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Immunology

Molecular chaperones involved in protein degradation in the endoplasmic reticulum: Quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum

(folding intermediates/half-life/subunit assembly/temperature sensitivity/substrate specificity)

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ABSTRACT **In the absence of immunoglobulin heavy-chain expression, some immunoglobulin light (L) chains are retained and degraded within the cell. We investigated the fate of two different nonsecreted murine L chains which exhibit different half-lives (50 min and 3–4 hr). Our results demonstrate that both nonsecreted L chains are quantitatively bound to BiP as partially oxidized molecules. The kinetics of L-chain degradation coincided with those of L-chain dissociation from BiP, which suggests that these two processes are functionally related. L-chain degradation does not depend on vesicular transport, indicating that these soluble proteins are degraded in the endoplasmic reticulum (ER). In contrast, secreted L chains, which interact only transiently with BiP, are completely oxidized and are not degraded even when they are artificially retained in the ER. Our data support the model that, by means of BiP interaction, the ER degradation mechanism has the potential to discriminate between partially and completely folded molecules.**

Subunits of most oligomeric membrane proteins that are exported to the cell surface of eukaryotic cells assemble in the endoplasmic reticulum (ER). When assembly cannot be achieved, subunits generally do not reach their final destination and are degraded intracellularly (reviewed in ref. 1). One of the best studied examples is the T-cell receptor complex, which consists of seven subunits. When individually expressed, isolated chains do not reach the medial Golgi compartment but undergo nonlysosomal degradation (2–5). Only when all chains are assembled into the complex is the receptor expressed on the cell surface (reviewed in ref. 6). The results of these and other (7, 8) studies led to the conclusion that a pre-Golgi compartment, possibly the ER itself, is a degradation site for unassembled protein subunits that fail to be exported on their own (9–12). Nonsecreted, unassembled immunoglobulin (Ig) light (L) chains produced by CH12κ cells exhibit a half-life of ≈50 min and are degraded in a pre-Golgi compartment suggested to be the ER (13), although the reported sensitivity to lowered temperatures is indicative of vesicular transport preceding the degradation process.

If nonlysosomal proteolysis of unassembled protein subunits occurs in the ER, an unresolved issue is how degradation substrates are selectively retained within an organelle that does not primarily function as a protein degradation compartment. A specific signal sequence for ER degradation of membrane proteins probably does not exist (14). Nonsecreted L chains are associated with the ER resident protein BiP (15–17). Since molecular chaperones are known to bind immature or malfolded polypeptides, it was tempting to speculate that an improper conformation is the common feature of putative ER degradation substrates. We therefore investigated the folding state as well as the precise degradation location of two different nonsecreted Ig L chains, the endogenous κ chains of NS1 cells (18) and the mutant λ_1FS62 L chains (16). We established stable L-chain transfectants of the NS1 cell line which allowed us to analyze the fate of the two different L chains in the same cellular environment. Our findings strongly support the notion that BiP mediates ER degradation of unassembled polypeptides, possibly by rendering unfolded portions of these substrates accessible to the action of the proteolytic machinery.

MATERIALS AND METHODS

Cell Culture, Biosynthetic Labeling, and Immunoprecipitation. J558L [secretable λ_1 (19)] and NS1 [nonsecretable κ (18)] cell lines were described previously (17). P3-X63Ag8 ($\gamma_1\kappa$) is a parent of NS1 (20). Biosynthetic labeling and immunprecipitation were performed as described (17).

Vectors and Stable Transfection. To construct pSVE2neoλ_1, the rearranged gene encoding murine wild-type λ_1 L chains was isolated from pA8-6λ_1 (gift of S. Weiss, Gesellschaft für Biologische Forschung, Braunschweig, Germany) as a 7.2-kb *Eco*RI fragment and inserted into the expression vector pSVE2neo (ref. 21; gift of T. Simon, Entwicklungslabor für Immunoassays, Freiburg, Germany). To construct pSVE2neoλ_{1FS62}, the variable (V) region-encoding exon of λ_{1FS62} was isolated from pJDEλ_1AV60FS62 (ref. 16; gift of Y. Argon, Duke University, Durham, NC) as a *Bam*HI fragment and inserted into pA8-6λ_1 so that the exon encoding the V region of wild-type λ_1 was exchanged. An *Eco*RI site introduced upstream of the gene allowed to excise the complete L-chain gene and to clone it as an *Eco*RI fragment into pSVE2neo. Stable transfections of myeloma cells were performed as described by Allen *et al.* (22).

Gradual *in Vitro* Reduction of L Chains. L chains were immunoprecipitated from lysates of 5×10^6 (J558L) or 2×10^7 (NSFS62) cells and divided into five aliquots. Immunoprecipitated proteins were solubilized in 50 μl of 5% SDS with 0, 0.3, 0.6, 1.2, or 20 mM 2-mercaptoethanol by boiling for 5 min (23). Ten microliters of 200 mM *N*-ethylmaleimide in 0.25 mM Tris·HCl, (pH 6.8) was added and, after 60 min at 4°C, 30 μl of a 3× concentrated nonreducing SDS/PAGE sample buffer was added. The samples were analyzed by Western blot; chemiluminescence detection was performed as described by the manufacturer (ECL system; Amersham).

Size Fractionation of Cellular Proteins and Western Blot Analyses. Size fractionation of cellular proteins and Western

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Abbreviations: ER, endoplasmic reticulum; L, light.

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blot analysis were performed as described (17) except that Sephacryl S-300 (Pharmacia LKB) was used.

RESULTS

Degradation of a Nonsecreted BiP Ligand Is Kinetically Linked to Dissociation of the BiP/Ligand Complex. Previous work showed that the kinetics of κ L-chain degradation in NS1 cells corresponded exactly to the kinetics of BiP/L-chain complex dissociation (17). To address whether this reflected a general principle, we investigated a mutant λ_1 L chain (λ_1FS62) which fails to be exported (16) and is a substrate for BiP that is structurally different from NS1κ L chain. A cell line producing this L chain (NSFS62) was generated by stable transfection of a κ L-chain loss variant of the NS1 cell line with an expression vector encoding the λ_1FS62 L chain.

Pulse–chase analyses and immunoprecipitation of either BiP or the L chains from NSFS62 cells lysed at various times after labeling were performed. L chains produced by the cells were quantitatively coprecipitated with BiP (Fig. 1*A*). The amount of labeled L chain either precipitated directly or coprecipitated with BiP decreased coordinately (Fig. 1*B*). Half of the newly synthesized λ_1FS62 L chains were degraded within 3–4 hr and the same time was needed to dissociate half of the L chains from BiP. Although these L chains seem to be much more stable than NS1 L chains ($t_{1/2} \approx 50$ min; see Fig. 3 and ref. 17), degradation also coincides with disappearance of the BiP/L-chain complex.

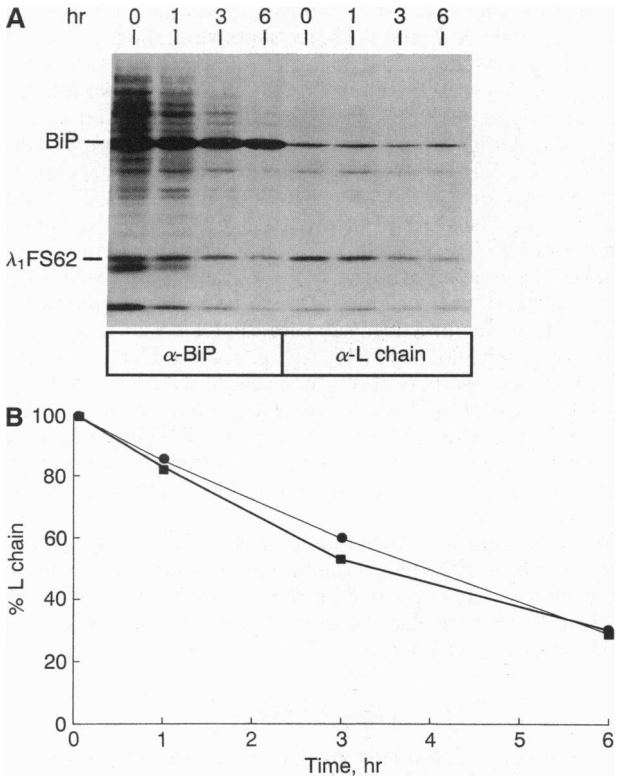

FIG. 1. Determination of the half-lifes of both the BiP/L-chain complex and the L chain in NSFS62 cells. NSFS62 cells (producing nonsecreted λ_1FS62 L chains) were pulse labeled with [^{35}S]methionine for 10 min and then incubated with nonradioactive methionine during a 0-, 1-, 3-, or 6-hr "chase." (*A*) Material immunoprecipitated with antibodies (α) to BiP or L chain was electrophoresed in an SDS/12.5% polyacrylamide gel and visualized by autoradiography. (*B*) The labeled L chains which were either coprecipitated with BiP (●) or immunoprecipitated directly (■) were quantified by scanning densitometry and plotted as percentage of the respective amounts detected directly after the pulse.

***In Vivo* Oxidation State of Wild-Type and Nonsecreted λ_1FS62 L Chains.** L chains consist of a variable and a constant domain, each of which is stabilized by one internal disulfide bond. A stepwise decrease in the electrophoretic mobility of originally completely oxidized murine κ L chains can be obtained by the addition of increasing amounts of a reducing agent (24). To analyze the oxidation states of murine wild-type λ_1 L chains, immunoprecipitated J558L L chains were exposed to various concentrations of 2-mercaptoethanol. Without reducing agent, most of the labeled L chains migrated as monomers with a greater electrophoretic mobility than the reduced form (20 mM mercaptoethanol) (Fig. 2 *Upper*). The additional bands revealed at lower mercaptoethanol concentrations (0.3–1.2 mM) presumably represented partially reduced L-chain molecules in which only either the variable or the constant domain was oxidized.

As with the wild-type protein, different oxidation forms of the nonsecreted mutant λ_1FS62 L chains were detected (Fig. 2 *Lower*). The L chains isolated in the absence of ATP migrated as a single species with a mobility corresponding to an intermediate between the completely reduced and the completely oxidized forms (compare first and last lanes in Fig. 2 *Lower*). Completely oxidized forms were detected only when the L chains were isolated from lysates that had been prepared in the presence of MgATP, which is known to induce dissociation of BiP/ligand complexes (25). This demonstrates that the λ_1FS62 L chains are capable of undergoing complete oxidation. However, since all cellular L chains are associated with BiP (see Fig. 1), these results suggest that BiP binding prevents complete oxidation of the partially oxidized λ_1FS62 L chains.

Nonsecreted NS1 L Chains Are Prevented from Complete Oxidation by Quantitative Association with BiP. In sharp contrast to the situation with λ_1FS62 L chains, only a small portion of the total cellular κ L chains coprecipitated with BiP isolated from NS1 cells (17). Moreover, different L-chain forms appeared to be present in NS1 cells: two distinct bands of 20 and 25 kDa, respectively, were resolved when the immunprecipitated L chains were analyzed by SDS/PAGE under nonreducing conditions (Fig. 3*A Left*, lane NR). In contrast, the reduced protein migrated at 30 kDa (Fig. 3*A, Left*, lane R). Thus, the two nonreduced forms presumably represent partially and completely oxidized species. The use of *N*-ethylmaleimide to prevent oxygen-mediated disulfide bond formation occurring during preparation of the cellular lysates led to a large increase in the recovery of the partially oxidized 25-kDa L-chain species (from 10% to >90%; Fig. 3*A Center*). This indicates that NS1 L chains also occur as partially oxidized BiP-bound molecules in the cells. Only the partially oxidized

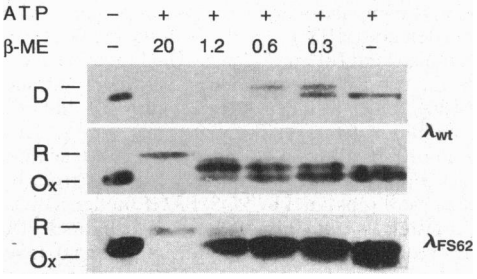

FIG. 2. Oxidation state of wild-type and mutant λ_1 L chains. Western blot analyses of wild-type (λ_{wt}) (*Upper*) and mutant FS62 (λ_{FS62}) (*Lower*) λ_1 L chains immunoisolated from cell lysates prepared in the absence or presence of 5 mM MgATP as indicated were performed. The L chains were analyzed either directly or after reduction at various concentrations of 2-mercaptoethanol (β-ME, 0.3–20 mM). The positions of L-chain dimers (D) and reduced (R) and oxidized (Ox) L-chain monomers are indicated. Note that the upper part of the λ_{wt} Western blot has been overexposed to visualize the chemiluminescent signals of the λ_{wt} dimers.

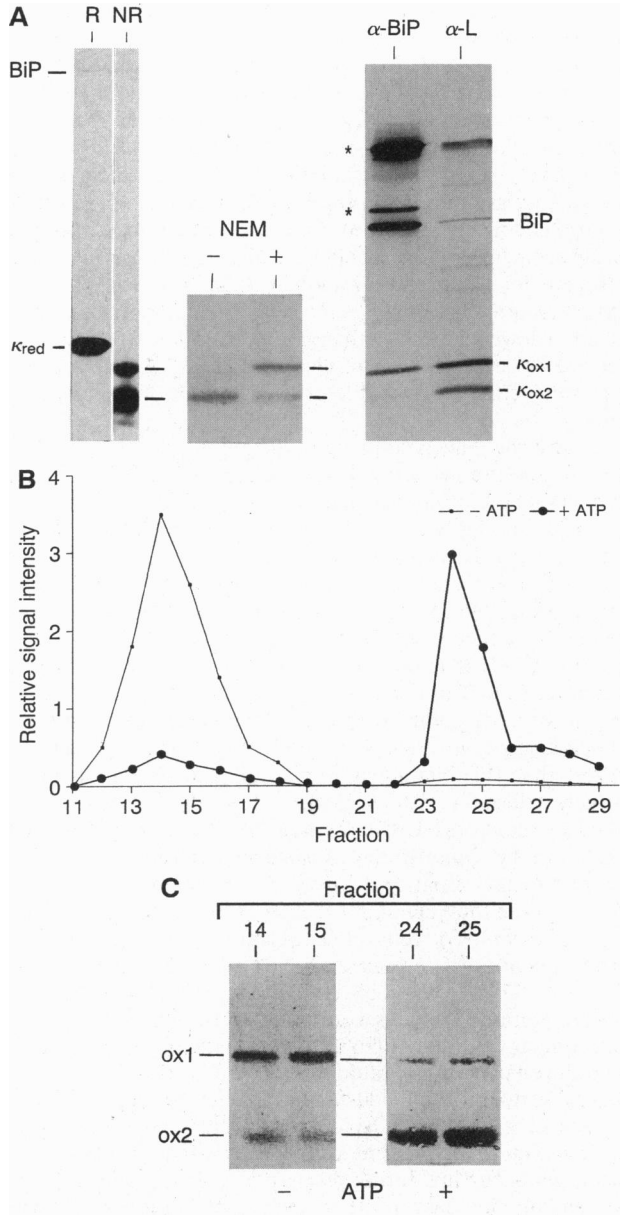

FIG. 3. Characterization of intracellular L chains in NS1 cells. (*A*) (*Left*) Biosynthetically labeled L chains were immunoprecipitated and analyzed by SDS/PAGE performed under reducing (R) or nonreducing (NR) conditions. (*Center*) SDS/PAGE analysis of nonreduced labeled L chains immunoisolated from lysates prepared with or without *N*-ethylmaleimide (NEM). (*Right*) Nonreducing Western blot analysis of immunoisolated BiP or L chains. The blot was developed with monoclonal rat anti-BiP in combination with goat anti-mouse κ L-chain antibodies, which also detect the rat anti-BiP antibody used for immunoprecipitation (stars). (*B*) Lysate (500 μl, 10^7 cells per ml) containing no or 5 mM MgATP was applied onto a Sephacryl S-300 column. Trichloroacetic acid-precipitated proteins from half of the 1-ml fractions were separated by SDS/PAGE under reducing conditions and transferred onto nitrocellulose membranes. The signals obtained after developing the blot with anti-L-chain reagent were quantified by scanning densitometry. (*C*) Indicated fractions were analyzed by nonreducing Western blots. The markers used were alcohol dehydrogenase (150 kDa, fraction 13) and carbonic anhydrase (30 kDa, fraction 23).

L-chain species coprecipitated with BiP (Fig. 3*A Right*). A low physical stability of the BiP/κ complex may cause it to fall apart during the immunoisolation procedure and lead to the artifactual appearance of completely oxidized L chains.

We redetermined the *in vivo* amount of BiP-bound NS1 L chains by using a different experimental approach that preserves the association of weakly bound polypeptides: cellular lysates were prepared in the absence or presence of MgATP and the proteins were separated by gel filtration. In the absence of ATP, most of the L chains were recovered in high molecular weight fractions (Fig. 3*B*) previously shown to contain the BiP/L-chain complex (17). In contrast, when the lysate contained ATP, most of the L chains were recovered in a low molecular weight peak corresponding to the size of L-chain monomers (Fig. 3*B*). The majority of the L chains recovered from the higher molecular weight fractions were partially oxidized (Fig. 3*C*, fractions 14 and 15), whereas the completely oxidized forms were enriched in the low molecular weight fractions (Fig. 3*C*, fractions 24 and 25). These results confirmed that all partially reduced L chains were associated with BiP and that the fully oxidized species appeared only after *in vitro* dissociation from BiP.

Nonsecreted L Chains Do Not Leave the ER Prior to Degradation. Monensin efficiently blocks the export of secretory molecules by inhibiting ER-to-Golgi and intra-Golgi transport steps and, in addition, affects lysosomal degradation (26, 27). To analyze the effect of monensin on L-chain degradation and on the export machinery in the same cell, we generated a stable transfectant of the NS1 cell line (NS1/λ_1) that, in addition to synthesizing its endogenous nonsecreted κ L chains, coexpresses a secreted wild-type λ_1 L chain. Monensin completely blocked secretion of λ_1 L chains in NS1/λ_1 cells (Fig. 4 *A* and *B*). In contrast, κ L-chain degradation was only slightly affected (Fig. 4 *C* and *D*). These results support the idea that L chains are degraded in a compartment close to or identical with the site of L-chain synthesis.

Examination of the temperature requirements for degradation provided additional evidence supporting the idea that the degradation of L chains does not depend on intracellular transport. These experiments rely on the observation that vesicular transport between Golgi stacks is inhibited at 20°C and ER-to-Golgi export is completely blocked at temperatures of 15–16°C (27). The two cell lines NS1 and NSFS62, expressing the two different nonsecreted Ig L chains, κNS1 and λ_1FS62, were subjected to pulse–chase analysis at various temperatures. To confirm that decreasing the temperature blocked the vesicular transport, Ig-secreting cells (P3-X63Ag8) were subjected to the same protocol. As expected, the rate of Ig secretion was drastically lowered between 26°C and 20°C and completely blocked at 16°C (Fig. 5 *A* and *D*, respectively). We chose a chase period of 6 hr because we found that after this time most of the L chains were degraded when the cells of both NS1 and NSFS62 lines were kept at 37°C (Fig. 5 *B* and *C*, respectively). Thus, this time point was suitable to measure the effect of temperature-dependent inhibition on the degradation process.

As seen from the Arrhenius plot (Fig. 5*D*), both L chains were degraded with linearly diminishing rate constants, whereas the transport rate depended on the temperature. These results confirm that a vesicular transport step is not required for degradation of Ig L chains.

DISCUSSION

The results presented here provide evidence for the involvement of molecular chaperones in the process of degradation of incompletely folded protein structures in the ER. One of the aims of this work was to investigate whether the correlation between the kinetics of L-chain degradation and BiP/L-chain complex dissociation previously observed in the NS1 cell line (17) also occurs with other L chains. If nonsecreted L chains interact with BiP until they are degraded, all intracellular L-chain molecules should be found in association with BiP. This is indeed the case as shown by size fractionation of the total cellular proteins (Fig. 3): essentially all NS1 κ L chains were eluted with fractions corresponding to the size of BiP/

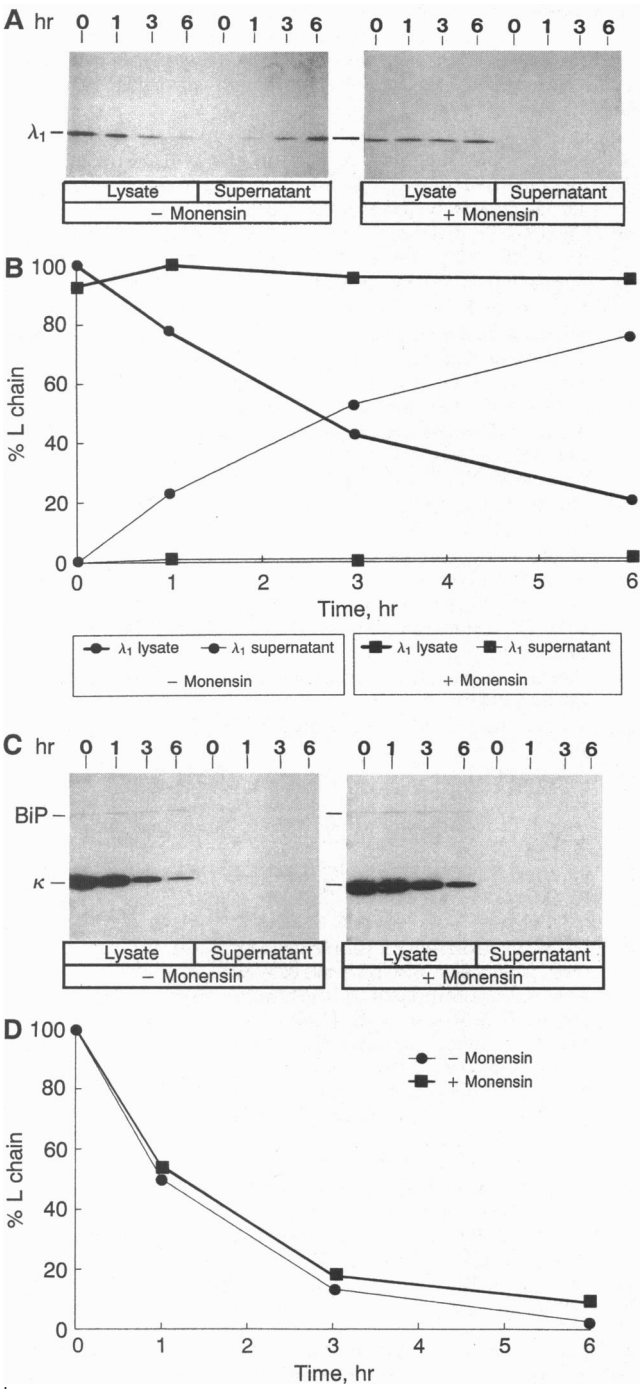

FIG. 4. Effect of monensin on the secretion of λ_1 L chains and on the degradation of κ L chains. NS1/λ_1 cells (producing nonsecreted κ and secreted λ_1 L chains) were pulse labeled for 10 min with [^{35}S]-methionine and chased for 0, 1, 3, and 6 hr, as indicated. L chains were immunoprecipitated with anti-λ (*A*) or anti-κ (*C*) antibodies from lysates or from corresponding cell culture supernatants. Where indicated, monensin was added 20 min prior to the pulse (7 μg/ml). The amount of λ_1 (*B*) or κ (*D*) L chains recovered at each time point of the chase is expressed as a percentage of the amount recovered directly after the pulse.

L-chain complexes that exhibited a molar stoichiometry. Based on these results, we estimate that approximately half of the cellular BiP pool in NS1 cells is involved in the interaction with L chains, which is in line with NS1 cells expressing approximately twice as much BiP as the parent cell line, which does not synthesize any Ig chains (15). Thus, the finding that only 10–15% of the NS1 κ L chains were coimmunoprecipitated

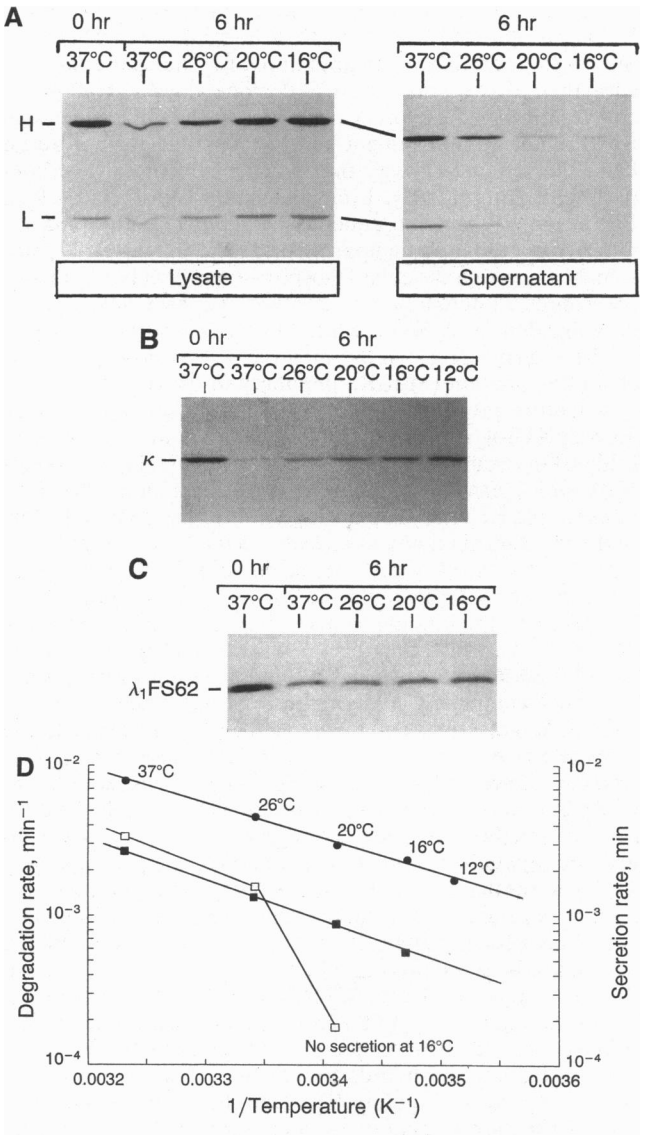

FIG. 5. Effect of temperature on the degradation of two different L chains. P3-X63Ag8 (producing secreted IgG_1), NS1 (producing nonsecreted κ), and NSFS62 (producing nonsecreted λ_1FS62) cells were pulse labeled for 10 min with [^{35}S]methionine and the chase was initiated by the addition of an excess of unlabeled methionine. All cells were cooled to 4°C and subsequently cultured at 37, 26, 20, 16, or 12°C, as indicated. Cells were lysed either directly after the pulse or after 6 hr of chase. (*A*) IgG_1 was immunoprecipitated from cellular lysates or cell culture supernatant of P3-X63Ag8. H, heavy chain. (*B* and *C*) κ λ_1FS62 L chains were immunoisolated from cellular lysates of NS1 or NSFS62 cells. (*D*) Radiolabeled proteins were analyzed by SDS/PAGE and the data of four independent experiments are summarized in an Arrhenius plot. The secretion rates (min^{-1}) of IgG (□ or the degradation rates (min^{-1}) of κ (●) and λ_1FS62 (■) were determined by comparing signals from microdensitometric scanning.

with BiP (17) is presumably due to a physical instability of this particular BiP/ligand complex. In contrast, the other nonsecreted L chains analyzed here (λ_1FS62; ref. 22) were quantitatively coimmunoprecipitated with BiP, indicating that different BiP/ligand complexes exhibit different physical stabilities. More importantly, the kinetics of the physiological BiP/L-chain complex dissociation and the degradation of the L chains were identical in the respective L-chain-expressing cells. These findings suggest a functional relationship between the processes of BiP dissociation and degradation of nonsecreted L chains. Whether or not the different half-lifes of the L chains directly reflect the different physical stabilities of the BiP/ligand complexes remains to be established.

The carboxyl-terminal tetrapeptide Lys-Asp-Glu-Leu allows BiP to be retrieved from a post-ER compartment. Therefore, the observation that the degradation of L chains is coincident with their dissociation from BiP alone does not allow the conclusion that these processes occur in the ER proper. The BiP/L-chain complex might be dissociated at a site different from the ER itself—e.g., the intermediate or salvage compartment. Alternatively, L chains could be dissociated from BiP in the ER and subsequently be rapidly transported to a putative degradation compartment. However, this would presumably involve a vesicular transport step which is excluded by the results of temperature-sensitivity experiments reported here. In addition, caffeine, which blocks transport from the ER to the intermediate compartment (28), had no effect on L-chain degradation (M.R.K., unpublished data).

A feature presumably common to all polypeptides undergoing pre-Golgi degradation is that they are not properly folded. One example is the asialoglycoprotein receptor subunit H2b, which exhibits an immature conformation (29). Both nonsecreted L chains are partially oxidized, suggesting that one of the two L-chain domains already assumes the typical Ig-fold structure stabilized by one internal disulfide bond, whereas the other portion of the molecule is still incompletely folded. Interestingly, BiP binding seems to prevent complete oxidation of the chains, which only occurs when the chaperone/ligand complex dissociates *in vitro*. BiP binding could serve to stabilize the incompletely folded state of a molecule to prevent aggregation reactions and to enable the proteolytic machinery to recognize these structures as degradation substrates *in vivo*. In favor of this model is the finding that wild-type λ_1 L chains, which interact only transiently with BiP (24), are fully oxidized *in vivo* and do not undergo degradation even if they are artificially retained inside the degradation compartment (Fig. 4). These results suggest that it is a conformational rather than a primary sequence information that plays the critical role in specifying ER-degradation targets. In fact, BiP may function as a mediator between protein folding and protein degradation in the ER. On the one hand, BiP binding to unfolded portions of a molecule may serve to stabilize the polypeptide chain in a folding- or assembly-competent conformation (C. R. Kaloff and I.G.H., unpublished data). On the other hand, BiP may assume a role in targeting ER degradation of unassembled subunits in case folding or assembly does not occur in time. However, it is not the BiP binding *per se* that determines the half-life of a protein, since the two different BiP ligands exhibit different half-lifes, even when they are expressed in the same cell (M.R.K., unpublished data). Rather, BiP could be required to render unfolded portions of a polypeptide chain accessible to the action of proteolytic enzymes. Nascent polypeptides could be protected from the immediate action of this proteolytic machinery by a transient interaction with additional molecular chaperones such as GRP94 or GRP170, which have been shown to coprecipitate with unassembled Ig chains in nonstoichiometric amounts (30–32).

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