receptor that differ only by a pentapeptide insertion in the exoplasmic juxtamembrane region of H2a. None of the H2a expressed in transfected cells mature to the plasma membrane, but up to 30% of H2b folds properly and matures to the cell surface. 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.
Unfolded forms of the H2b subunit are degraded in the ER while folded forms of the protein can mature to the cell surface. There are eight cysteines in the exoplasmic domain of the protein, forming four disulfide bonds in the folded protein. Mutation of the disulfide bond nearest to the transmembrane region (C1 mutant) does not prevent proper folding of the protein in cells. Mutations of the other three disulfides prevent proper folding of the protein and all of these three mutant proteins are degraded in the ER. A normal (~20%) fraction of the C1 mutant protein exits the endoplasmic reticulum to be processed in the Golgi complex and it does so at a faster rate compared to the wild-type. The same fraction of newly-made C1 mutant and wild-type proteins (~80%) remain in the ER, but the mutant protein is degraded more quickly. Furthermore, the folded form of this mutant protein is more resistant to unfolding by dithiothreitol than the wild-type. Together with the H1 subunit, the H2b C1 mutant protein can form a functional cell-surface receptor with similar binding affinities and uptake kinetics for natural ligands as that of the wild-type receptor. Therefore, two cysteines in the wild-type protein actually retard the folding and processing of the protein.

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H2b Ala82Pro and Ala82Gly mutants have metabolic fates similar to that of the wild-type H2a. 

TLCK, TPCK and Iodoacetamide block ER degradation of both wild-type H2 and mutants that do not form the 35 kD fragment.

TLCK and TPCK do not inhibit formation or degradation of the 35 kD fragment.

Discussion

There are at least two pathways for the ER degradation of the H2 subunit of the asialoglycoprotein receptor.

The endoprotease that cleaves H2 to generate the 35 kD fragment has properties similar to signal peptidase.

Conformational differences in the juxtamembrane region may explain the difference in metabolic fate between H2a and H2b.

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Chapter 3: Enhanced Folding and Processing of a Disulfide Mutant of the Human Asialoglycoprotein Receptor H2b Subunit

Preface

Abstract

Introduction

Materials and Methods

Materials

Cell surface iodination

Cyanogen bromide digestion

Mutagenesis of ASGP receptor H2b cDNA

Cell transfection and culture

Metabolic labeling, immunoprecipitation, and enzyme digestions

Gel electrophoresis, fluorography and scanning densitometry

Immunofluorescence microscopy

Ligand binding, uptake and degradation assays

Results

A disulfide bond (C1) connects the H2 protein between Cys157 and Cys171.

Mutant H2b proteins lacking the C1 disulfide bond show enhanced rate of folding in the ER and Golgi processing.

The H2b C1 mutant is expressed on the cell surface and together with the H1 subunit, can form a functional receptor.

Discussion

Acknowledgments

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Chapter 1

Protein Degradation and Folding in the Endoplasmic Reticulum
This thesis is based on research using the human asialoglycoprotein (ASGP) receptor to study protein degradation and folding in the endoplasmic reticulum (ER) of mammalian cells. This introductory chapter will discuss the basic molecular biology of the ASGP receptor, and the current knowledge of protein degradation and folding in the ER.

The Human Asialoglycoprotein Receptor as a Model System for Studying Protein Degradation and Folding in the ER

The human asialoglycoprotein receptor is a membrane glycoprotein that is normally expressed only on the sinusoidal (basolateral) surface of hepatocytes. It functions to bind and remove desialylated glycoproteins with exposed terminal galactoses on their carbohydrate side chains (asialoglycoproteins) from the circulatory system. It is also known as the hepatic lectin because of its binding specificity for the carbohydrate moiety of glycoproteins. This receptor-ligand system is a good example of receptor-mediated endocytosis via coated pits. The receptor is constitutively endocytosised and recycled while the ligands are degraded in lysosomes (Spiess, 1990). The molecular parameters of binding and endocytosis have been well characterized from in vitro studies using hepatoma HepG2 cells and desialylated orosomucoid (α-1 acid glycoprotein), an abundant plasma protein in mammals (Schwartz et al., 1981; Schwartz et al., 1982; Schwartz et al., 1982). About 500,000 receptors are expressed by each cell and the dissociation constant for binding of ligand is about $10^{-9}$ M. Although the receptor shows specific binding to asialoglycoproteins, its exact physiological role is still unclear. Transgenic mice in which the receptor has been inactivated demonstrate no obvious phenotype although they lack the ability to remove large quantities of injected asialo-orosomucoid (Ishibashi et al., 1994).

The ASGP receptor is a hetero-oligomer consisting of two types of subunits, H1 and H2, which are 60% homologous in amino acid sequence (Spiess and Lodish, 1985; Spiess et al., 1985). They are type II transmembrane proteins and the polypeptide chain of each subunit consists of four main regions: a short cytosolic amino-terminal, a single transmembrane section serving as an uncleaved signal anchor sequence, followed by a "stalk"
domain and the Ca\(^{2+}\) dependent galactose binding domain in the exoplasmic carboxy-terminal region (Figure 1). The amino acid sequences of H1 and H2 can be aligned for most of their length except for a short 18 amino acid insert in the cytosolic amino-terminal of H2. 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. The two H2 forms are alternatively spliced variants which differ by the presence of a 15-bp miniexon in H2a (Lederkremer and Lodish, 1991). Both H1 and H2 are required to make a functional receptor capable of binding asialoglycoproteins although each subunit by itself has the ability to bind to galactose at low affinities. The functional receptor complex has a minimum stoichiometry of \((H1)_3(H2)_1\) (Henis et al., 1990; Lodish, 1991). Besides the human receptor, highly homologous ASGP receptors with identical binding characteristics have also been cloned and analyzed in rat and mouse (Halberg et al., 1987; Takezawa et al., 1993). Another homologous protein is the chicken hepatic lectin which binds to terminal N-acetyl-glucosamine residues of glycoproteins after both sialic acid and galactose residues have been removed (Drickamer, 1981).

The ASGP receptor belongs to a family of animal proteins known as the C-type lectins with the characteristic of requiring calcium ions for ligand binding. All members of this family share sequence homologies in the carbohydrate recognition domain (CRD). 18 invariant and 32 conserved residues can be found in all members within this domain of about 130 amino acids (Drickamer, 1988). Members of this family include many proteins from a wide variety of animals. Examples include: the serum/liver mannose binding proteins, pulmonary surfactant protein, cartilage/fibroblast proteoglycan core proteins, tetranection, pancreatic stone/thread protein, sea urchin lectin, acorn barnacle lectin, and sea raven antifreeze protein. All these proteins show high homologies in the CRD. The IgE receptor also shows homology to this family in the ligand binding domain although it recognizes the protein, not carbohydrate moiety of its ligand (Ludin et al., 1987; Vercelli et al., 1989). Invariant residues in the CRD include four cysteines forming two disulfide linkages which have been determined chemically in some members and observed by x-ray crystallography in the rat mannose binding protein (Weis et al., 1991). Two cysteines in the "stalk" region of the protein that are nearer the CRD are also conserved in other C-type lectins and presumed to form a disulfide bond based
on biochemical data obtained in homologous proteins (Fuhlendorff et al., 1987; Giga et al., 1987; Ng and Hew, 1992). However, the two cysteines on the exoplasmic side nearest to the transmembrane region are only found in the mammalian ASGP receptors but not in homologous C-type lectins. The role of the cysteines in the folding and processing of the H2 protein will be examined in Chapter 3.

As the ASGP receptors are type II transmembrane proteins, the transmembrane region normally serves as uncleaved signal anchor sequence. It directs the protein to be inserted into the ER in the orientation of amino-terminus on the cytosolic side, carboxy-terminus on the exoplasmic side, but it is not cleaved off itself, unlike signal sequences found in type I transmembrane or soluble secretory proteins. However, if the 40 residue amino-terminal sequence preceding the transmembrane region of the H1 subunit is deleted, the transmembrane region is cleaved off co-translationally to yield a soluble exoplasmic domain (Schmid and Spiess, 1988). As we will discuss in Chapter 2, the complete H2 subunit is also cleaved in a similar region to produce a soluble exoplasmic fragment and signal peptidase is most probably involved in the cleavage. The “stalk” regions of the ASGP and IgE receptors between the transmembrane region and the CRD have amino acid sequences that suggest the formation of α-helical coiled-coil structures. Analysis of residues 65 to 143 in the rat ASGP receptor major subunit shows a region of characteristic heptad repeats (Beavil et al., 1992). Although there is no direct structural evidence, presence of this motif suggests that the stalk region may be involved in interaction of the subunits to form the functional oligomeric receptor.

In hepatocytes, the H1 subunit is synthesized as a 40 kD core-glycosylated form which matures to 46 kD after processing of its two N-linked oligosaccharides in the Golgi complex. Likewise, H2 matures from a 43 kD core-glycosylated form in the ER to become 50 kD in size after processing of its three N-linked sugars. In transfected fibroblasts expressing only the H1 subunit, about 50% of the synthesized core precursor exit the ER to the cell surface. However, expression of the H2 subunit by itself in transfected fibroblasts lead to its rapid degradation in a pre-Golgi compartment (100% of H2a, 80% of H2b). The degradation of the ASGP receptor H2 subunit in the ER will be discussed in more detail in a later section and also in Chapter 2.
ASGP receptors contain Ca\(^{2+}\) in their folded structure. Treatment of cells with Ca\(^{2+}\) ionophores or Ca\(^{2+}\)-ATPase inhibitors prevent folding of the protein in the ER. In vivo folding intermediates of H1 have also been observed based on their relative mobilities on non-reducing SDS-PAGE. The Golgi processed form of the protein is resistant to DTT unfolding while the protein which is still in the ER is sensitive to DTT unfolding, suggesting a folding step that is required for the protein to exit the ER (Lodish et al., 1992). About 20 to 30 percent of H2b protein expressed in transfected 3T3 fibroblasts mature to the cell surface while the rest are degraded in the ER. In the presence of TLCK and TPCK, a large fraction of H2b is not degraded and remains in the ER. Analysis of the undegraded proteins showed that these proteins migrate more slowly on non-reducing SDS-PAGE (Wikström and Lodish, 1993). This observation suggests that the fraction of protein which accumulates in the presence of TLCK/TPCK are unfolded and this portion is normally degraded in the ER while the folded fraction matures to the cell surface. Therefore, this system is a good model for studying the relationship between protein degradation and folding in the ER.

The Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a cellular organelle in eukaryotic cells that was first observed and described during the initial visualization of an intact chicken embryo fibroblast under the electron microscope fifty years ago (Porter et al., 1945; Porter and Thompson, 1947). Its name was derived from the observed lacework of an extended reticulate, luminal tubular network within the cytoplasm (Palade and Porter, 1954; Porter, 1953). This membrane-bound organelle is also physically linked to the nuclear envelope. The ER can be structurally divided into the rough ER, which has large number of ribosomes closely associated with the membranous network, and the smooth ER, which does not have such attachments. It is now established that the rough ER is the site of synthesis of membrane and secretory proteins (Klausner, 1989; Palade, 1975). Therefore, the ER is the first destination of these proteins as they pass through what is known as the secretory pathway. This pathway starts with the ER and proceeds onto the Golgi complex, the trans Golgi network, secretory vesicles and terminates at the cell surface or organelles within the secretory system (e.g. lysosomes). The smooth ER,
without associating ribosomes, is the site of synthesis of membrane lipids and glycolipids and also contain enzymes such as cytochrome P-450 which are involved in oxidation/hydroxylation reactions for the detoxification of xenobiotics.

The synthesis of proteins that are destined to be secreted or targeted to cell surface or various organelles within the secretory system begins on cytosolic ribosomes. As the nascent polypeptide chains of these proteins begin to emerge during the translation process, their signal sequences are recognized by signal recognition particles which direct the whole ribosome complex with the nascent polypeptide chain to dock onto the endoplasmic reticulum before further chain elongation. The polypeptide chain is now inserted into the ER membrane co-translationally and the amino-terminal signal sequence is usually cleaved off by signal peptidase (Dalbey and Von Heijne, 1992). The whole polypeptide chain will pass through the membrane lipid bilayer if it is a soluble non-membranous protein or it will be anchored into the membrane if it is a membrane protein. This complex scheme of transport of proteins across the membrane is still not well understood and is currently under intense investigation (reviewed by Gilmore, 1993; Jungnickel et al., 1994; Ng and Walter, 1994).

The translation process is only the beginning in the maturation of a protein molecule to its final functional form. This maturation process involves the attainment of the correct three-dimensional structure and chemical modifications or cleavage of the polypeptide chain (reviewed by Rowling and Freedman, 1993). Some of these processes begin co-translationally and are completed as membrane and secretory proteins pass through the secretory pathway. Folding of the nascent protein chains, oligomerization of multimers and some covalent modifications of the proteins occur in the ER. These include addition and modification of carbohydrate side chains to asparagine residues (N-linked glycosylation) or serine/threonine residues (O-linked glycosylation), hydroxylation of proline or lysine residues, γ-carboxylation of glutamate residues and addition of glycosylphosphatidylinositol (GPI) anchors. Therefore, the rough ER is not only the site of synthesis of membrane and secretory proteins, it is also where the maturation of the nascent polypeptide chain to their final functional form begins. As these co- and post-translational
processes within the ER do not proceed without errors, the ER performs quality control functions to prevent undesired products from reaching their final destinations. Proteins which fail to be processed properly cannot become functional, will fail the quality control test and generally will not be transported out of the ER to the Golgi complex (reviewed by Doms et al., 1993; Hurtley and Helenius, 1989; Pelham, 1989; Rowling and Freedman, 1993). Therefore, proteins in the ER that fail to fold, oligomerize (in cases of multimeric proteins) or attain the appropriate chemical modifications are retained in the ER and degraded.

**Protein Degradation in the Endoplasmic Reticulum**

In any quality control system, a mechanism is required for the removal of products which are defective. The translational process is not perfect and will produce a small fraction of truncated or mutated proteins. In the ER, not all the proteins which have been synthesized undergo post-translational processes without flaws. A portion of the proteins will be misfolded, a fraction of glycoproteins will fail to obtain the proper sugar side chains and others will not get the appropriate covalent modifications. Subunits of multimeric complexes may not be synthesized in proportional amounts resulting in the excess of certain subunits which cannot oligomerize. The quality control system should ensure that only fully functional proteins reach their final destination as proteins with errors cannot perform the necessary functions and may have harmful effects on normal metabolism. The errors and imperfections of the synthesis and maturation processes in the ER produce unwanted proteins. They cannot exit the ER and have to be removed to prevent their accumulation in the ER. Therefore, the existence of a proteolytic system within or closely associated with the ER is not only logical but necessary as only proteins which have been processed correctly are transported to the Golgi complex. The role of this degradation system in the quality control scheme would be to specifically recognize defective proteins in the ER for degradation but not the properly matured proteins. The degradation system must be finely tuned so that nascent chains that have not yet been fully processed are not unnecessarily degraded. For proteins with processing errors in the ER, it may not be economical or selective enough to utilize the secretory system to transport these all the way to the lysosomes for
degradation. It is also probably better to retain such proteins in the ER for rapid degradation as transport of such proteins along the secretory pathway may affect the efficiency of post-ER processing reactions for the normal proteins. Rescue systems may exist in the ER to correct the errors so that the efficiency of the maturation processes may be maximized. However, such recovery systems may be energetically less efficient than direct degradation of unwanted proteins and cannot be perfect themselves. A machinery for protein degradation should exist in or be associated with the ER. Furthermore, protein turnover is an important part of the regulation of cellular metabolism and it is controlled by both the rate of protein synthesis and degradation. Since many proteins are associated with the ER, a degradation system should be present to allow regulation of the levels of these proteins according to the needs of the cell.

The two most extensively studied protein degradation mechanisms in eukaryotic cells are that of the ubiquitin/proteasome proteolytic system and the lysosomal/vacuolar proteolytic system. Many short-lived cytosolic and nuclear proteins are targeted for degradation by the covalent linkage of ubiquitin, a 76 amino acid highly conserved polypeptide, to the target proteins. The ubiquitinated proteins are then degraded by a large 26S proteasome complex (reviewed by Ciechanover, 1994; Hochstrasser, 1995). The lysosomal/vacuolar degradation system degrades many proteins in the endocytic and secretory pathways (Knop et al., 1993). It is also involved in the process of autophagocytosis or autophagy whereby cellular organelles or cytosolic proteins are sequestered by membranous structures to form autophagic vacuoles which are then fused with lysosomes and degraded. The membrane that forms the autophagic vacuoles is actually derived from the ER (Dunn, 1990). However, this degradation system degrades mainly long-lived proteins and is probably not specific enough for selective degradation of proteins within the ER.

The only proteolytic system that is well characterized in the ER is that of signal peptidase (Dalbey and Von Heijne, 1992; Lively, 1989; Muller, 1992). Eukaryotic signal peptidases are multimeric membrane-associated complexes (Baker and Lively, 1987; Evans et al., 1986) which remove amino-terminal signal peptides from most secreted proteins and type I transmembrane
proteins. The signal peptides are usually amino-terminal sequences of 13-20 amino acids in length, containing characteristic positively charged amino acids near the amino-terminus followed by a long hydrophobic central region and a more polar carboxy-terminus (von Heijne, 1990). The proteolytic cleavage occurs co-translationally and the cleavage site is generally carboxy-terminal to small, uncharged amino acids (with the amino acid at -1 and -3 positions being small, uncharged and usually not proline) (von Heijne, 1985; von Heijne, 1986). It is not clear if the signal peptidase complex or a separate proteolytic system is responsible for the further degradation of the signal peptide which has been cleaved off the polypeptide chain. Although the signal peptidase system clearly plays an important role in the removal of signal peptides, its function in the overall quality control scheme of the ER is not clear.

The existence of a proteolytic system within the ER other than the signal peptidase complex has long been suspected but not proven. In a series of studies by Klausner and co-workers using the T-cell receptor as a model system, molecular details of such a degradation system began to emerge (Bonifacino et al., 1989; Chen et al., 1988; Lippincott et al., 1988). The functional T-cell receptor consists of an oligomer of seven different subunits. (α, β, γ, δ, ε, ψ, ζ). When the α, β or δ subunits were expressed alone without the other subunits in transfected fibroblasts, they were synthesized properly on ribosomes, inserted into the ER and became core-glycosylated. However, the proteins were degraded with rapid kinetics with half lives of 30-60 minutes following a lag of about 15-30 minutes. None of the proteins acquired complex carbohydrates, indicating that they were not processed by Golgi enzymes. Inhibitors that blocked lysosomal protein degradation did not prevent the degradation process, indicating that lysosomes were not involved. Immunofluorescence and immunoelectron microscopy studies localized the expressed proteins mainly with the ER compartment. Furthermore, Brefeldin A, which disrupted normal ER to Golgi transport, did not prevent the degradation process. Although the degradation process was inhibited at low temperatures (16-20°C), it could proceed in the absence of vesicular transport in permeabilized cells (Stafford and Bonifacino, 1991). These observations suggest the existence of a proteolytic system within the ER that targets unassembled subunits of oligomeric proteins for rapid degradation. This is a specific process as ε or ζ subunits expressed alone in fibroblasts are quite
stable. In 2B4 T mutant cells which do not synthesize the δ chain, the α and β subunits are also rapidly degraded in the ER, indicating that these two subunits require a full complement of the subunits for maturation to the cell surface. However, the γ subunit undergoes rapid degradation in the ER when expressed by itself but it is stabilized when it is co-expressed and assembled with only the ε chain.

Another well studied example of protein degradation is the H2 subunit of the ASGP receptor. Expression of the H2 subunit by itself in fibroblasts, without the H1 subunit, leads to its rapid degradation (100% of H2a, 80% of H2b) in a non-lysosomal, pre-Golgi event (Amara et al., 1989; Lederkremer and Lodish, 1991; Shia and Lodish, 1989; Wikström and Lodish, 1991). The protein is synthesized, properly inserted into the ER and core-glycosylated normally but never become resistant to endoglycosidase H and the composition of the carbohydrate chains of the protein isolated from cells suggest that they have not been processed by Golgi enzymes. Instead, it is quickly degraded in the ER, after a lag of 30 to 60 minutes, with a half life of about 30 minutes and the degradation is not affected by agents that inhibit lysosomal degradation. About 50% of the H1 subunit expressed alone in fibroblasts mature to the surface and co-expression of H1 and H2b allows most of H2b to be expressed on the surface. During the ER degradation of the H2 protein, a 35 kD fragment that can be immunoprecipitated by anti-serum against the carboxy-terminus of the H2 protein is detected. The size of this fragment suggests that it is formed by a proteolytic cleavage near the transmembrane region on the exoplasmic side. It was thought that this could be an intermediate in the process of the ER degradation of this protein, whereby the degradation is initiated by a proteolytic cleavage to give an intermediate carboxy-terminal fragment that is then further degraded. The degradation process and the formation of this 35 kD fragment can occur in semi-permeabilized cells depleted of ATP and cytosol, conditions that prevent vesicular transport, showing that the cleavage occurs in the ER without the need for vesicular transport to another organelle (Wikström and Lodish, 1992). As discussed in Chapter 2, we show that there are two separate pathways for the degradation of the protein in the ER, one involving formation of the 35 kD intermediate while the other does not. The degradation pathway which is independent of cleavage is inhibited by the protease inhibitors, tosyl-lysine
chloromethyl ketone (TLCK) and tosyl-phenylalanine chloromethyl ketone (TPCK) and also calpain inhibitors (appendix I). The occurrence of a degradation intermediate and availability of inhibitors make this system an attractive candidate for further molecular characterization of ER degradation.

There have been many other reports of proteins which undergo the phenomenon of “ER degradation” with characteristics similar to that of the degradation of the T-cell receptor subunits and ASGP receptor H2 subunit in the ER. Examples are α-1 antitrypsin PiZ variant (Le et al., 1992), truncated ribophorin I (Tsao et al., 1992), truncated influenza hemagglutinin (Doyle et al., 1986), truncated β-hexoaminidase which causes Tay-Sachs disease (Lau and Neufeld, 1989), mutant lysozyme (Omura et al., 1992), transferrin receptor which failed to be N-glycosylated (Hoe and Hunt, 1992), unassembled HLA-DR β monomers (Cotner, 1992), unassembled immunoglobulin light chains (Gardner et al., 1993), mutant LDL receptor (Esser and Russell, 1988), acetylcholine receptor subunits (Claudio et al., 1989), fibrinogen subunits (Roy et al., 1992) and mutant GPI-linked proteins with uncleaved GPI signals (Field et al., 1994). Most of these are mutant forms of the protein or unassembled subunits of multimeric proteins and they cannot fold or oligomerize correctly. Therefore, they cannot pass the quality control test of the ER and cannot be exported to the Golgi complex. Their rapid degradation in the ER would be advantageous to the secretory system which has to process large quantities of protein for export within limited space and resources.

One example of protein degradation in the ER with biomedical implications is that of the cystic fibrosis transmembrane regulator (CFTR) (Cheng et al., 1990). This is a membrane protein that functions as a cAMP-regulated chloride channel and its functional absence in epithelial cells lead to the clinical symptoms. A predominant CFTR mutant is that of ΔF508 in which the normal phenylalanine at residue 508 is deleted. The result of this deletion is that all of the mutant protein is degraded within the ER and none matures to the cell surface. Therefore, the deletion of one amino acid residue leads to the inability of the protein to fold proper in the ER and it is degraded within the organelle (Ward and Kopito, 1994; Yang et al., 1993). Interestingly, at low temperatures, the mutant protein is capable of folding properly and exits the ER to mature to the cell surface. Another interesting example of ER
degradation with clinical significance involves the down regulation of CD4 molecules in cells infected by the human immunodeficiency virus (HIV). The HIV gp160 product, which is further processed to produce viral envelope proteins, binds to the immature CD4 molecules in the ER and the complexes are retained in the ER. The Vpu product induces the degradation of CD4 in the ER, releasing the gp160 to be further processed in the secretory pathway while reducing the amount of CD4 that matures to the cell surface (Geleziunas et al., 1994; Willey et al., 1992). The Vpu product is an accessory membrane protein found only in HIV-1 strains and it is not essential for viral replication. However, its presence enhances viral particle release from infected cells, probably by stimulating the degradation of CD4 to release gp160 for further processing. This represents an example where a protein is retained and degraded in the ER after being bound by a foreign protein.

However, the ER degradation system is not omnipotent because some improperly processed proteins are not degraded in the ER. T-cell receptor complexes which lack only the ζ chain are transported through the Golgi and degraded in the lysosomes (Letourneur and Klausner, 1992; Sussman et al., 1988). LDL receptors in mutant Chinese Hamster Ovary (CHO) cells that exhibit defects in oligosaccharide processing in the ER are degraded rapidly. However, the degradation is inhibited by lysoosmotropic agents, suggesting that the degradation occurs in lysosomes (Hobbie et al., 1994).

Detailed studies have also been done on the regulated degradation of proteins located on the ER, the best examples being the HMG-CoA reductase (Chun et al., 1990; Lecureux and Wattenberg, 1994; Meigs and Simoni, 1992), and ethanol inducible cytochrome P450 2E1 (Eliasson et al., 1992). Besides mammalian cells, protein degradation in the ER has also been described in yeast (Saccharomyces cerevisiae) cells (Finger et al., 1993; Hampton and Rine, 1994; McCracken and Kruse, 1993). Most of the examples described involved expression of abnormal proteins in cultured cells in vitro and therefore indicate the presence of degradation systems in the ER that respond to unnatural situations. However, as discussed above, the normal secretory machinery is not perfect and the same degradation mechanisms are presumably involved in the fine tuning of protein secretion in vivo during normal cellular metabolism. For multimeric complexes like the T-cell receptor, the proportions of each
subunit synthesized in the ER cannot be expected to be incorporated into fully functional complexes at 100% efficiency and the excess individual subunits would be degraded. Indeed, most of the receptor proteins never reach the cell surface but are degraded (Minami et al., 1987). The erythropoietin receptor is another example where only a small fraction of the synthesized proteins ever reach the cell surface, with most of them being degraded in the ER or in the lysosomes (Hilton et al., 1995; Neumann et al., 1993).

**Molecular Determinants of Selective ER Degradation**

The hallmarks of selective degradation of proteins within the ER are as follows: there is usually a lag period before the degradation process proceeds with rapid kinetics. The degradation is often energy (ATP) dependent but does not require vesicular transport, is insensitive to inhibitors of lysosomal proteases, and is selective for only certain proteins. As mentioned above, many of the proteins that undergo rapid degradation in the ER are mutated proteins which presumably cannot fold properly. The other examples are subunits of multimeric complexes which are expressed alone and thus could not be assembled into the functional oligomeric complexes. Therefore, the ER has a machinery that can recognized unassembled subunits to target them for degradation while subunits which have oligomerized properly continue in the maturation process. However, the studies on the T-cell receptor subunits show that the recognition process is more subtle in that only certain subunits that are expressed alone without the others in fibroblasts undergo degradation in the ER. Furthermore, different subunits have different oligomerization requirements to gain stability. For example, complexes of αβ or βδ subunits undergo rapid ER degradation but εγ or αδ associations are quite stable (Bonifacino et al., 1989; Lippincott et al., 1988; Wileman et al., 1990).

There are examples of misfolded protein aggregates that remain in the ER for long periods of time without being degraded (Hurtley et al., 1989). This indicates that retention in the ER by itself does not lead to degradation. Furthermore, ER resident proteins are quite stable and have long half-lives and additions of ER retention signals (KDEL sequence at the carboxy-terminus) to secreted proteins do not necessarily lead to degradation in the ER (Le et al., 1990). Therefore, there must be molecular determinants within the proteins that are recognized by the degradation system. A series of experiments on the
T-cell receptor subunits suggest that the transmembrane region of the subunits (α and β chains) that undergo ER degradation is the molecular determinant for degradation (Bonifacino et al., 1990; Bonifacino et al., 1990; Wileman et al., 1990). Constructs of the α subunit which lack the transmembrane region are stable in the ER and chimeric constructs in which the transmembrane region of the interleukin-2α receptor (which normally does not exhibit ER degradation) was replaced by that from the α chain, become susceptible to ER degradation. Analysis of the transmembrane regions of the α and δ chains indicated that the unusually present charged residues were responsible for conferring susceptibility to ER degradation. The transmembrane domain of the α chain contains two positively charged residues while the δ chain has a negatively charged residue in the transmembrane region. It was hypothesized that the charged residues would be shielded from the hydrophobic environment in the multimeric protein complex but would be exposed and destabilize the protein when each subunit was expressed alone (Bonifacino et al., 1991; Wileman et al., 1993). However, the ε chain also has a negatively charged residue in the transmembrane region but did not exhibit ER degradation. Further studies showed that the exact placement of the charged residues within the transmembrane region and the overall length of the transmembrane region in relation to the charged residues were important in conferring sensitivity to ER degradation (Lankford et al., 1993). However, an alternative viewpoint has suggested that the T-cell α subunits were rapidly degraded in the ER because they could not be properly inserted into the membrane when expressed alone and thus are translocated into the ER lumen as soluble proteins and then rapidly degraded (Shin et al., 1993).

For the asialoglycoprotein H2 subunit, none of the H2a subunit matures to the cell surface in transfected cells but 20% of the H2b subunit can mature to the cell surface though the two proteins are identical except for a five amino acid insertion in H2a near the transmembrane region on the exoplasmic side of the protein. This strongly suggests that the pentapeptide is the signal for ER degradation and that the juxtamembrane region and not the transmembrane region, is the important determinant for ER degradation. This subject is examined and discussed in more detail in Chapter 2.
Many reports of ER degradation have indicated that ATP depletion, inhibition of protein synthesis and low temperatures can inhibit the process. However, it is not clear if these conditions actually affect the structures of the protein substrate to alter their susceptibility to degradation or decrease the activities of the protease involved in the degradation process, or both. The γ chain of the human T-cell receptor is quite stable when it is expressed without the other subunits but it is rapidly degraded in the ER when cells are treated with DTT, a reducing agent (Young et al., 1993). This is can be due to the unfolding of the protein that makes it more susceptible to degradation, or the reducing conditions may activate cysteine proteases in the ER. The degradation of the T-cell receptor β and δ subunits in the ER is accelerated when cells are treated with Ca\(^{2+}\) ionophores or Ca\(^{2+}\)-ATPase inhibitors to deplete Ca\(^{2+}\) in the ER (Wileman et al., 1991). Ca\(^{2+}\) may be required for the folding of these proteins and the depletion of Ca\(^{2+}\) can prevent folding and enhance the susceptibility of the proteins to degradation. Another possible explanation is that Ca\(^{2+}\) concentration in the ER may be a regulating factor for proteolysis within this organelle.

The role of processing of the N-linked carbohydrates in determining degradation of proteins in the ER has also been examined. Asparagine to glutamine mutations on N-linked glycosylation (Asn-X-Ser/Thr) sites prevented N-linked glycosylation in the transferrin receptor and lysozyme and led to their degradation in the ER. Although some proteins do not need carbohydrate side chains for folding, it appears that others cannot fold properly without them and so are degraded in the ER (Helenius, 1994). The presence of N-linked sugars in human chorionic gonadotropin also facilitates the formation of correct disulfide bonds and therefore the folding of the protein (Feng et al., 1995). Nascent N-linked sugars undergo trimming in the ER. Inhibitors of the trimming process have been shown to affect the folding and secretion of glycoproteins. Studies of yeast prepro-α-factor expressed in mammalian cells showed that deoxynojirimycin, which inhibits ER glucosidase, accelerates the degradation of the protein in the ER while deoxymannojirimycin, which inhibits mannosidases, blocked the degradation (Su et al., 1993). Glucosidase inhibitors have also been shown to accelerate the degradation of unassembled major histocompatibility complex (MHC) class I heavy chains in the ER (Peyreras et al., 1983; Moore and Spiro, 1993). These results indicate that
specific modifications of the carbohydrate side chains can affect the susceptibility of the proteins to degradation in the ER. Again, the question is whether these are direct effects that affect the susceptibility of the protein substrates to degradation or whether the inhibitors affect the degradation machinery.

It is interesting to note that in reports where inhibitors were effective in blocking the ER degradation process, the inhibition of degradation did not lead to formation of the mature protein nor exit of the protein from the ER to the Golgi complex. Therefore, it can be hypothesized that the degradation process in the ER is at least a two step process. An initial recognition step determines which proteins are unfit for exit to the Golgi and thus should be destined for degradation in the ER would be followed by the actual proteolysis. The presence of protease inhibitors will block the degradation process, presumably by the inhibition of the protease function, but the recognition step would not be affected and the protein would still remain in the ER. The recognition system would need to selectively recognize unfolded or misfolded proteins and unassembled subunits to target them for degradation. Molecular chaperones in the ER may perform this role and this is discussed in a later section. The segregation of the proteins destined to be degraded into a specialized region of the ER may also be coupled to the targeting mechanism.

Possible Sub-compartment of ER Specialized for Degradation

All the examples of “ER degradation” generate strong evidence that the degradation process is in a compartment that is “pre-Golgi”, i.e., the degradation process does not require vesicular transport and is not inhibited by the usual inhibitors of lysosomal protein degradation. The main evidence for the actual degradation process occurring within the ER is from the glycosylation pattern of the target proteins. The sugar side chains of the proteins are processed only by ER resident enzymes but not by Golgi resident enzymes. The observations that there is a time lag in the degradation process and that the degradation of some proteins is inhibited at low temperatures (18°C) and also by depletion of ATP and inhibition of protein synthesis have raised the possibility that a transport process may be involved in the degradation process. However, in cells which have been permeabilized by streptolysin O or hypotonic shock and depleted of ATP and cytosol, degradation
of the T-cell receptor and ASGP receptor subunits in the ER are not inhibited (Stafford and Bonifacino, 1991; Wikström and Lodish, 1992). Addition of ATP or cytosol back to the permeabilized cells does not enhance the degradation processes. GTPγS, which blocks ER to Golgi transport, also does not inhibit degradation. Therefore, under conditions that prevent vesicular transport from the ER to Golgi, the proteins still undergo rapid degradation. However, it is possible that non-vesicular transport within the ER network itself may be required for the degradation process.

After transfected fibroblasts expressing the ASGP receptor H2a subunit were treated with cycloheximide, which stopped protein synthesis and caused the accumulation of the 35 kD cleavage fragment, immunofluorescence localization of the H2a protein showed a punctuate pattern of staining (Wikström, 1992). This suggested that the undegraded proteins or cleavage fragments may be accumulating in a specific compartment. This compartment may be a specialized region of the ER for protein degradation. There have been suggestions of subcompartments within the ER specialized for various functions including storage of calcium ions, storage of luminal protein aggregates (e.g. zymogens in pancreatic cells), and intermediate compartments between the ER and Golgi involved in retrieval of ER resident proteins. Expression of exogenous or mutated proteins have also led to formation of protein aggregates which accumulate in localized luminal domains of the ER (reviewed by Sitia and Meldolesi, 1992). Studies on albumin and vesicular stomatitis virus glycoprotein secretion show that these proteins are first concentrated in certain regions of the ER before export to the Golgi, suggesting that regions of the ER may be specialized for regulating ER to Golgi transport (Balch et al., 1994; Mizuno and Singer, 1993). Therefore, it may be possible that a specialized region of the ER may be involved in the process of ER degradation where the proteins targeted for degradation are first segregated. Although there is no concrete morphological evidence for such an ER sub-compartment, its existence is logical as it can separate unwanted proteins targeted for degradation from other proteins in the ER which have passed the quality control tests and immature proteins that are still undergoing the process of folding and post-translation modifications. The concentration of proteases with their substrates into compartments should also increase the efficiency and rate of degradation.
Proteases Involved in ER Degradation

No specific protease has so far been identified that is responsible for degradation of any specific proteins in the ER. The only clues as to their identity are from inhibitor studies. Different examples of ER degradation show sensitivity to different inhibitors, indicating that more than one type of protease exist in the ER and that different substrates are degraded by different proteases. The degradation of the ASPR receptor H2 subunit is inhibited by TLCK, TPCK (Wikström and Lodish, 1991) and calpain inhibitors (Appendix I). The degradation of the T-cell receptor subunits and HMG-CoA reductase are sensitive to cysteine protease inhibitors but the exact profile of inhibition by a panel of inhibitors differ in the two cases (Inoue and Simoni, 1992). Other examples also point to various protease inhibitors as being effective in blocking degradation of various proteins in the ER but each system is sensitive to a unique panel of inhibitors (Fra and Sitia, 1993). The only general observation is that cysteine protease inhibitors (sulfhydryl agents) affect many of the ER degradation models being studied. This indicates that cysteine proteases may be involved or reactive thiol groups are important in the degradation process. Several different proteases are likely to be found in the ER and each may have specific substrates. Therefore, there are likely to be several different pathways for degradation of proteins in the ER. Some proteins may be degraded by more than one pathway while others may be substrates to only one specific protease. Two proteins with protease activities, ER60 and ERp72, have been isolated from the ER (Urade and Kito, 1992; Urade et al., 1992; Urade et al., 1993). Amino acid sequences of these proteins show homology to protein disulfide isomerase (PDI) and their protease activities are inhibited by cysteine protease inhibitors. They demonstrate the ability to degrade ER resident proteins (PDI and calreticulin) in vitro but their roles in the degradation of any of the proteins exhibiting ER degradation is unknown. ERp72 also demonstrates chaperone-like functions (Nigam et al., 1994).

Studies of protein translocation in the ER of yeast cells have identified an ubiquitin conjugating enzyme located in the ER, suggesting possible role of ubiquitin in the degradation of ER associated proteins (Sommer and Jentsch, 1993). The ubiquitin conjugating enzyme, designated UBC6, was found to be an integral membrane protein localized in the ER. UBC6 loss of function
mutants suppresses a defect caused by a mutant SEC61. SEC61 is a component of the ER protein translocation complex and its mutant form is presumably degraded by a process mediated by UBC6. This raises the possibility that the ubiquitin degradation system may be involved in the degradation process of some proteins in the ER. However, the catalytic site of UBC6 is on the cytosolic side and ubiquitin is only found in the cytosol. Therefore, other degradation systems should exist to degrade proteins within the ER lumen. Mutant carboxypeptidase yscY and mutant proteinase yscA in yeast cells have been reported to undergo degradation in the ER (Finger et al., 1993). Genetic and biochemical characterization of mutant yeast strains that show inability to degrade the mutant proteins in the ER may provide important information on the ER degradation mechanism in yeasts.

**Protein Folding in the ER**

All proteins must attain proper three dimensional structures for their normal functions. The process whereby the newly synthesized linear polypeptide chains attain their final native molecular conformation is commonly called protein folding. *In vitro* studies showed that the basic information for the correctly folded state of proteins are contained within the protein sequences themselves (Anfinsen, 1973). However, the mechanism in which the one dimensional sequence information is decoded into the three dimensional structure of proteins is still not understood. Protein folding within living cells occur at a much greater speed and efficiency than under *in vitro* conditions, suggesting the importance of the local intra-cellular environment and participation of other cellular factors in optimizing the folding process (Gething and Sambrook, 1992). The folding of proteins produced in eukaryotic cells which are destined to be exported out of the cell or proteins targeted to cell surface membrane or organelles of the secretory system occur within the ER (Rowling and Freedman, 1993). Proteins that fold within the ER are exposed to conditions different from that of cytosolic proteins. The ER has a more oxidizing environment (Hwang et al., 1992) and contains a much higher concentration of calcium ions compared to the cytoplasm (Somlyo et al., 1985). The redox buffer maintaining the oxidation state of the ER compartment is glutathione. The ratio of reduced to oxidized glutathione (GSH/GSSG) within the ER averages 2:1 while the ratio in cytosol is about 65:1. As a result of the
more oxidizing redox potential in the ER, covalent disulfide linkages are formed by secretory or exoplasmic domains of membrane proteins but not by cytosolic proteins. Secretion of some proteins are inhibited when the calcium ions in the ER are depleted by treatment of cells with Ca\(^{2+}\) ionophores or Ca\(^{2+}\)-ATPase inhibitors (Lodish and Kong, 1990; Lodish et al., 1992). This is probably due to the requirement of Ca\(^{2+}\) for folding of certain proteins in the ER. Therefore the more oxidative and high Ca\(^{2+}\) environment of the ER provide conditions which are not found in the cytosol of the cell and are conditions required for proper folding of many secreted and membrane proteins. Furthermore, it has been shown in E. coli that disulfide bonds are actively prevented from being formed in the cytosol by the indirect action of thioredoxin reductase to maintain a reduced cytosolic environment (Derman et al., 1993).

**Disulfide Bond Formation and Protein Folding**

Formation of intrachain disulfide bonds is necessary for proper folding of many secreted and membrane proteins before they can be transported out of the ER to be further processed in the Golgi complex. Disulfide bond formation in the ER of immunoglobulin chains is a co-translational event (Bergman and Kuehl, 1979; Bergman and Kuehl, 1979) but it occurs post-translationally in other proteins. Nascent polypeptides of proteins which contain disulfide bonds in the mature folded form (e.g. influenza hemagglutinin, vesicular stomatitis virus G protein, ASGP receptor, albumin) are unable to attain a folded structure and do not exit the ER when cells are treated with a reducing agent, dithiothreitol (DTT) (Lodish and Kong, 1993; Tatu et al., 1993). The blockage of folding by DTT is reversible as proteins can refold on the removal of DTT, suggesting that these proteins can fold post-translationally (Braakman et al., 1992; Braakman et al., 1992). Once the proteins are folded and exit the ER, they become resistant to DTT unfolding. However, proteins which do not contain disulfide bonds (e.g. α-1 antitrypsin) are folded and secreted normally in the presence of DTT. Mutational studies of influenza hemagglutinin (HA) in which the cysteine residues were replaced with alanine or serine residues also showed that the disulfide bonds were needed for proper folding of the protein in the ER (Segal et al., 1992). In other proteins (e.g. IgM, transferrin receptor), inter-chain disulfide bonds are required for proper oligomerization of the functional protein (Morgan and Peters, 1985; Roth and Koshland, 1981).
The formation of intrachain disulfide linkages has been used as an indicator of the protein folding process of bovine pancreatic trypsin inhibitor (BPTI). By analyzing the disulfide linkages of folding intermediates which were trapped by iodoacetate or formic acid, the folding pathway of BPTI was determined. (Creighton, 1992; Weissman and Kim, 1991). The folding pathway of BPTI in vitro illustrates certain general principles of protein folding. Only a few species of disulfide linked forms are detected (6 out of possible combination of 75 for a protein with 6 cysteines), indicating that the folding pathway does not sample all the possibilities but only a small subset of conformations. The pathway is not random but ordered and sequential with defined disulfide bonds forming one after another. Disulfide bonds also undergo intramolecular rearrangements. A non-productive pathway leading to a kinetic trap also exits. More efficient trapping techniques demonstrate that intermediates with non-native disulfide bonds are of low abundance, suggesting that the productive folding pathway proceeds mainly by formation of native disulfide bonds. Similar studies done on the folding of ribonuclease T1 and ß-lactalbumin also demonstrated the same principles (Ewbank and Creighton, 1993; Pace and Creighton, 1986). In general, the in vitro folding studies show that protein folding occurs in pathways and only a limited number of folding intermediates are formed.

From studies of cultured cells, in vivo folding intermediates have been observed in influenza hemagglutinin, H1 subunit of the ASGP receptor, retinol binding protein and immunoglobulin chains (Braakman et al., 1992; Kaji and Lodish, 1993; Lodish et al., 1992; Valetti and Sitia, 1994). The folding intermediates were analyzed by differential mobilities of various disulfide bonded folding intermediates on non-reducing SDS-PAGE. Moreover, folding intermediates but not the mature form of the above mentioned proteins could be unfolded by DTT treatment of cells expressing the proteins. The intracellular folding pathway of the β subunit of the human chorionic gonadotropin (HCG) has been characterized (Bedows et al., 1993; Huth et al., 1992). Folding intermediates were trapped with iodoacetate, purified by immunoprecipitation, and separated by reverse HPLC for tryptic mapping. Disulfide bond formation is post-translational and only populations with native linkages are detected. Within a domain of the protein, formation of the disulfide bonds is sequential and therefore the folding process follows a specific pathway.
These principles are similar to those deduced from studies of protein folding \textit{in vitro}. The pathway of formation of interchain disulfides in the assembly of fibrinogen in cells has also been determined (Zhang and Redman, 1994). It clear that many secreted and membrane proteins require proper disulfide linkages for maintaining the functional native structure. Cysteine residues may not only be needed for forming critical disulfides, but can also modulate the assembly, retention and degradation of proteins. For example, the carboxy-terminal cysteine of \( \mu \) chain of IgM molecules is needed for assembly of pentameric IgM molecules (Fra et al., 1993). It is also needed for the retention and degradation of unassembled subunits in the ER. However, there have been reports that showed that certain cysteine residues in several secreted proteins may not be critical for folding and exit from the ER. For instance, mutation of certain cysteine residues in \( \beta \)HCG (Suganuma et al., 1989) and lysozyme (Omura et al., 1992) actually increased the rate of secretion of the protein. Removal of the disulfide bond in bovine growth hormone also did not affect its secretion or function (Chen et al., 1992). Therefore, the importance of disulfide bond formation in the folding and secretion of proteins appear to vary among proteins.

**Folding Catalysts and Molecular Chaperones in ER**

Protein folding in cells occurs at much higher rates than that observed \textit{in vitro} because the \textit{in vivo} process is also facilitated by other proteins in the cell. The proteins that facilitate the folding process can be divided into the enzymes that catalyze the actual folding process and molecular chaperones that bind to unfolded/misfolded proteins to prevent non-productive aggregation (reviewed by Jaenicke, 1993). Many of the chaperones were first discovered as heat shock proteins (hsp) whose expression in cells are greatly increased under stress conditions. Chaperones which have been well studied for their role in folding of cytosolic proteins include members of the hsp90, hsp70 and hsp60 families (Craig et al., 1993). Chaperones are also found in the ER where they are believed to assist the folding of secretory and membrane proteins. Some of the known ER chaperones include heavy chain binding protein (BiP/GRP78), endoplasm (GRP94) and calnexin. Folding enzymes found in the ER include peptidyl prolyl cis-trans isomerase (PPI) and protein disulfide isomerase (PDI). PDI, BiP and GRP94 are soluble ER resident proteins that contain the KDEL
sequence at their carboxy-terminus and are thus constantly retrieved from the cis-Golgi back to the ER by the KDEL receptor (Nilsson and Warren, 1994). They are also present in high concentrations in the ER, suggesting crucial roles for these proteins in the secretory process.

PDI is thought to catalyze the formation, isomerization and breakage of disulfide bonds of proteins that fold in the ER (Freedman et al., 1994). The protein shows sequence homology to thioredoxin and contains two active site sequences of -CXXC- which are presumably involved in disulfide exchange reactions. Depletion of PDI from microsomes used for in vitro translation experiments results in the inability of synthesized proteins to form native disulfide bonds and this can be rescued by reconstitution of purified PDI (Bulleid and Freedman, 1988). Therefore, PDI facilitates the formation of disulfide bonds during the folding process to produce the native protein. Direct in vivo evidence for this function is still lacking but addition of purified PDI does enhance the rate of BPTI folding in vitro, particularly the refolding of kinetically trapped intermediates (Weissman and Kim, 1993). Cross-linking studies in cultured cells show that PDI is bound to immunoglobulins and misfolded lysozymes in the ER (Otsu et al., 1994; Roth and Pierce, 1987). PDI also binds to a protein without disulfide bonds (D-Glyceraldehyde-3-phosphate dehydrogenase) and assists in its folding (Cai et al., 1994). Therefore, PDI may also have chaperone functions by binding to unfolded proteins to prevent aggregation. PDI can catalyze the in vitro unfolding of retinol binding protein with DTT and therefore it may allow unfolding of misfolded proteins with non-native disulfides in vivo (Kaji and Lodish, 1993). In vitro experiments with lysozyme showed that at low stoichiometric ratios compared to the substrate, PDI actually acts as an anti-chaperone and causes the aggregation of proteins but it acts like a chaperone at high concentrations (Puig and Gilbert, 1994). This may explain the need for high concentrations of PDI present in the ER. Over-expression of PDI in yeast cells can increase secretion of certain heterologous proteins which form disulfide bonds, presumably by increasing the folding efficiency (Robinson et al., 1994). Deletion of the PDI homologue in yeast is lethal but its essential function in yeast does not appear to be in the isomerase catalytic site (La Mantia and Lennarz, 1993). It is possible that PDI serves other unique essential roles in the ER while there are other proteins in the ER with redundant functions of catalyzing disulfide exchanges. It is
interesting that PDI is identical to the β subunit of prolyl-4-hydroxylase which catalyzes proline hydroxylation of collagen but PDI does not appear to contribute any catalytic property to the enzyme (Vuori et al., 1992).

PPI is a family of proteins that are important in catalyzing the cis-trans isomerization of peptidyl-proline bonds in almost all proteins (Schmid et al., 1993). This is because newly synthesized proteins all have trans oriented peptidyl-proline bonds but the mature native proteins usually have the cis configuration. Often, the cis-trans isomerization is the rate limiting step in protein folding. Some members of this family contain amino-terminal signal sequences and are expected to be found in the secretory pathway (Hasel et al., 1991; Price et al., 1991). In vitro studies show that PPI enhances folding of ribonuclease T1 and immunoglobulin light chains by two orders of magnitude. The in vivo importance of PPI has been demonstrated in the Drosophila system where mutations of the ninaA gene, a member of the PPI family, lead to reduced amounts of functional rhodopsin, most likely due to the inability of rhodopsin to attain the mature folded state (Stamnes et al., 1991). Cyclosporin A, which inhibits cyclophilin, a cytosolic PPI, also inhibits the folding of transferrin receptor in the ER, most likely by inhibiting a PPI in the ER (Lodish and Kong, 1991).

BiP was originally identified as an ER resident protein that binds to heavy chain immunoglobulin chains that are expressed in the absence of light chains (Morrison and Scharff, 1975). It also binds to many other proteins in the ER that are unfolded or misfolded and only releases the proteins if they become folded. The release is dependent on ATP hydrolysis (Flynn et al., 1991; Haas, 1994; Munro and Pelham, 1986). The role of BiP in protein folding in the ER is thought to be mainly in binding of unfolded or misfolded proteins with exposed hydrophobic domains. Such binding will prevent aggregation of these proteins and perhaps allow them to refold into the native state. Whether BiP itself actively assists the folding process is unclear. However, as discussed in a later section, aggregate formation may be part of the normal folding process of some proteins and BiP has been found in association with the complexes (de Silva et al., 1993; Marks et al., 1995). Therefore, the role of BiP in protein folding and assembly may be more complex and dynamic. From studies of the yeast homologue KAR2, BiP is also thought to be involved in the translocation
of protein precursors across the ER membrane as temperature sensitive mutants of KAR2 demonstrate defects in protein translocation at restrictive temperatures (Nguyen et al., 1991; Vogel et al., 1990). One possible role of BiP or other chaperones would be to facilitate protein translocation across membranes by keeping the nascent chains in an unfolded state without aggregation (Craig et al., 1993).

Calnexin (p88) is a membrane bound chaperone protein found in the ER (Bergeron et al., 1994; Ou et al., 1993). Its amino acid sequence shows that it does not belong to any of the Hsp families. It is an integral membrane protein and binds selectively to N-glycosylated membrane and soluble proteins. It does not bind to proteins without carbohydrate side chains and binds only to polypeptides with nascent carbohydrate side chains containing only one glucose at the terminus of the sugar side chain. Calnexin will bind to the monoglucosylated protein that is unfolded and release it only after it all the glucose residues are removed and the protein becomes folded. Therefore, its main role in assisting the folding process appears to be in binding unfolded glycoproteins to retain them in the ER, and to release the proteins for export to the Golgi after folding in the ER is complete. Furthermore, it has been postulated that calnexin may be modulating a deglucosylation/reglucosylation cycle involved in the folding of glycoproteins in the ER (Hammond et al., 1994; Hebert et al., 1995). In this scheme, nascent polypeptides are N-glycosylated co-translationally but remain unfolded. Two of the three terminal glucose residues of the nascent sugar side chain are removed by glucosidase I and II and then calnexin binds to the protein that is still unfolded. If the protein becomes folded and the final glucose is removed by glucosidase II, calnexin releases the protein to exit the ER. However, if the removal of the glucose is not accompanied by proper folding, UDP-glucose:glycoprotein glucosyltransferase will add back the glucose residue to the sugar side chain of the unfolded protein to allow calnexin to rebind the molecule and prevent its exit to the Golgi. In this scheme, only properly folded glycoproteins can exit the ER as any unfolded forms of the protein will be re-glucosylated and retained by calnexin. Therefore, this process is performing a quality control function of the ER and may explain the role of the complex residue adding and trimming reactions that occur on the sugar side chains of glycoproteins in the ER.
Each of the chaperones plays a unique role in facilitating protein folding and possibly cooperate with each other. BiP and GRP94 have been shown to bind sequentially to immunoglobulin chains in the ER (Melnick et al., 1994). BiP and calnexin have both been shown to interact with newly made VSV G proteins, class I heavy chains and thyroglobulin (Hammond and Helenius, 1994; Jackson et al., 1994; Kim and Arvan, 1995). Chaperones have also been implicated in important roles in the degradation of proteins in the ER. Many of the mutant proteins destined for degradation in the ER are associated with BiP or other chaperones. It is possible that chaperones may be the recognition molecules that select misfolded proteins and target them for degradation in the ER but there is little evidence to support this hypothesis. Studies of degradation of immunoglobulin light chain degradation in the ER showed that the kinetics of degradation coincided with the kinetics of release from BiP, suggesting that the two processes may be linked (Knittler et al., 1995).

**High Molecular Weight Aggregates in Folding and Assembly of Proteins in the ER**

Nascent polypeptide chains entering the ER are not completely folded and may have exposed hydrophobic surfaces. Therefore they may aggregate to form mixed complexes, similar to aggregates formed by unfolded and misfolded proteins *in vitro*. It has been shown that glycosylation inhibitors or mutations which prevent glycosylation can cause formation of protein aggregates in ER and this was presumed to be due to inability of glycoproteins to fold in the absence of sugar side chains (Gibson et al., 1979; Hurtley et al., 1989; Marquardt and Helenius, 1992). Therefore, large protein aggregates in the ER were thought to be non-productive results of misfolded proteins which could not be efficiently handled by chaperones. DTT treatment of HepG2 cells induces heterotypic aggregation of newly made proteins in the ER, suggesting a role for thiol groups in the formation of aggregates (Sawyer et al., 1994). Only α1-antitrypsin, which does not contain disulfide bonds, did not aggregate. However, recent studies on the folding of vesicular stomatitis virus G protein, thyroglobulin and MHC class II chains in the ER have revealed the involvement of high molecular weight complexes as transient intermediates in the normal processing of the proteins in the ER (de Silva et al., 1993; Kim et al., 1992; Marks et al., 1995). Detection of these complexes in the normal
folding process of various proteins has raised the possibility that these are formed as part of the folding pathway in the ER. The complexes were formed by non-covalent and covalent bonds, possibly disulfide linkages. However, not all proteins can form high molecular weight aggregates (e.g. HA). It is suggested that the formation of large complexes may represent an early reversible event for the normal assembly and folding of some proteins. Complex formation may also be used for regulating exit of proteins from the ER. Formation of large complexes may actually facilitate folding and oligomerization. By only allowing folded oligomers to leave the complex to exit the ER, it can perform a regulatory function. The association of chaperones (e.g. BiP) with such aggregates also suggests that the roles of chaperones in the ER may be more complex than simply binding to misfolded proteins to prevent aggregation.

Epilogue

The above introduction shows that many questions remain unanswered in the processes of protein degradation and folding in the ER and the relationship between these processes. The following chapters will describe the use of the ASGP receptor as a model system to further analyze and perhaps provide some insights into these processes. In Chapter 2, we determine the cleavage site in the H2 protein that leads to the formation of the 35 kD fragment. Mutations of amino acids around the cleavage site suggests that signal peptidase may be involved in the cleavage. However, degradation of the protein in the ER occurs in mutants that are not cleaved to form the 35 kD fragment. This suggests the presence of another pathway for degradation that is not dependent on the cleavage process. The pathway that does not involve the initial cleavage step is inhibited by TLCK and TPCK but the pathway that goes through the 35 kD intermediate is not inhibited by these inhibitors. We also show the importance of the conformation of the juxtamembrane region of the protein in determining its metabolic fate in the ER. In Chapter 3, we analyze the importance of the disulfide bonds in H2b for the folding and degradation of the protein. Mutation of the disulfide bond nearest to the transmembrane region leads to enhanced folding of the protein. The fraction of protein that exits the ER is similar to that of the wild-type but it exits the ER more quickly and the fraction that remains in the ER is degraded more quickly.
than that of the wild-type. This mutant can also form a functional receptor when co-expressed with H1. Mutation of the other disulfide bonds in the protein caused all of the protein synthesized to be degraded in the ER but the rate of degradation is similar to that of the fraction of wild-type protein that is retained in the ER. Chapter 4 will discuss the implications of these studies and prospects for future research in these topics.
Figure 1: Structure of the Asialoglycoprotein Receptor Subunit
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Chapter 2

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

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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. Nonlysosomal, pre-Golgi degradation of unassembled asialoglycoprotein receptor subunits: a TLCK- and TPCK-sensitive cleavage within the ER. J Cell Biol. 113:997-1007.) Here we have generated mutants of H2 which 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 N-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, uncharged amino acid residues. Substitution of the residues N- or C-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.

The abbreviations used are: ER, endoplasmic reticulum; ASGP, asialoglycoprotein; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; Endo H, endoglycosidase H, SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; ATZ, anilinothiazoline; PCR, polymerase chain reaction.

Introduction

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 (Hurtley and Helenius, 1989; Lodish, 1988; 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 Bonifacino and Lippincott, 1991; Klausner and Sitia, 1990) 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 (Bonifacino et al., 1989; Chen et al., 1988; Lippincott et al., 1988; Wileman et al., 1990). Monomeric HMG-CoA 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 Ca^{2+}-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 (Bischoff et al., 1988; Bischoff and Lodish, 1987; Spiess and Lodish, 1985; Spiess et al., 1985). The polypeptide chain of each subunit consists of four main domains: a short cytosolic N-terminal segment, a single transmembrane segment that also functions as an uncleaved signal anchor sequence, a "stalk" domain and, at the very C-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 subunit, expressed without H2 in NIH 3T3 fibroblasts, matures 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 minutes 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 \textit{in vitro} translation system in the presence of pancreatic microsomes. We examined by mutational analyses of the amino acid residues N- and C-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 pathway 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).

\textbf{Materials and Methods}

\textit{Materials}

Materials were purchased from sources previously listed (Amara et al., 1989; Wikström and Lodish, 1991; Wikström and Lodish, 1992). In addition, reagents and enzymes for \textit{in vitro} transcription and translation were obtained from Promega (Madison, WI). Reagents and enzymes for PCR reactions were from Perkin Elmer Cetus (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 (Bedford, MA). The Sequenase 2.0 kit for DNA sequencing was from United States Biochemical (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 $^{35}$S-Cys or $^3$H-Leu as radiolabel, in a total reaction volume of 12.5 µl. At the end of the reaction, microsomes were isolated by centrifugation at 165,000g for 15 minutes through a cushion of 0.5M sucrose, 10 mM Tris, pH 7.4, 150 mM NaCl. The pelleted microsomes were solubilized using lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 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.

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 H2a S79W mutant was generated with the following 4 primers:

Primer 1: 5’-CCTCAGAGCAACCTCAG-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 to 3); primer 2: 3’-CACCAGTAGACACACTGA-5’, corresponding to bp 213 to 230 (antisense); primer 3: 5’-GTGGTCATCTGTGTGACTGGGTGGCAAAGTGAG-3’, corresponding to bp 225 to 257 (mutant bases are underlined); primer 4: 3’-ATGTTGTGTGCTTTGGGG-5’, corresponding to bp 673 to 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 \textit{Xma} I and \textit{Dra} III, 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 minute at 94°C, 1 minute at 50°C and 2 minutes 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.

\textit{Cell Culture}

NIH 3T3 cells expressing wild-type H2a (2-18 cells) and H2b (2C cells) were kind gifts of Drs. L. Wikström and G. Lederkremer (Lederkremer and Lodish, 1991; Shia and Lodish, 1989). 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 \textit{Bam} H1 and \textit{Eco} R1 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.

\textit{Metabolic labeling, Immunoprecipitation and enzyme digestions.}

Confluent or near confluent (80%) cells in 100 mm or 60 mm diameter tissue culture dishes were labeled with $^{35}$S-Cysteine or $^{3}$H-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).

\textit{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 laser microdensitometer as previously described (Lodish and Kong, 1991).

Radiosequencing analysis of the N-termini of the 35 kD fragment

Limited N-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 pre-wet Immobilon-P paper at 0.5 A for 30 minutes 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 model 470A protein sequencer. The ATZ-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; Wikström and Lodish, 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 N-terminal amino acid sequence of the 35 kD fragment. 3T3 fibroblasts were pulse labeled with 3H-leucine and chased for 3 hours 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, $^3$H-Leucine was used as the radioactive label. The fragments were subjected to N-terminal sequencing by automated Edman degradation.

Figure 1a 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. Figure 1b 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 $^3$H-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 $^3$H-leucine 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 (Figure 1c). 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 H2b is at the position where the 5 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 Figure 2 shows that when H2a mRNA (synthesized by *in vitro* transcription of the corresponding cDNA) is subjected 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 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 show that the full length and 35 kD fragment 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. Figures 3a and 3b show that the 35 kD fragments formed in vitro from both H2b and H2a mRNA have the N-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 Figure 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 hours 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 N-termini of the 35 kD fragment formed by these mutant proteins. As the residues surrounding the cleavage sites are small, polar, and non-charged, 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 N-terminal to the cleavage site (Ser81) or the C-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 normally 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 Ia, 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 two-fold 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 two-fold 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 C-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. Figure 4 shows that the N-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 exhibits increased amounts of 35 kD fragments, showed that the putative cleavage sites are the same as wild-type (Figure 4).

Six mutants of Ala82 in H2b were also analyzed (Table Ib). No mutant showed any significant change in the amount of 35 kD fragment formed during in vitro translation (Column 3). However, analyses of the N-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 N-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 Ic). 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 which 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 Figure 5. Both the H2b S81G and the H2b S81W mutants are synthesized as 43 kD core-glycosylated precursors (lanes 1 and 3, respectively, solid arrow). During a chase of 3 hours 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 \textit{in vitro} translations generate no detectable amount of 35 kD fragment in transfected fibroblasts. All mutants which generated a normal or above-normal amount of 35 kD fragment during \textit{in vitro} translations formed, in transfected cells, approximately normal amounts of a 35 kD fragment. We did not determine the N-termini of the 35 kD fragments generated in cells expressing the mutant proteins, and we assume that they are identical to those generated during \textit{in vitro} translations in the presence of microsomes (summarized in Figure 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.

\textit{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 is an obligatory step in ER degradation, we would expect the rate of ER degradation of H2 mutant proteins which 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 hours. The amount of the 43 kD core-glycosylated ER precursor was monitored at intervals (Figure 6).

Figure 6a shows a comparison of the H2a wild-type with seven different mutants. After a lag of 30 to 60 minutes, the core-glycosylated precursor of the wild-type H2a protein is degraded rapidly (half-life of ~ one hour) with kinetics similar to those previously described (Amara et al., 1989). After 4 hours of chase, less than 10% of the pulse labeled precursor remains. The core-glycosylated precursors of the five mutants which 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 which 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.

Figure 6b shows that the rate of ER degradation of the core-glycosylated precursors of several mutants H2b proteins which 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, about 25-40% of the pulse-labeled protein acquires complex oligosaccharides by 4 hours 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 fibroblasts although H2a differs from H2b only by having 5 extra amino acids inserted between Ser81 and Ala82. All ER core-glycosylated H2a is degraded in the ER while, as noted, ~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 (Figure 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.

Figure 6c 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 (Figure 6c, 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 that of wild-type H2a rather than H2b. Equally significantly, 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 (Figure 6c, Table II) although this mutant has a glutamate residue at position 82, as does wild-type H2a (Figure 1c). Therefore, the difference in metabolic fates between wild-type H2a and H2b is not caused by the presence of glutamate (the most N-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 (Figure 1c). This mutant has the same metabolic fate as wild-type H2a (Figure 6a); 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; Wikström and Lodish, 1992). Figure 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 hours of chase in the absence of any inhibitors (lanes 2 and 7). The presence of 100 μM TLCK (lanes 3 and 8) or 20 μM TPCK (lanes 4 and 9) in the chase medium inhibits ER degradation, as shown by the accumulation of the 43 kD core-glycosylated protein. The presence of 30 μM 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 which do not generate any 35 kD fragment (T77W, G78W, S79W, S79P) as well as H2a mutants that do (G78P, G82A).
**TLCK and TPCK do not inhibit formation or degradation of the 35 kD fragment.**

Figure 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 (lane 1, solid arrow; see Wikström and Lodish, 1991). The cycloheximide was then removed and the chase continued for up to 3 hours without any inhibitor (lanes 2, 4, 6) or in the presence of 100 μM TLCK (lanes 3, 5, 7). The 35 kD fragment is degraded rapidly both in the absence of inhibitors and in the presence of 100 μM 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.

Figure 9a 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 (lane 1, solid arrow) is inhibited by 100 μM TLCK (lanes 5-7) compared to lanes 2-4 (chase without any inhibitor). When the chase medium contained both 100 μM 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.

Figure 9b shows that up to 400 μM of TLCK or 200 μM 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 pre-incubated in the translation mixture for 30 minutes 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 sulphydryl 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 which do not generate a 35 kD fragment have similar rates of ER degradation to that of the H2 wild-type and H2 mutants which do generate a 35 kD fragment (Figure 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 which do not generate the 35 kD fragment in transfected fibroblasts 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 (Figure 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 (Figure 8). We also found that the 35 kD fragment does accumulate in cells when both TLCK and cycloheximide are present (Figure 9a). 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 significantly inhibited by TLCK or TPCK (Figure 9b). Therefore, TLCK and TPCK neither block the formation nor the degradation of the 35 kD fragment totally. 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. Cycloheximide inhibits the degradation of the 35 kD fragment and probably also inhibits the cleavage independent pathway as presence of cycloheximide causes accumulation of the 35 kD fragment. Presence of both cycloheximide and TLCK/TPCK also inhibits overall degradation to a greater degree than either reagent alone. However, the non-specificity of the reagents do not allow firm conclusions to be drawn. Nevertheless, Figure 10 shows a model for the ER degradation of H2 that illustrates the two pathways of degradation that is consistent with the observed results. Inoue and Simoni (1992) showed that the degradation of the T-cell receptor α subunit and the HMG-CoA 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, S81R, S81K) 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 N-termini of the 35 kD fragments, we have localized the most likely proteolytic cleavage sites in both H2a and H2b (Figure 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 N- and C-terminal to the cleavage sites are all small neutral or small polar amino acids (Thr, Gly, Ser, Ala). One proteolytic enzyme in the ER membrane that is known to have preferences for such amino acids N- and C-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 optimal signal peptidase cleavage site should be between Ser81 and Glu82, but the experimentally deduced sites are T77/G78 and G78/S79. However, the experimentally determined cleavage sites agree with von Heijne's -1, -3 rule which states that residues at these positions should be small and uncharged and should not be prolines.

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 N-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 N-terminal cytosolic domain of H1 may modulate the accessibility of the potential cleavage site in the transmembrane region. When the N-terminal cytosolic domain is removed, the site becomes accessible and the protein is cleaved. The site of cleavage of the mutant H1 lacking the N-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 C-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 N- and C-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 C-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 a 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 a 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 N-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 C-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 N-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 \( \beta \)-turns or breaking \( \alpha \) 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 N- or C-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 5 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 N-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 N-termini of the various fragments analyzed are due to differential
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 (Figure 6c). 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 introductions 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 to 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 residues
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., 1991; Bonifacino et al., 1990; 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 (Figure 10).

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Figures

Figure 1. **Determination of the cleavage site of H2b and H2a that generates the 35 kD fragment in NIH 3T3 fibroblasts.**

Ten million 3T3 cells expressing either H2b (a) or H2a (b) were pulse labeled with 0.3 mCi/ml of $^3$H-Leu for 30 minutes and chased in unlabeled medium containing 0.5 mM cycloheximide for 3 hours. Cells were lysed in lysis buffer, immunoprecipitated with the anti-carboxyl terminal H2 antiserum, and 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 N-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: $^*$ indicates deduced cleavage site(s). The underlined section is the putative transmembrane region. The residues in H2a represented in shadow are not found in H2b.
(a) 

![Graph showing CPM vs. cycle number with amino acid sequence AQLAELRSLEK]

(b) 

![Graph showing CPM vs. cycle number with amino acid sequence SQSEGHRG]

(c) 

H2b: CFSLLALSFNILLVICVTGSQSAQLQAELR

H2a: CFSLLALSFNILLVICVTGSQSEGHRGAQLQAELR
Figure 2. **Formation of the 35 kD fragment during *in vitro* translation of H2 mRNA and in transfected cells.**

Lanes 1 & 2: 3T3 cells expressing H2a were pulse labeled with $^{35}$S-Cys for 30 minutes, chased for 2 hours with 1mM 2,4-dinitrophenol, 5 mM 2-deoxyglucose and 0.5 mM cycloheximide, and then lysed in lysis buffer. Lanes 3 & 4: H2a mRNA was translated with $^{35}$S-Cys in a rabbit reticulocyte lysate with dog pancreatic microsomes for 2 hours. 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.
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 hours with $^3$H-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 N-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.
(a) CPM

(b) CPM
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 $^3$H-Leu in rabbit reticulocyte lysates with dog pancreatic microsomes for 2 hours. 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 N-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 $^3$H-Leucines were determined and data extrapolated to determine the cleavage site. Boldface indicates the mutant residue. ^ indicates deduced cleavage site(s). The underlined section is part of the putative transmembrane region.
H2b Wild type: VICVTG SQV TGS AQLQAELR

H2b Mutants:

S81I: VICVTG SQI AQLQAELR
S81R: VICVTG SQR AQLQAELR
S81G: VICVTG SQG AQLQAELR
S81A: VICVTG SQA AQLQAELR

A82R: VICVTG SQS RQLQAELR
A82P: VICVTG SQS PQLQAELR
A82G: VICVTG SQS GQLQAELR
A82T: VICVTG SQS TQLQAELR
A82E: VICVTG SQS EQLQAELR
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 $^{35}$S-Cys for 15 minutes, chased with 0.5 mM cycloheximide in unlabeled medium for 3 hours, 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 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 hours.
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 $^{35}$S-Cys for 15 minutes and then chased in unlabeled medium for up to 4 hours. 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.
Figure 7. **Inhibition by 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 $^{35}$S-Cys for 15 minutes and then chased in unlabeled complete medium containing various inhibitors for 4 hours. Immunoprecipitates of cell lysates were analyzed by SDS-PAGE and fluorography. Lanes 1 & 6: Pulse labeled only. Lanes 2 & 7: Chased in medium without any inhibitors. Lanes 3 & 8: Chased in medium with 100 μM TLCK. Lanes 4 & 9: Chased in medium with 20 μM TPCK. Lanes 5 & 10: Chased with 30 μM iodoacetamide (IAA). The G78R mutant does not form the 35 kD cleavage product. The solid arrow indicates the position of the core-glycosylated 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 hours without any inhibitor.
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 $^{35}$S-Cys for 15 minutes and then chased in unlabeled complete medium containing 0.5 mM cycloheximide for 5 hours (lane 1). Cycloheximide was then removed and medium without any inhibitor (lanes 2, 4, 6) or medium with 100 μM TLCK (lanes 3, 5, 7) was added and the chase continued for one (lanes 2, 3), two (lanes 4, 5) or three (lanes 6, 7) more hours. 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.
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 $^{35}$S-Cys for 15 minutes and then chased in unlabeled complete medium containing various inhibitors for up to 5 hours. Immunoprecipitates of cell lysates at 0, 100, 200 and 300 minutes of chase were analyzed by SDS-PAGE and fluorography. Lane 1: Pulse labeled only. Lanes 2 to 4: Chased in medium without any inhibitor. Lanes 5 to 7: Chased in the presence of 100 μM TLCK. Lanes 8 to 10: Chased in the presence of 100 μM TLCK and 0.5 mM cycloheximide. Lanes 11 to 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 the position of the 35 kD fragment.
Chase Times: 0 min, 100 min, 200 min, 300 min

No inhibitor | TLCK only | TLCK + Cycloheximide | Cycloheximide only

1 2 3 4 5 6 7 8 9 10 11 12 13

46 kD

30 kD
H2a mRNA was translated with $^{35}$S-Cys in a rabbit reticulocyte lysate with dog pancreatic microsomes for 2 hours in the absence of inhibitor (lane 1) or in the presence of TLCK (lanes 2 to 5) or TPCK (lanes 6 to 9). Inhibitors were added to the translation mixture and the reactions were incubated for 30 minutes 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.
Figure 10. **Model illustrating the two pathways for ER degradation of H2.**
35 kD fragment

blocked by cycloheximide, ATP depletion.
Not blocked by TLCK, TPCK

DEGRADED

DEGRADED

H₂ precursor

ER LUMEN

CYTOSOL

signal peptidase?

Not blocked by TLCK, TPCK.
Table I. **Formation of 35 kD fragment by H2 mutants during *in vitro* translation and in transfected fibroblasts.**

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 $^{35}$S-Cys in rabbit reticulocyte lysates in the presence of dog pancreatic microsomes for 2 hours. 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 $^{35}$S-Cys for 15 minutes, chased with 0.5 mM cycloheximide in unlabeled medium for 3 hours, 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. n.d.- not determined.
Table I(a): H2b wild type and Ser81 mutants:

<table>
<thead>
<tr>
<th>Species:</th>
<th>Sequence:</th>
<th>Amount of 35 kD fragment formed in vitro: (wild type=1)</th>
<th>Formation of 35 kD fragment in fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2b wild type</td>
<td><strong>VICVTGSQS</strong>&lt;sup&gt;81&lt;/sup&gt; <strong>AQLQAEILR</strong>&lt;sup&gt;82&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
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<tr>
<td>H2b Ser 81 Mutants:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ser81Ile</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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Table I(b): H2b wild type and Ala82 mutants:

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<th>Formation of 35 kD fragment in fibroblasts</th>
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<td>H2b wild type</td>
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<td>+</td>
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<tr>
<td></td>
<td>AQLQAELR</td>
<td></td>
<td></td>
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<tr>
<td>H2b Ala 82 Mutants:</td>
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<td></td>
<td></td>
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<tr>
<td>Ala82Pro</td>
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Table I(c): H2a wild type and Mutants:

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<th>Formation of 35 kD fragment in fibroblasts</th>
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<td>H2a Mutants:</td>
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<td>Thr77Trp</td>
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<td>+</td>
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<tr>
<td>Glu82Ala</td>
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<td>+</td>
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Table II. Fraction of pulse labeled H2b protein acquiring complex oligosaccharides after a 4 hour chase.

3T3 cells expressing H2b wild-type or mutants were pulse labeled with 0.3 mCi/ml of $^{35}$S-Cys for 15 minutes and then chased in unlabeled medium for 4 hours. 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.

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<th>Species</th>
<th>Fraction complex glycosylated at 4 hours</th>
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<td>H2b S81W</td>
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<tr>
<td>H2b S81N</td>
<td>0.40</td>
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<td>H2b S81P</td>
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<tr>
<td>H2b S81G</td>
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<td>H2b S81A</td>
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<tr>
<td>H2b A82P</td>
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Chapter 3

Enhanced Folding and Processing of a Disulfide Mutant of the Human Asialoglycoprotein Receptor H2b Subunit
Preface

A major portion of this chapter has been submitted and accepted for publication in the Journal of Biological Chemistry as:


The unpublished sections are mainly Figures 2(b), 2(c) and 6(a) to 6(d) and the text associated with these figures.
Abstract

Unfolded forms of the H2b subunit of the human asialoglycoprotein receptor, a galactose specific C-type lectin, are degraded in the endoplasmic reticulum (ER) while folded forms of the protein can mature to the cell surface (1). There are eight cysteines in the exoplasmic domain of the protein, forming four disulfide bonds in the folded protein. We have constructed double cysteine to alanine mutants for each of the four disulfide bonds and examined the folding and metabolic fate of each of the mutants in transfected 3T3 fibroblasts. We find that mutation of the two cysteines nearest to the transmembrane region (C1) does not prevent proper folding of the protein while mutations of the other three disulfides prevent proper folding of the protein and all of the mutant proteins are degraded in the ER. A normal (~20%) fraction of the C1 mutant protein exits the endoplasmic reticulum and is processed in the Golgi complex and it does so at a faster rate compared to the wild-type. Furthermore, the folded form of this mutant protein is more resistant to unfolding by dithiothreitol than the wild-type. The C1 mutant protein is expressed on the cell surface and can form a functional receptor with the H1 subunit with similar binding affinities for natural ligands as that of the wild-type receptor. The same fraction of newly-made mutant and wild-type proteins (~80%) remain in the ER, but the mutant protein is degraded more quickly. Thus, the presence of the C1 disulfide bond in the wild-type receptor both reduces the rate of protein folding and exit to the Golgi, and also slows the rate of ER degradation of the portion (~80%) of the receptor that never folds properly.

This work was supported by Grant CDR 88-03014 from the National Science Foundation to the M.I.T. Biotechnology Process Engineering Center.

The abbreviations used are: ER, endoplasmic reticulum; ASGP, asialoglycoprotein; DTT, dithiothreitol; PBS, phosphate buffered saline; SHPP, N-succinimidyl-3-(4-hydroxyphenyl)propionate; CRD, carbohydrate recognition domain; CNBr, Cyanogen bromide; Cy3, cyanine dye Cy3.18; Endo H, endoglycosidase H, DME, Dulbecco’s Modified Eagle medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; BiP, heavy-chain binding protein.
Introduction

The asialoglycoprotein (ASGP) receptor, also known as the hepatic lectin, is a type II integral membrane protein that is normally expressed only on mammalian hepatocytes. This Ca\textsuperscript{2+}-dependent lectin binds to terminal galactose residues on sugar side chains; these ligands are removed from the circulation by receptor mediated endocytosis via coated pits and degraded in lysosomes (2, 3, 4). The functional human ASGP receptor is a hetero-oligomer consisting of two types of subunits, H1 and H2, with a minimum stoichiometry of (H1)\textsubscript{3}(H2)\textsubscript{1} (5). H1 and H2 are 60% homologous in amino acid sequence (6, 7) and both subunits are required for a functional receptor (8, 9). The polypeptide chain of each subunit consists of four main domains: a short cytosolic amino-terminal segment, a single hydrophobic transmembrane segment that also functions as an uncleaved signal anchor sequence, followed by an exoplasmic "stalk" domain and, at the very carboxy-terminus, the Ca\textsuperscript{2+}-dependent carbohydrate recognition domain (CRD). 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. In HepG2 human hepatoma cells, most of the H2 expressed on the cell surface is H2b (10). Besides the human receptor, highly homologous ASGP receptors with identical functions have also been cloned and analyzed in rat (11) and mouse (12). The ASGP receptor belongs to a family of animal proteins known as the C-type lectins with the characteristic of requiring calcium ions for ligand binding (13). All members of these family share sequence homology in the carbohydrate recognition domain (CRD). Within this domain of about 130 amino acids, 18 invariant and 32 conserved residues are found in all members (13).

The important role of disulfide bonds in determining and maintaining tertiary and quaternary structures of secreted proteins and exoplasmic domains of membrane proteins has long been recognized. These proteins form disulfide bonds between cysteine residues due to the more oxidative redox state of the endoplasmic reticulum (ER) compared to the cytosol (14). The formation of disulfide bonds in newly synthesized proteins occurs within the ER and is catalyzed by the ER resident enzyme protein disulfide isomerase (15). Intracellular disulfide-bonded folding intermediates of the human chorionic gonadotropin β subunit have been identified (16) and disulfide-bonded in vivo
folding intermediates have also been reported for influenza hemagglutinin (17), retinol binding protein (18) and the ASGP receptor H1 subunit (19). Studies on the influenza hemagglutinin have shown that all the disulfide bonds are required for proper folding and protein maturation (20). When cells are treated with a reducing agent, dithiothreitol (DTT), which disrupts disulfide bonds, newly-made proteins that contain disulfide bonds, e.g. albumin and the ASGP receptor, are retained within the ER, while proteins which do not have disulfides are secreted normally in the presence of DTT (21).

Depletion of Ca\(^{2+}\) in the ER by treating cells with Ca\(^{2+}\)-pump inhibitors or Ca\(^{2+}\) ionophores also blocks exit of the ASGP receptor from the ER and unfolded proteins accumulate (1, 19). Therefore, proteins which are misfolded or cannot attain a properly folded state in the ER cannot exit this organelle to the Golgi complex and are ultimately degraded in the ER (22, 23).

When expressed without H1 in transfected fibroblasts, about 80% of ASGP receptor H2b subunits are degraded in the ER (10, 24, 25). The unfolded portion of the newly-made H2b protein is selectively degraded within the ER while the 20% that is folded matures to the cell surface (1). Therefore, we felt it important to analyze further the relationship between folding and degradation of H2b within the ER. In this study, we determine the importance of each of the four disulfide bonds in the folding and degradation of the H2b protein by specifically mutating each pair of cysteine residues and observing the metabolic fate of the mutant proteins in transfected fibroblasts. We find that disruption of three of the disulfide bonds results in the inability of the protein to fold properly and these cannot exit the ER and are degraded. However, mutation of the two cysteines nearest the transmembrane region (C1 mutation) actually causes the protein to fold faster than the wild-type and the mutant protein exits the ER to the Golgi complex more rapidly than does the wild-type. Furthermore, the C1 H2b mutant will form a functional receptor with H1 and bind a natural ligand with similar affinity to the wild-type receptor.

**Materials and Methods**

**Materials.** - Materials and chemicals were purchased from Sigma (St. Louis, MO) or sources previously listed (9, 10, 24, 26). Iodination reagents were purchased from Pierce (Rockford, IL). Reagents for PCR reactions and *Pfu* polymerase were from Stratagene (La Jolla, CA). Restriction enzymes
were from New England Biolabs (Beverly, MA). The Sequenase 2.0 kit for DNA sequencing was from United States Biochemical (Cleveland, OH). Cy3 labeled goat-anti-rabbit antibody was from Jackson Immunoresearch Labs (West Grove, PA).

Cell surface iodination. - NIH 3T3 cells expressing wild-type or mutant H2b were cell surface iodinated using water soluble Bolton Hunter reagent (sulfo-SHPP) as previously described (10, 27).

Cyanogen bromide digestion. - NIH 3T3 cells expressing wild-type or mutant H2b which had been treated with 0.1M iodoacetamide and cell surface iodinated were lysed in detergent and immunoprecipitated by antiserum against the carboxy-terminus of the H2 protein. The immunoprecipitate was washed and a portion was digested for 12 hours by 50 mg/ml cyanogen bromide in 70% formic acid at room temperature in the dark. Products were analyzed on SDS-PAGE under non-reducing or reducing (50 mM DTT) conditions.

Mutagenesis of ASGP receptor H2b cDNA. - Four mutations of the H2b protein were made corresponding to the four putative disulfide linkages (C1 to C4, see Figure 1), and each mutant has both cysteines of the disulfide bond mutated to alanine residues. The substitution mutations of H2b were introduced by overlap extension PCR (28) using Pfu polymerase for primer extension. The concentrations of reagents and enzymes used in the PCR reactions were according to the manufacturer's recommendations. The double mutations were made sequentially and all mutations were verified by double-stranded dideoxy sequencing using the Sequenase 2.0 kit from United States Biochemical Corp.

Cell transfection and culture. - NIH 3T3 cells expressing wild-type H2b (2C cells) were kind gifts of Dr. G. Lederkremer (Tel Aviv University) (10). NIH 3T3 cells expressing H2 mutants were generated by using a calcium phosphate transfection protocol (29), using the pMEX-neo mammalian expression vector containing mutant H2 cDNAs subcloned into the Bam H1 and Eco R1 sites in the multicloning site of the vector. Colonies resistant to G418 were subcloned and tested for expression of H2 protein by metabolic labeling. Wild-type H1 cDNA subcloned into the pCB7 expression vector was similarly transfected into 3T3 cells expressing wild-type or C1 mutant H2b and selected by hygromycin resistance. 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 mm or 60 mm diameter tissue culture dishes were labeled with \(^{35}\)S-Cysteine using techniques previously described (24, 26). Antisera against the carboxy terminus of the ASGP receptor H2 subunit (8) were kind gifts of Drs. L. Wikström and G. Lederkremer. Immunoprecipitation, and Endo H and N-glycanase digestions of cell lysates were done as previously described (24, 26).

**Gel electrophoresis, fluorography and scanning densitometry.** - Immunoprecipitates were subjected to SDS-PAGE using 0.75mm thick 10% or 12% Laemmlı gels and analyzed by autoradiography or fluorography using 20% 2,5-diphenyloxazole as previously described (30). Autoradiograms and fluorograms were quantitated with a Molecular Dynamics laser microdensitometer as previously described (31).

**Immunofluorescence microscopy.** - Immunofluorescence localization of H2b wild-type and cysteine mutant proteins on the cell surface of transfected 3T3 fibroblasts were done as previously described (10). Basically, live 3T3 cells expressing wild-type or mutant proteins were reacted at 4°C first with a primary rabbit antibody against the carboxy-terminus of the H2 protein followed by a secondary Cy-3 labeled goat anti-rabbit antibody. Cells were washed with cold PBS, fixed with methanol/acetone, and visualized with a Zeiss epifluorescence microscope.

**Ligand binding, uptake and degradation assays.** - Orosomucoid was desialylated with immobilized neuraminidase (Sigma) and radioiodinated with Iodobeads (Pierce) as previously described (9, 32, 33). Saturation binding, uptake, and degradation assays were performed as previously described (9, 32, 33). Binding assays were done with radioligand (specific activity = \(10^7\) cpm/\(\mu\)g) concentrations in the range of 20 ng/ml to 5 \(\mu\)g/ml. Non-specific binding was measured in the presence of 100 fold excess unlabeled ligand and typically represented less than one-tenth of the total binding. Degradation products were measured as radioactivity remaining in the culture medium after it was precipitated with trichloroacetic acid, oxidized with potassium iodide / hydrogen peroxide, and extracted with chloroform (32).

**Results**

*A disulfide bond (C1) connects the H2 protein between Cys157 and Cys171.* - Figure 1 shows that the exoplasmic domain of the ASGP receptor H2b subunit contains eight cysteine residues, at positions 157, 171, 172, 185,
200, 275, 287 and 295, that are conserved in man, rat and mouse. The six cysteines nearest to the carboxy-terminus are conserved in C-type lectins and are predicted to form three disulfide bonds, based on biochemical and crystallographic data obtained on homologous proteins (34, 35, 36, 37). They are Cys172/185, Cys275/287, Cys200/295, and are labeled here as C2, C3 and C4 disulfides, respectively. The two cysteines nearest to the transmembrane region, Cys157 and Cys171, are not conserved in other C-type lectins.

To determine if a disulfide is formed between these two cysteines (referred to as the C1 disulfide), we take advantage of the methionine residue at position 159, between these two cysteines, that is only one of two methionines in the whole molecule, excluding the translation initiation residue site. Met159 is also the only methionine in the exoplasmic domain (Figure 1). The mature complex glycosylated H2b protein is ~50 kD, with a core protein mass of ~35 kD and three N-linked sugar side chains adding ~5 kD each to the total mass. In the experiment leading to Figure 2, proteins in H2b transfected fibroblasts were reacted with iodoacetamide to block free sulphydryl groups and then radioiodinated with the membrane impermeable Bolton Hunter reagent (sulfo-SHPP). The cells were then dissolved in detergent and immunoprecipitated with an antiserum against the carboxy-terminus of the H2 protein. We chose to analyze only the mature, cell surface, form of the H2b protein to avoid ambiguities that can arise in the intracellular forms which may not be fully folded or are misfolded with non-native disulfides. If there is a C1 disulfide bond, on digestion of the immunoprecipitated mature cell surface H2b protein by cyanogen bromide, which specifically cleaves after methionine residues, a ~27 kD fragment (with two N-linked sugar side chains) and a ~17 kD fragment (with one N-linked sugar side chain), should be detected only in reducing conditions but not under non-reducing conditions. Figure 2 shows the 50 kD full length complex glycosylated H2b expressed on the cell surface of stably transfected 3T3 fibroblasts analyzed under non-reducing (lane 1) and reducing (lane 3) conditions. After digestion with cyanogen bromide, a portion of the 50 kD protein is decreased in size to about 44 kD due to cleavage at Met56 but no smaller fragments were observed under non-reducing conditions (lane 2). However, under reducing conditions, a ~27 kD fragment (which corresponds to the carboxy-terminal fragment with two N-linked sugar side chains) and a ~17 kD fragment (which corresponds to the amino-terminal fragment with one N-linked sugar side chain) are observed (lane 4). These were
not observed under non-reducing conditions (lane 2). These observations strongly suggest that there is a reducible intrachain disulfide linking Cys157 and Cys171. It is possible that the C1 disulfide bond is actually between Cys157 and Cys172 while the C2 disulfide is between Cys171 and Cys185 but the main conclusion is that Cys157 is disulfide bonded. In summary, the intrachain disulfides of the H2b protein are presumed to be: Cys157/171 (C1), Cys172/185 (C2), Cys275/287 (C3), and Cys200/295 (C4).

The results shown in Figure 2a shows that there is most probably a disulfide bond between Cys157 and Cys171 of the H2b protein. One of the cysteine mutants we constructed is the C1 mutant which has both of these residues mutated to Ala. As shown in a later section, this mutant H2b protein can mature to the cell surface. Therefore, a cyanogen bromide digestion of the cell surface C1 mutant protein should produce the 17 kD and 27 kD fragments under both non-reducing and reducing conditions. Figure 2b shows the 50 kD full length complex glycosylated H2b C1 mutant expressed on the cell surface of stably transfected 3T3 fibroblasts analyzed under non-reducing (lane 1) and reducing (lane 3) conditions. After digestion with cyanogen bromide, a ~27 kD fragment (which corresponds to the carboxy-terminal fragment with two N-linked sugar side chains) and a ~17 kD fragment (which corresponds to the amino-terminal fragment with one N-linked sugar side chain) are observed under both non-reducing (lane 2) and reducing conditions (lane 4). This result strengthens the conclusions drawn from results of Figure 2a. Another cysteine mutant of H2b, the C171A mutant, in which Cys171 is mutated to Ala, is also Golgi processed and presumably matures to the cell surface as described in a later section. Figure 2c shows the results of a cyanogen bromide digestion experiment on cell surface labeled H2b C171A protein. The 17 kD and 27 kD fragments are observed in both reducing and non-reducing conditions, suggesting that at least a large fraction of the cell surface form of this mutant protein does not have a disulfide bond between Cys157 and Cys172. Therefore, the wild-type protein most likely has a disulfide bond between Cys157 and Cys171, and not Cys157 and Cys172.

Mutant H2b proteins lacking the C1 disulfide bond show enhanced rate of folding in the ER and Golgi processing. To examine the importance of each of the 4 intrachain disulfides in the H2b protein, we constructed double cysteine to alanine mutants which individually disrupted each of the disulfide bonds (C1 to C4 mutants) and studied the metabolic fate and folding of each of the
mutants in stably transfected 3T3 fibroblasts. Figure 3, lanes 1, 7, 13, 19 and 25, show that on pulse labeling with $^{35}$S-cysteine, the wild-type and C1 to C4 mutants of H2b are all synthesized as a 43 kD precursor (open arrow), which can be deglycosylated to a 35 kD core protein with endoglycosidase H (data not shown). Lanes 2 to 6 show that in the subsequent chase period, a fraction of the wild-type precursor is converted to a 50 kD mature form (solid arrow), which is resistant to Endo H (data not shown). Densitometric scans of this pulse chase experiment (Figure 4a) shows that about 20% of the wild-type precursor becomes complex glycosylated (i.e., Endo H resistant). This Golgi processing proceeds with a half time of about 90 minutes with most of the mature forms appearing by 3 hours. After an initial lag of 60 minutes, ~80% of the Endo H sensitive forms of H2b are degraded with a half life about 60 minutes, consistent with previous reports (1, 10). Figure 3, lanes 8 to 12 shows that a fraction of the C1 mutant precursor is also converted to the 50 kD mature form that is Endo H resistant (data not shown). Furthermore, densitometric scans (Figure 4b) shows that although the fraction of the C1 mutant precursor that attains complex glycosylation is similar to the wild-type (~20%), the Golgi processed forms appear much faster than the wild-type. Substantial amounts of the complex glycosylated form of the C1 mutant protein has already appeared by 30 minutes and the maximum level is reached by 60 minutes. The Endo H sensitive form of the C1 mutant also disappears much faster than that of the wild-type, with a half life of less than 30 minutes.

Figure 3, lanes 14 to 18, 20 to 24 and 26 to 30 show that the C2, C3 and C4 mutants do not form any detectable complex glycosylated mature forms. All the pulse labeled proteins remain as 43 kD forms (Endo H sensitive, data not shown). A ~55 kD polypeptide that appears in all lanes is unlikely to be a processed form of the mutant H2b receptor since it is present in the pulse-labeled samples and its mobility is not affected by N-glycanase digestion (data not shown). We do not know the identity of this co-immunoprecipitated protein. The immunoprecipitated 35 kD polypeptide that appears in the chase periods comprises the carboxy-terminus of the H2b protein; it is produced by a proteolytic cleavage in the exoplasmic domain near the membrane-spanning region (24, 25). We consistently observe that more of this fragment is produced in cells expressing the C2, C3 and C4 mutants, consistent with the finding that no detectable fraction of these proteins matures out of the ER. Figure 4c shows that the kinetics of degradation of the Endo H sensitive form
of the C4 mutant is similar to that of the wild-type. Similar densitometric scans show that the C2 and C3 mutants also have similar kinetics of degradation compared to the wild-type H2b (data not shown).

On non-reducing SDS-PAGE, less compact forms of a protein should experience more hydrodynamic resistance, and thereby migrate more slowly than more compact forms of the same protein. Formation of disulfide bonds in a protein should allow it to attain more compact forms and migrate faster under non-reducing conditions on SDS-PAGE than non-compact forms without disulfide bonds. Previous studies on folding of the ASGP receptor subunits showed folding intermediates that were separable by non-reducing SDS-PAGE (1, 19). Figure 5, lanes 1 and 2 show that, as judged by their differences in mobility on non-reducing SDS-PAGE, pulse labeled H2b wild-type and C1 mutant proteins have a less compact and presumably unfolded structure (shaded arrow) but that they attain a more compact structure (lanes 6 and 7, striped arrow) after a 30 minute chase period. However, if the cells are treated with 5 mM DTT for another 5 minutes at the end of the 30 minute chase, a fraction of the wild-type H2b protein is “unfolded” to a less compact form (lane 11) while almost all of the C1 mutant protein remains as the more compact species (lane 12). Lanes 3 to 5 show that the C2, C3 and C4 mutants are also synthesized as less compact unfolded forms, similar to the wild-type (lane 1, shaded arrow). After 30 minutes, the C2 and C4 mutant (lanes 8 and 10) also attain a more compact or more folded form (striped arrow) similar to the wild-type (lane 6). However, most of the C3 mutant remains as the less compact unfolded form after 30 minutes (lane 9). On treatment with 5 mM DTT, only a fraction of the wild-type is unfolded to the less compact form (lane 11) but all of the C2, C3 and C4 proteins are unfolded to the less compact form (lanes 13 to 15). The differences in the band intensities observed reflect the variability of sample detection in non-reducing gels and so the results are not quantitatively significant. On reducing SDS-PAGE, all the bands migrate to the same positions (data not shown) and therefore results under non-reducing conditions reflect differential oxidative isoforms of the proteins. Experiments in which longer chase times were done (up to 3 hours) showed identical results concerning the relative mobilities and DTT sensitivities of the wild-type and mutant proteins (data not shown). We conclude that the C2, C3 and C4 disulfides are required for proper folding of the protein. If any of these disulfides are missing, the protein cannot attain its properly folded form and cannot exit
the ER and is all degraded within the ER. However, the absence of the C1 disulfide bond enhances protein folding and processing.

The C1 mutant, with both Cys157 and Cys171 mutated to alanine, showed enhanced folding and processing compared to the wild-type. The metabolic fate and folding of single residue mutants at Cys157 or Cys171 were also examined. Figure 6a, lanes 1 and 7, show that, after pulse labeling of transfected fibroblasts expressing either the C157A (lane 1) or C171A (lane 7) single mutant, both were synthesized as core-glycosylated 43 kD precursors (open arrow). The C157A mutant does not appear to acquire any complex sugars and all the precursor proteins are degraded in the ER during the chase period (lanes 2-6). The Endo H sensitive precursor of the C157A mutant appears to be degraded at a slightly faster rate compared to that of the wild-type, as shown in the densitometric scans in Figure 6c compared to Figure 4a. Figure 6a, lanes 8-12, show that about 35% of the C171A precursor becomes complex glycosylated (solid arrow) after 3 hours of chase. Densitometric scans (Figure 6d) show that the kinetics of the degradation of the precursor and the appearance of the complex glycosylated form of the C171A mutant are similar to that of the wild-type.

Figure 6b, lanes 3 and 4 show that, on non-reducing SDS-PAGE, pulse labeled C157A and C171A mutants have a less compact and presumably unfolded structure (shaded arrow) similar to the wild-type (lane 1) and C1 double mutant (lane 2) proteins. All of them attain a more compact structure (lanes 5 to 8, striped arrow) after a 30 minute chase period. When the cells are treated with 5 mM DTT for another 5 minutes at the end of the 30 minute chase, a large fraction of the wild-type H2b protein is “unfolded” to a less compact form (lane 9) while almost all of the C1 mutant protein remains as the more compact species (lane 10). Lane 11 shows that the C157A mutant is also partially unfolded by DTT treatment and the fraction that is “unfolded” is similar to the wild-type protein. Lane 12 shows that only a minor fraction of the C171A protein can be unfolded by DTT treatment.

*The H2b C1 mutant is expressed on the cell surface and together with the H1 subunit, can form a functional receptor.* - The data from Figure 3 shows that a similar fraction of the wild-type and C1 mutant H2b proteins becomes Golgi processed. Previous studies showed that wild-type H2b protein is expressed on the cell surface of transfected fibroblasts (10). This is confirmed in Figure 7a, which shows binding of an antibody against the carboxy-terminus of the H2
protein to the surface of transfected fibroblasts expressing wild-type H2b protein. A similar immunolabeling experiment (Figure 7b) shows that the C1 mutant protein is also detected on the cell surface of transfected fibroblasts. Figure 7c shows that none of the C4 mutant protein can be detected on the cell surface of transfected fibroblasts. These results are consistent with the pulse chase experiments in which no complex glycosylated forms of the C4 protein could be detected. Similar negative results were obtained for cells expressing the C2 and C3 mutants (data not shown).

A functional ASGP receptor requires both the H1 and H2 subunits and it is possible that the C1 disulfide bond is required to form the functional receptor complex with H1. To determine if the H2b C1 mutant can form a functional receptor, we stably transfected cDNA encoding the H1 subunit into 3T3 fibroblasts expressing the wild-type or C1 mutant. Both cell lines showed specific and calcium dependent binding to a well-studied ligand for the ASGP receptor, human asialo-orosomucoid (data not shown) while cell lines expressing only the H2b wild-type or C1 mutant protein did not show any specific binding (data not shown). Figure 8a shows the Scatchard plot of a binding study at 4°C, employing cells expressing H1 and wild-type H2b, and radiiodinated asialo-orosomucoid. The results indicate a dissociation constant of 11 nM and about 600,000 surface receptors per cell. Figure 8b shows the Scatchard plot of a similar binding study employing cells expressing H1 and C1 mutant H2b. The results indicate a binding constant of 9 nM and about 120,000 surface receptors per cell. These levels of expression are within the range of that exhibited by human hepatoma HepG2 cells (32). The different levels of surface expression may be accounted for by different levels of H2 protein expression in the two cell lines used here. Pulse label studies with the two cell lines show that they express equal levels of the H1 protein but that the wild-type H2b is expressed at about a five-fold higher level than the C1 mutant (data not shown). However, both cell lines show specific ligand binding and similar binding affinities; and the values are similar to those of wild-type receptors naturally expressed in cultured HepG2 cells (32).

Figures 9a and 9b show that both the cell lines expressing H1 and wild-type H2b, and H1 and C1 mutant H2b are capable of endocytosis and degradation of asialo-orosomucoid. With both cell lines, cell associated radioactivity plateaus after about 30 minutes of incubation at 37°C and degradation products appear in the medium within 30 minutes. The rates of
total ligand uptake and degradation are similar in both cell lines - ~3.5 pg of ligand per min per µg cell protein over 4 hours at 37°C. It is interesting that although the cell line expressing H1 and H2b C1 receptors has about five fold fewer surface receptors than one expressing H1 and wild-type H2b (as determined by the saturation binding experiments), they show a similar rate of ligand endocytosis and degradation. This may indicate that the turnover rate of the endocytic cycle of the mutant receptor is higher than that of the wild-type receptor.

We conclude that the absence of the C1 disulfide bond in the H2b protein does not prevent its transport to the cell surface; it can form a functional receptor when co-expressed with the H1 subunit. This receptor binds to a natural ligand, asialo-orosomucoid, with an affinity similar to that of the wild-type receptor. The mutant receptor can also perform its natural functions of receptor mediated endocytosis, ligand degradation, and presumably receptor recycling. Therefore, it is unlikely that the these two cysteines are required for the formation of a functional receptor complex.

Discussion

A large fraction of the ASGP receptor H2b subunit expressed in transfected fibroblasts in the absence of the H1 subunit is degraded in the ER. Depletion of calcium ions in the ER causes all of H2b to be retained in a misfolded state in the ER and eventually is degraded there (1). When cells expressing the H1 subunit are depleted of calcium ions or treated with DTT, all of the H1 protein is also retained in and degraded in the ER (19). Studies on the T-cell receptor subunits also showed that the ER degradation process is enhanced in the presence of DTT (38) or depletion of calcium ions (39), conditions that prevent proper folding of proteins. Therefore, proper folding and formation of disulfide bonds in secretory or membrane proteins are normally required for transport out of the ER.

As we expected, three of the disulfide bonds (C2, C3 and C4) in the H2b protein are essential for proper folding of the protein as absence of any one of them prevents transport of the protein out of the ER to the Golgi and all of the newly-made protein is degraded in a pre-medial Golgi compartment. As judged by non-reducing SDS-PAGE and sensitivity to DTT, the C2, C3 and C4 mutants cannot fold as well as the wild-type as only the wild-type protein can attain resistance to DTT unfolding (though only a fraction of it can do so).
These observations confirm the previous deduction that only folded forms of the H2b protein can exit the ER to the cell surface (1). Presumably, a folded form of the H2b protein that is resistant to DTT unfolding, a conformation attained by a fraction (~20%) of the wild-type protein, is the prerequisite for ER to Golgi transport and appearance on the plasma membrane. An interesting result from these assays is that the C3 mutant cannot attain any compact structure after a 30 minute chase period, while the C2 and C4 mutants can attain some compact structure(s) although these are all sensitive to DTT unfolding (Figure 5). This suggests that the C3 disulfide bond plays a more important role in attaining or maintaining a folded structure of the H2b protein than do the C2 and C4 disulfide bonds, although the C3 disulfide bond is formed by two cysteines that are very close in amino acid sequence positions (Cys275 and Cys287) relative to the C2 and C4 disulfide bonds.

Surprisingly, mutation of the two cysteines nearest to the transmembrane region to alanine residues does not prevent maturation of the protein to the cell surface. In contrast, the mutant protein is folded more rapidly than the wild-type as all of the mutant protein that attains a compact structure (as judged by mobility on non-reducing SDS-PAGE) within 30 minutes is resistant to unfolding by DTT while less than half of the compact form of the wild-type protein is resistant to DTT unfolding. Furthermore, the mutant protein is processed more rapidly than the wild-type in that it is transported to the Golgi complex more quickly. However, the proportion of the mutant protein that becomes Golgi processed (~20%) is about the same as that of the wild-type. The portion of the mutant H2b protein that remains in the ER is degraded at a higher rate than the wild-type.

The metabolic and folding studies of the C157A and C171A single cysteine mutants suggest that Cys171 is not required for the correct folding of the protein and transport of the protein from the ER to the Golgi complex as a normal fraction of the C171A mutant is processed in the Golgi. The rate of appearance of the complex glycosylated forms of C171A and the rate of degradation of the core-precursor in the ER are similar to that of the wild-type. A smaller fraction of the compact form of the C171A mutant is "unfolded" by DTT compared to that of the wild-type protein (Figure 6b), suggesting that the mutant may actually fold slightly better. Mutation of Cys157 does not appear to affect folding (as compared to the wild-type on the non-reducing SDS-PAGE)
but none of the C157A mutant protein exits the to be processed in the Golgi. Therefore, the compact form of C171A protein observed on non-reducing SDS-PAGE may be misfolded. However, firm conclusions cannot be drawn from studies of these single cysteine mutants as such mutations often lead to formation of non-native mixed disulfides during protein folding.

We speculate that when present, the C1 cysteines cause the protein to be misfolded and to be bound by chaperones in the ER such as protein disulfide isomerase or BiP. The binding would cause the protein to remain in the ER for longer periods, during which a fraction of them may refold to form the native disulfide bonds after which the protein is exported to the Golgi complex. As a result, ER to Golgi transport of the protein is delayed and degradation in the ER is also slowed down. In the C1 mutant these cysteines are absent. The misfolded proteins would not be formed and the protein would not bind chaperones. Therefore, the C1 mutant would fold faster and exit the ER quicker while the fraction that is unable to fold would remain in the ER and would be quickly degraded. This hypothesis indicates that binding of H2 to certain chaperones might delay both folding and ER degradation. It is consistent with our observation that the C2, C3 and C4 mutant proteins are degraded at the same rate as the fraction of the wild-type that remains in the ER, as all these proteins would be misfolded and bound to chaperones. We have no direct evidence for this hypothesis as we have not been able to detect binding of specific chaperones to the wild-type or mutant H2b proteins.

We have previously demonstrated that degradation of the H2 subunit in the ER occurs via two pathways, one which involves an initial cleavage of the protein (most likely by signal peptidase) to form a 35 kD carboxy-terminal fragment as a degradation intermediate, and another pathway which does not go through this intermediate. The pathway that is not dependent on the formation of the 35 kD intermediate is inhibited by the protease inhibitors TLCK or TPCK and is probably the major pathway (25). In the presence of TLCK or TPCK, unfolded forms of the H2b protein accumulate in the ER, therefore suggesting that it is the unfolded forms of H2b that are degraded in the ER (1). The degradation of the C1 mutant protein in the ER is also inhibited in the presence of TLCK or TPCK (Yuk and Lodish, unpublished observations); this is consistent with the notion that the unfolded fraction of the C1 mutant protein that remains in the ER is degraded via the major TLCK and TPCK-sensitive degradation pathway. Relative to wild-type H2b, more of
the 35 kD carboxy-terminal fragment was produced from the C2, C3 and C4 mutants. Its higher abundance may indicate that a larger fraction of each of these mutants is degraded via the pathway that goes through this intermediate rather that the pathway that does not involve cleavage to the 35 kD fragment.

We find that the H2b C1 mutant protein is capable of forming a functional receptor when co-expressed in fibroblasts with the wild-type H1 subunit. The H1/H2b C1 mutant receptor binds a natural ligand with an affinity similar to that of the wild-type receptor and is capable of catalyzing ligand endocytosis and degradation. The rate of endocytosis and recycling of the mutant receptor actually appears to be slightly higher than the wild-type. This may indicate that the mutant protein exhibits a higher rate of turnover in the secretory/endocytic pathway. Another possibility is that access to the coated-pits is the rate limiting step for rate of endocytosis and that this process is saturated at low numbers of receptors. Therefore, the rate of endocytosis and recycling would be similar for the wild-type and the C1 mutant even though they have a five fold difference in the number of cell-surface receptors. Importantly, the two C1 cysteines in the H2 protein are not required for proper receptor function. In contrast, studies on the influenza hemagglutinin have shown that all the disulfide bonds are required for proper folding and secretion of the protein (20). However, when certain cysteine residues are mutated in the β subunit of human chorionic gonadotropin (40) and human lysozyme (41), the rate of secretion is enhanced. Our study also demonstrates that loss of a disulfide linkage can lead to enhanced folding and formation of a fully functional protein.

The presence of the C1 cysteines in the wild-type ASGP receptor H2 subunit may hinder protein folding and processing. That they have been selected in evolution suggests that higher efficiency in folding and processing may not always be advantageous. Perhaps the presence of the C1 cysteines serve to reduce the turnover rate of a receptor whose expression need not be highly regulated. Nevertheless, it would be interesting to study the role of the C1 cysteines during the folding of the protein by determining and comparing the folding pathways of the wild-type and C1 mutant proteins.

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and encouragement.

References
Figures

Figure 1. Schematic diagram of putative exoplasmic disulfide bonds of the human asialoglycoprotein receptor H2b subunit. C, cysteine residues in the exoplasmic domain; M, methionine residues; Cyto, cytosolic domain; TM, transmembrane domain; Exo, exoplasmic domain; numbers above the cysteine and methionine residues refer to their position in the amino acid sequence; C1 to C4 refer to the putative disulfide bonds. * indicates position of N-linked glycosylation sites. Diagram is not to scale.
Figure 2. **Cyanogen bromide digestion of (a) H2b wild-type, (b) C1 mutant and (c) C171A mutant protein.** Fibroblasts expressing (a) wild-type or (b) C1 mutant or (c) C171A mutant H2b protein were treated with 0.1M iodoacetamide in PBS on ice for 5 minutes and then cell surface proteins were radio-iodinated with the water-soluble Bolton-Hunter reagent as described in the text. Cells were lysed and immunoprecipitated with an antiserum against the carboxy-terminus of the H2 protein and the immunoprecipitated protein was reacted in 70% formic acid with (lanes 2 and 4) or without (lanes 1 and 3) 50 mg/ml cyanogen bromide for 12 hours. After the reaction, the proteins were subjected to SDS-PAGE under non-reducing (lanes 1 and 2) or after reduction with 50 mM DTT (lanes 3 and 4).
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Lanes:

- Lane 1: 69 kD
- Lane 2: 46 kD
- Lane 3: 30 kD
- Lane 4: 19 kD
- Lane 5: 14 kD
The image shows a gel electrophoresis experiment with four lanes labeled 1 to 4. The gel contains bands at various molecular weights:

- 69 kD
- 46 kD
- 30 kD
- 19 kD
- 14 kD

A table is present indicating the conditions for DTT and CNBr treatments:

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Figure 3. Metabolic fate of H2b wild-type and cysteine mutant proteins in stably transfected 3T3 fibroblasts. 3T3 cells expressing wild-type (lanes 1-6) or mutant H2b proteins (C1 mutant, lanes 7-12; C2 mutant, lanes 13-18; C3 mutant, lanes 19-24; C4 mutant, lanes 25-30) were pulse labeled with 0.3 mCi/ml of $^{35}$S-Cys for 15 minutes (lanes 1, 7, 13, 19 and 25) and then chased in unlabeled medium for up to 4 hours (lanes 2-6, 8-12, 14-18, 20-24, 26-30). Cell lysates from various times of chase were immunoprecipitated with an antiserum against the carboxy-terminus of the H2 protein and then subjected to SDS-PAGE under reducing conditions. Open arrow indicates the high mannose precursor form of the protein. Solid arrow indicates the complex glycosylated form of the protein.
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Lanes: 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Cells expressing:

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Lanes: 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Figure 4. Kinetics of ER degradation and Golgi processing of H2b wild-type and cysteine mutant proteins in transfected 3T3 fibroblasts. The 43 kD high mannose precursors and 50 kD complex glycosylated forms of the H2b wild-type or mutant proteins in the fluorograms from Figure 3 were quantitated by scanning densitometry, normalized to amount of precursor after each pulse, and plotted against time of chase. (a) Rate of loss of high mannose forms and appearance of complex glycosylated forms of wild-type protein. (b) Rate of loss of high mannose forms and appearance of complex glycosylated forms of C1 mutant protein. (c) Rate of loss of high mannose forms of C4 mutant protein.
Figure 5. **Folding of H2b wild-type and cysteine mutant proteins in transfected 3T3 fibroblasts.** 3T3 cells expressing wild-type (lanes 1, 6 and 11) or mutant H2b proteins (C1 mutant, lanes 2, 7, 12; C2 mutant, lanes 3, 8, 13; C3 mutant, lanes 4, 9, 14; C4 mutant, lanes 5, 10, 15) were pulse labeled with 0.3 mCi/ml of $^{35}$S-Cys for 10 minutes (lanes 1 to 5) and then chased in unlabeled medium for 30 minutes (lanes 6 to 15). Where indicated, 5 mM of DTT was added to the medium at the end of the chase period for 5 minutes (lanes 11 to 15). All samples were treated with 0.1M iodoacetamide before the cells were lysed. Cell lysates were immunoprecipitated with an antiserum against the carboxy-terminus of the H2 protein, treated with N-glycanase to remove the sugar side chains, and then subjected to SDS-PAGE under non-reducing conditions. Shaded arrow indicates the less compact form of the protein. Striped arrow indicates the more compact form of the protein.
Pulse 10' wt C1 C2 C3 C4

Chase 30' wt C1 C2 C3 C4

Chase 30' 5 mM DTT 5'

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Figure 6(a). **Metabolic fate of H2b C157A and C171A single cysteine mutant proteins in stably transfected 3T3 fibroblasts.** 3T3 cells expressing H2b C157A mutant (lanes 1-6) or C171A mutant proteins (lanes 7-12) were pulse labeled with 0.3 mCi/ml of $^{35}$S-Cys for 15 minutes (lanes 1 and 7) and then chased in unlabeled medium for up to 4 hours (lanes 2-6, 8-12). Cell lysates from various times of chase were immunoprecipitated with an antiserum against the carboxy-terminus of the H2 protein and then subjected to SDS-PAGE under reducing conditions. Open arrow indicates the high mannose precursor form of the protein. Solid arrow indicates the complex glycosylated form of the protein.

Figure 6(b). **Folding of H2b wild-type, C1 double cysteine mutant, and single cysteine mutants (C157A and C171A) in transfected 3T3 fibroblasts.** 3T3 cells expressing wild-type (lanes 1, 6 and 11) or mutant H2b proteins (C1 mutant, lanes 2, 7, 12; C2 mutant, lanes 3, 8, 13; C3 mutant, lanes 4, 9, 14; C4 mutant, lanes 5, 10, 15) were pulse labeled with 0.3 mCi/ml of $^{35}$S-Cys for 10 minutes (lanes 1 to 5) and then chased in unlabeled medium for 30 minutes (lanes 6 to 15). Where indicated, 5 mM of DTT was added to the medium at the end of the chase period for 5 minutes (lanes 11 to 15). All samples were treated with 0.1M iodoacetamide before the cells were lysed. Cell lysates were immunoprecipitated with an antiserum against the carboxy-terminus of the H2 protein, treated with N-glycanase to remove the sugar side chains, and then subjected to SDS-PAGE under non-reducing conditions. Shaded arrow indicates the less compact form of the protein. Striped arrow indicates the more compact form of the protein.
(a)

<table>
<thead>
<tr>
<th>Cells expressing:</th>
<th>C157A Mutant</th>
<th>C171A Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chase Time (hrs):</td>
<td>0 0.5 1 2 3 4</td>
<td>0 0.5 1 2 3 4</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Pulse 10'</th>
<th>Chase 30'</th>
<th>Chase 30' 5 mM DTT 5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt C1 157 C 171 A</td>
<td>wt C1 157 C 171 A</td>
<td>wt C1 157 C 171 A</td>
</tr>
</tbody>
</table>

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12
Figures 6(c) and 6(d). **Kinetics of ER degradation and Golgi processing of in transfected 3T3 fibroblasts.** The 43 kD high mannose precursors and 50 kD complex glycosylated forms of the proteins in the fluorograms from Figure 6(a) were quantitated by scanning densitometry, normalized to amount of precursor after each pulse, and plotted against time of chase. (c) Rate of loss of high mannose forms of C157A mutant protein. (d) Rate of loss of high mannose forms and appearance of complex glycosylated forms of C171A mutant protein.
(c) C157A Mutant

Endo H Sensitive Form

Endo H Resistant Form

(d) C171A Mutant

Endo H Sensitive Form

Endo H Resistant Form
Figure 7. **Immunofluorescence localization of H2b wild-type and cysteine mutant proteins on the surface of transfected 3T3 fibroblasts.** Live 3T3 cells expressing (a) wild-type, (b) C1 mutant or (c) C4 mutant H2b protein were reacted at 4°C first with a primary rabbit antibody against the carboxy-terminus of the H2 protein followed by a secondary Cy-3 labeled goat anti-rabbit antibody. Cells were washed, fixed and visualized by fluorescence microscopy.
Figure 8. Saturation binding of $^{125}$I-asialo-orosomucoid to transfected 3T3 fibroblasts expressing (a) H1 and wild-type H2b proteins and (b) H1 and C1 mutant H2b proteins. Cells were incubated with various concentrations of radiolabeled ligand for 2 hours at 4°C with or without excess unlabeled ligand. Total and non-specific binding were measured as described in the text and their difference, the specific binding, was analyzed by Scatchard plots. B, specific bound ligand; B/F, ratio of specific bound ligand to free ligand. $K_d$, dissociation constant.
(a) Wild type

Kd = 11 nM

(b) C1 Mutant

Kd = 9 nM
Figure 9. **Uptake and degradation of $^{125}\text{I}$-asialo-orosomucoid by transfected 3T3 fibroblasts expressing** (a) H1 and wild-type H2b proteins and (b) H1 and C1 mutant H2b proteins. Cells were incubated with 2 µg/ml of radiolabeled ligand for various times at 37°C. At each time point, the medium was analyzed for $^{125}\text{I}$ degradation products and the cell-associated $^{125}\text{I}$ was also determined as described in the text.
(a) Wild type

(b) C1 Mutant
Chapter 4

Conclusion and Prospectus
This thesis discusses two aspects of the regulation of the secretory pathway in mammalian cells - the degradation and folding of proteins in the endoplasmic reticulum (ER) - using the human asialoglycoprotein (ASGP) receptor as a model system. From studies of degradation of the ASGP receptor H2 subunit in the ER, we conclude that there are two pathways for this process, and that the juxtamembrane conformation of the protein is likely to be a molecular determinant for degradation. The existence of multiple pathways for degradation should not be surprising. Just as proteins undergo various post-translational modifications, we can expect them to be degraded in more than one way. Since several types of chaperones exist in the ER, there are most likely to be several different proteases to be found in the ER, each with different substrate specificity. The existence of multiple proteases in the ER would be analogous to the variety of proteases present in lysosomes. The different effects of various protease inhibitors on ER degradation of various proteins also indicate the presence of a number of different proteases responsible for degradation. Furthermore, we expect the degradation and quality control systems to have different characteristics in different tissues as each cell type and the proteins they produce have unique secretory requirements.

To date, no specific protease has been identified in the ER that is responsible for degradation of a specific protein. We originally believed that the ASGP receptor might provide a system for identification of the protease(s) involved in ER degradation of this protein. This is because the degradation pathway that does not go through the 35 kD intermediate can be inhibited by two protease inhibitors, TLCK and TPCK. These compounds are covalent inhibitors of serine and cysteine proteases and they exhibit specific interactions with proteases in vitro. To determine if these compounds reacted with a limited number of proteins in cells, cultured fibroblasts were pre-incubated with TLCK or TPCK and the cells were then lysed by homogenization or detergent. The cell extracts were immunoblotted with antiserum specific for the tosyl group present in TLCK and TPCK. The results showed that a large number of cellular proteins, in both cytosolic and microsomal fractions, reacted with TLCK and TPCK (unpublished observations). Therefore these compounds are probably not suitable for
specific labeling of proteins for further characterization. Most other non-protein protease inhibitors are also not very specific in their actions. Therefore, while various ER degradation models show sensitivities to different protease inhibitors, indicating that each system utilize a unique set of protease(s), the inhibitors themselves probably do not provide useful handles for initial identification of the proteases.

It was previously thought that the degradation of the ASGP receptor H2 subunit in the ER occurs in one pathway via two steps. First the protein is cleaved near the transmembrane region on the exoplasmic side to produce a 35 kD fragment which is then further degraded. As discussed in Chapter 2, we find that this is only one of the two pathways that the protein can be degraded in the ER. We also have indirect evidence that signal peptidase is the enzyme responsible for the cleavage process to generate the 35 kD fragment. Furthermore, in a collaborative project which I participated in, it was shown that some of the fragment could be further Golgi processed and secreted into the extracellular medium (see Appendix II). To confirm that signal peptidase is responsible for the cleavage process, we would need to use a defined \textit{in vitro} biochemical system similar that used for studies of protein translocation in the ER (Gorlich and Rapoport, 1993). An assay system of membranes reconstituted with purified individual protein components should enable us to determine if signal peptidase is required for the cleavage of the H2 protein produced by \textit{in vitro} translation. The other pathway of degradation does not involve any observed cleavage product, but is inhibited by TLCK and TPCK. Since iodoacetamide and calpain inhibitors also block this degradation pathway, cysteine proteases or active thiol groups are probably involved.

The identification of proteases involved in degradation of various proteins in the ER would be important in clarifying the molecular details of the processes. The use of yeast genetic systems could be one approach towards identification of ER proteases. The phenomenon of ER degradation has been described in yeast (Finger et al., 1993). Complementation analysis of mutants of yeast that show defects in the ER degradation should lead to identification of genes required for this process. Biochemical analysis of their gene products should provide information on the mechanism of the degradation process. Homologues of genes in yeast that are needed for ER degradation could then be
identified in mammalian cells and further characterized. However, yeast cells probably have different regulatory systems in their secretory pathway and may not have the same types of degradation mechanisms in the ER compared to mammalian cells. When the ASGP receptor H2 subunit is expressed in yeast cells, the protein remains in the ER, as judged by the glycosylation pattern of the protein. However, it is very stable in the yeast cells and is not cleaved to form the carboxy-terminal fragment (unpublished observations). As yeast cells do not form complex tissue structures compared to mammalian cells, they also have different quality control requirements. Yeast cells can dispose of their unwanted proteins by secreting them into the environment but mammals cannot do that as the secretion of unfolded proteins into the circulation may provoke immune responses. Therefore, yeast cells may not have the same level of complexity as mammalian cells in the ER degradation systems. Nevertheless, the yeast system could be a good starting point to analyze the basic requirements for degradation of proteins in the ER.

Biochemical approaches may also be used to identify ER proteases if the degradation processes can be observed in a defined in vitro system. Subcellular extracts of ER could be tested for degradation activity on purified protein substrates. If specific proteolytic activity could be observed, the extracts could be further fractionated till a defined protease activity is determined. This endeavor would involve extensive testing of suitable reaction conditions to mimic the micro-environment of ER and provide optimal conditions for the substrate-enzyme reaction. This is challenging for membrane proteins such as the ASGP receptor as it may be difficult to identify a suitable detergent to keep the protein in solution but at the same time still allow access of the enzyme to the substrate. Another issue that has not been resolved is the recognition mechanism for selecting proteins for ER degradation. There should be ER proteins that specifically recognize misfolded and unassembled proteins to target them for degradation. This is an important consideration for setting up an assay system to identify components needed for degradation if the process requires both the recognition molecules and proteases to work in concert. The proteases themselves may perform the recognition function. Chaperones in the ER may also perform this function but direct evidence for this is lacking. The chaperones are known to bind to unfolded proteins to prevent aggregation. Whether the binding will target the
proteins for degradation or prevent the degradation to give the proteins more time to fold properly is still an open question. The overall scheme for such recognition is likely to be complex and involve various proteins, many of which have yet to be identified.

The conformation of the juxtamembrane region of the H2 protein is probably important for determining the metabolic fate of the protein, as shown by the different metabolic fate of the H2a and H2b subunits. To fully understand this, structural determination of this region of the protein in both H2a and H2b by biophysical methods would be necessary. The crystal structure of the carbohydrate recognition domain (CRD) of the homologous mannose binding protein has been determined (Weis et al., 1991; Weis et al., 1992) and allowed us to understand the structural basis for ligand binding and specificity. Sequences of both H1 and H2 subunits show the presence of heptad repeats within the “stalk” region which may form α-helical coiled coils (Beavil et al., 1992). Such structures might allow the interaction of the subunits to form the functional oligomeric receptor. It would also determine the stoichiometry of the oligomeric complex and the orientation of the CRD for binding to the ligand. Determination of the structure of the “stalk” region (between the transmembrane domain and the CRD) of the ASGP receptor or homologous proteins by x-ray crystallography or NMR would provide more concrete information on these matters. Moreover, if conformational differences in the juxtamembrane region between H2a and H2b can be confirmed by structural studies, it would also enhance our knowledge of how protein structures determine their processing in the secretory pathway.

The folding of the ASGP receptor H2 subunit in cells has been analyzed by studying the importance of the disulfide bonds in the folding and maturation of the protein. Disruption of each of three (out of a total of four) disulfide bonds lead to the inability of the protein to fold properly. The mutant proteins are all retained and degraded in the ER. However, mutant proteins lacking the disulfide linkage closest to the transmembrane (C1 mutant) can still exit the ER to be processed in the Golgi. The rate of exit is actually faster than the wild-type though the proportion of synthesized protein that exits the ER is similar to that of the wild-type. Within cells, this mutant protein is also more resistant to DTT unfolding than the wild-type. Different folding intermediates
of retinol binding protein (RBP) have been shown to show differential
sensitivity to DTT unfolding (Kaji and Lodish, 1993b). Unfolding of RBP by
DTT is also dependent on cellular factors in the microsomes and one such
factor is probably PDI (Kaji and Lodish, 1993a). Therefore, the ASGP receptor
H2b C1 mutant may fold to a different conformation compared to the wild-type
or it may not interact with unfolding factors within the ER. The mutant H2b
C1 protein can form a functional receptor together with the H1 subunit and
therefore the cysteines are not required for subunit interactions. Therefore,
certain cysteine residues that form disulfide bonds can actually retard the
folding and processing of proteins. However, their presence in wild-type H2
proteins suggests that they regulate the efficiency of folding of the protein. By
decreasing the folding efficiency and speed of exit of the protein from the ER, it
may serve to reduce the turnover rate of the protein whose expression does not
need to be highly regulated. It may also allow the protein to remain in the ER
for a longer period of time so that it can oligomerize more efficiently with the
other subunits to form the functional receptor. Homologous C1 cysteines are
also found in the H1 subunit. It would be interesting to test if mutations of
these residues in H1 have the same effect as that in H2.

The mechanism of folding of many proteins within cells is still largely
unresolved. Intermediates are formed during the folding of the ASGP receptor
in cells, as shown by differential mobilities on non-reducing SDS-PAGE and
sensitivities to DTT unfolding. However, the molecular basis for the
conformational changes are still unknown and the pathway of disulfide bond
formation has not been determined. To understand the folding pathway, these
folding intermediates would have to be trapped and isolated from cells in
substantial quantities for biochemical analysis to determine their molecular
conformations. As there are four disulfide bonds in the ASGP receptor, there
should be more disulfide intermediates than have been observed. Perhaps
formation of some of them are rapid and the intermediates are short-lived. It
may be necessary to perform pulse chase studies with chase periods in the
order of seconds to trap these intermediates with iodoacetamide. These can
then be resolved on non-reducing SDS-PAGE or HPLC for tryptic mapping.
Such studies would allow the pathway of formation of the disulfide bonds to be
determined, both in wild-type and mutant proteins. This information would
allow the understanding of the importance of various cysteine residues in the
folding pathway, the existence of non-native disulfides (if any), and explain the results observed in my preliminary studies of folding of the protein in cells. It would also be important to identify the various folding catalysts and chaperones in the ER that are involved in the folding process. Co-immunoprecipitation and cross-linking studies of the folding intermediates could shed light on this matter. Again, the conclusive determination of the factors required for efficient folding would involve the construction of a defined in vitro assay system in which all necessary components could be identified, their functions defined, and the folding pathway reproduced as it is observed in living cells. Another area of interest would be to determine if the processing of the H2 and H1 proteins in the ER involve formation of high molecular complex aggregates. For such studies, immunoprecipitates from cells or in vitro translation could be analyzed on sucrose gradients. If such aggregates are detected, their kinetics of formation and dissociation could be analyzed by pulse chase experiments. Co-immunoprecipitations would determine if chaperones are associated with the aggregates and redox conditions can be manipulated (for e.g., by DTT) to determine if they play a role in aggregate formation.

From the studies discussed in this thesis and research in other model systems, it is obvious that the quality control systems in the ER are complex and much needs to be learned. Each cell type has a different secretory requirement and each protein has a unique maturation pathway within the secretory system related to their ultimate function. However, as molecular details emerge from studies of different systems, we can expect to see important principles emerge that explain the need for complexity of the overall system and the unique requirements of individual proteins.
Literature Cited


Appendix I
Preface

Appendix I is a summary of a collaborative research work between Dr. Drorit Neumann and Dr. Gerardo Lederkremer of Tel Aviv University and myself. This study examines the effect of calpain inhibitors on the degradation and secretion of membranous and secreted forms of the erythropoietin receptor and the asialoglycoprotein receptor. My main contribution is to show that calpain inhibitors can inhibit the degradation of a mutant ASGP receptor H2a protein which is not cleaved to form the 35 kD carboxy-terminal fragment.
Blocking intracellular degradation of the erythropoietin and asialoglycoprotein receptors by calpain inhibitors does not result in the same increase in the levels of their membrane and secreted forms

Drorit Neumann, Gerardo Z. Lederkremer, Ming Huam Yuk and Harvey F. Lodish

Submitted for publication in the Biochemical Journal

Abstract

The erythropoietin receptor (EPO-R), a type one membrane glycoprotein is degraded mainly in lysosomes or endosomes; the asialoglycoprotein receptor (ASGP-R) H2a subunit, a type two membrane glycoprotein, is degraded exclusively in the endoplasmic reticulum (ER). This study describes compounds that inhibit intracellular degradation of these receptors in an efficient manner. However, the levels of cell surface expression and also secretion of their soluble exoplasmic domains were not enhanced to the same extent. The calpain inhibitors N-acetyl-leucyl-leucyl-norleucinal (ALLN) and N-acetyl-leucyl-leucyl-methional (ALLM) inhibited EPO-R degradation profoundly. After 3 hours of chase, using Ba/F3 cells and NIH 3T3 fibroblasts expressing the EPO-R, virtually most of the receptor molecules are degraded, whereas, 80% of the pulse labeled receptor, remains intact in the presence of the inhibitor. EPO-R cell surface expression was elevated 1.5 fold after 1 hour of incubation with ALLN. In the absence of protein synthesis ALLN caused accumulation of non-degraded EPO-R molecules in endosomes and lysosomes, as determined by double immunofluorescence labeling of NIH 3T3 cells, expressing EPO-R. In Ba/F3 cells expressing a soluble EPO-R, ALLN treatment increased secretion of the soluble exoplasmic domain of the EPO-R 2-5 fold. Similarly, in NIH 3T3 cells singly transfected with the ASGP-R H2a subunit cDNA, ALLN inhibited degradation of the ASGP-R H2a subunit precursor as well as the degradation of the 35 kDa proteolytic fragment corresponding to the receptor ectodomain by 3-6 fold. Yet, accumulation of secreted proteolytic fragment in the medium was augmented in the presence of ALLN by only 1.75 fold. In cells expressing the G78R mutant of the ASGP-R
H2a subunit, which is not cleaved to the 35 kDa fragment, degradation of the precursor was inhibited. Overall, our data suggest the involvement of cysteine proteases located in the ER, as well as in post Golgi compartments, in degradation of the EPO-R and the ASGP-R H2a subunit. The much lower effect of the inhibitory compounds on cell surface and secreted forms of the EPO-R and ASGP-R H2a subunit illustrates the complexity and the tight regulation of cellular localization and stability of membrane proteins.
Appendix II
Preface

Appendix II is a summary of a collaborative research work between Dr. Gerardo Lederkremer and members of his laboratory at Tel Aviv University and myself. This study examines the secretion of the 35 kD carboxy-terminal fragment of the asialoglycoprotein receptor H2a subunit into the extracellular medium. My main contribution is in determining the N-terminal sequence of the secreted fragment by radiosequencing and thereby determining the putative proteolytic cleavage site that leads to the formation of the fragment.
Membrane bound versus secreted forms of human asialoglycoprotein receptor subunits: Role of a juxtamembrane pentapeptide

Gerardo Z. Lederkremer, Ming Huam Yuk, Michael Ayalon, Sandra Tolchinsky and Harvey F. Lodish

Submitted for publication in the Journal of Biological Chemistry

Abstract

The H2a alternatively spliced variant of the human asialoglycoprotein receptor H2 subunit differs from the H2b variant by the presence of an extra pentapeptide, EGHRG, in the exoplasmic domain next to the membrane-spanning segment. This pentapeptide causes retention and degradation in the endoplasmic reticulum (ER) when H2a is expressed without the H1 subunit in 3T3 cells. In contrast, a significant portion of H2b, expressed without H1, is processed through the Golgi and reaches the cell surface. To study the H2a and H2b polypeptides in HepG2 cells (which co-express both H1 and H2) we have generated an antipeptide antibody which specifically recognizes H2a. We found that in metabolically labeled HepG2 cells, H2a is rapidly cleaved to a 35 kD fragment, comprising the entire ectodomain, most of which is secreted into the medium. Using the same antibody we found that 3T3 cells expressing H2a alone, without H1, also secrete a portion of the fragment, though much of it is degraded intracellularly. The cleavage site for the secreted fragment was on the exoplasmic side next to the transmembrane segment, between Gly 78 and Ser 79, N-terminal to the pentapeptide. No membrane bound H2a exits the ER, indicating that the pentapeptide can function as a signal for ER retention and degradation of the membrane form but does not hinder secretion of the cleaved soluble form. Secretion could be completely blocked by incubation with cycloheximide, which causes accumulation of endo H sensitive 35 kDa intracellular H2a fragments. H2a does not participate in a membrane bound receptor complex with H1 as is the case for H2b. H2a is therefore not a subunit of the receptor but a precursor for a soluble secreted form of the protein; signal peptidase is probably responsible for the cleavage to the soluble fragment. H2b produces two and a half-fold less intracellular fragment than
H2a and none was secreted. Therefore, the juxtamembrane sequence regulates the function of the transmembrane domain of a type II membrane protein as either as a signal-anchor sequence (H2b) or as a cleaved signal sequence which generates a secreted product (H2a).