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Building a multichain receptor: Synthesis, degradation, and assembly of the T-cell antigen receptor

(membrane proteins/intracellular sorting)

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ABSTRACT **The murine T-cell antigen receptor consists of at least seven chains and six different proteins. The two clonotypic chains α and β are glycoproteins of 40–45 kDa present as a disulfide-linked heterodimer. Four clonally invariant chains include δ (a 26-kDa glycoprotein), γ (a 21-kDa glycoprotein), ε (a 25-kDa protein), and ζ (a 16-kDa protein). ζ is found in the complex as a disulfide-linked homodimer. The clonotypic chains and the invariant chains form a noncovalent complex on the cell surface. We have developed antibodies against each of the chains and used them to examine the assembly of the mature complex in the murine antigen-specific T-cell hybridoma 2B4. Pulse–chase studies of metabolically labeled cells demonstrate that many of the chains are synthesized in great excess over the amount assembled into the mature complex. These excess chains, either as free components or as partially assembled complexes, are rapidly degraded. This degradation most likely takes place in the lysosomes. The complete complex is quite stable with a long half-life. A specific hierarchy of partially assembled complexes can be discerned.**

Numerous cell surface receptors are multisubunit complexes including the acetylcholine receptor, the IgE Fc receptor, and the T-cell antigen receptor. The complete and precise assembly of these components is a formidable problem, but one that has been solved by the cell to ensure proper function of these critical cellular elements. In studies of the acetylcholine receptor (1, 2), it was established that only about 30% of the newly synthesized α chain is assembled into mature receptor. The unassembled α chains are rapidly degraded (1, 2). A similar set of observations about the B-cell antigen receptor (surface IgM) has correlated assembly of multisubunit complexes with dramatic stabilization of the components of the complexes (3). Studies of the assembly of erythrocyte cytoskeletal proteins, α and β spectrin, revealed that unassembled chains were rapidly degraded, in contrast to the fully assembled complex (4). The selective stabilization imparted by complex assembly may be critical to the mechanism whereby the cell ensures the existence of only stoichiometrically assembled protein complexes.

Because selective catabolism may be fundamental to the selective survival of properly assembled complexes, the cell must possess mechanisms whereby unassembled, partially assembled, or incorrectly assembled complexes can be recognized. The following two major systems for intracellular proteolysis have been extensively studied: lysosomes and cytosolic ATP-dependent proteolytic pathways. Degradation by each of these is selective. Lysosomes are generally regarded as the major site of degradation of membrane proteins but they can also be responsible for the catabolism of cytosolic proteins and the content of other intracellular organelles (5). ATP-dependent, cytosolic, neutral protease systems have been implicated in the selective degradation of cytosolic proteins (6). Denatured proteins when microinjected into the cell (7) or cytosolic proteins after the biosynthetic incorporation of amino acid analogues (8) are rapidly degraded by these systems. The exact signals utilized to target proteins for cytosolic breakdown remain unknown. To use multichain protein complexes to investigate such targeting signals, it is crucial to determine the catabolic system(s) involved in the degradation of the unassembled components (9).

The T-cell antigen receptor provides an excellent model system for studying the relationship of subunit assembly, degradation, and expression on the plasma membrane. The antigen-recognition element of the T-cell antigen receptor exists in the plasma membrane as a multichain complex of at least seven chains (10–13). We have characterized (12) those chains in the murine T-cell receptor complex. In this study we employ antibodies directed against different chains of the complex to examine the assembly of the receptor. From our observations we formulate a hierarchy of associations and model the relationship between assembly and catabolism.

MATERIALS AND METHODS

Cells and Antibodies. The antigen-specific T-cell hybridoma 2B4 was maintained as described (14). The A2B4-2 monoclonal antibody binds the T-cell antigen receptor on the 2B4 cell (15). The R9 antiserum binds the δ chain (gp26) of the murine T-cell antigen receptor (16). The rabbit antiserum designated 124 specifically binds the ζ chain (p16) and was raised against purified ζ chain from 2B4 cells. Antiserum 127 was raised in rabbits against purified ε and is specific for this chain. All antisera are able to precipitate the intact complex.

Biosynthetic Labeling of the Cells. 2B4 cells were metabolically labeled with [^{35}S]methionine. For pulse–chase experiments cells were incubated for 1 hr at 37°C in RPMI 1640 supplemented with 20% (vol/vol) fetal calf serum without methionine. Thereafter, [^{35}S]methionine (specific activity, 1200 Ci/mmol; 1 Ci = 37 GBq) was added at 200–400 μCi/ml and incubated either for 10 or for 30 min. After the pulse period, the labeled cells were resuspended in chase medium that contained excess unlabeled methionine. At appropriate time intervals, the pulse was terminated by rapidly cooling the cells to 4°C. The cells were then treated with 10 mM iodoacetamide for 10 min at 4°C. Iodoacetamide was removed by washing the cells with ice-cold phosphate-buffered saline followed by lysis in a buffer containing 0.5% Triton X-100, 300 mM NaCl, 50 mM Tris·HCl (pH 7.6), and protease inhibitors as described (12).

Immunoprecipitation and Electrophoresis. Immunoprecipitations were performed with the indicated monoclonal antibodies or rabbit antisera adsorbed to protein A-Sepharose. Before immunoprecipitation, cell lysates were precleared twice, first with control serum adsorbed to protein A-Sepharose and then with protein A-Sepharose. Immunoprecipitates were eluted by boiling in NaDodSO$_4$ sample buffer

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with or without 3% (vol/vol) 2-mercaptoethanol. Details regarding one-dimensional NaDodSO$_4$/PAGE using the buffer system of Laemmli and two-dimensional diagonal gel electrophoresis have been described (12). Multiple exposures were used to insure linearity of the intensities over the wide range required. Band intensities were determined by scanning densitometry using a Hoeffer densitometer and computer analyzed using the Appligration II data analysis program (Dynamic Solutions, Pasadena, CA).

Percoll Density Fractionation of the Cells. For Percoll density gradients, the 2B4 cells were homogenized in 0.20 M sucrose/1 mM EDTA/0.01 M Tris·HCl, pH 7.6 (hypotonic homogenization buffer), by 30 up-and-down strokes in a tight Dounce homogenizer. After restoration of tonicity, the homogenates were centrifuged for 10 min. The resultant supernatants were subsequently centrifuged on a sucrose cushion at 3500 × g for 20 min. Then the final supernatant was layered onto 20% (vol/vol) Percoll and centrifuged at 8000 × g in a Beckman 70.1Ti rotor to a final $\omega^2 t$ value of 4.55×10^9 rad^2/sec. The resultant gradients were fractionated, and immunoprecipitation was carried out. The lysosomal marker enzyme hexosaminidase was assayed according to Hall *et al.* (17) in aliquots of gradient fractions in the presence of 0.5% Triton X-100.

RESULTS

The T-Cell Antigen Receptor Is a Seven-Chain Complex of Distinct Stoichiometry. The T-cell antigen receptor on human and murine T cells is a multicomponent structure. The clonotypic receptor molecules consist of disulfide-linked α and β chains, each a 40- to 45-kDa glycoprotein. The α and β structures on human T cells have been shown to be noncovalently associated with three integral membrane proteins termed the T3 complex (10, 18). Analogous structures are also associated with the heterodimeric α-β chains in murine T-cell antigen receptors (12, 13, 19, 20). At least four different polypeptides can be coimmunoprecipitated with a monoclonal antibody that recognizes the α chain of the antigen receptor present on a pigeon cytochrome c-specific T-cell hybridoma (2B4) (12). These molecules include two endoglycosaminidase-F-sensitive glycoproteins of 26 kDa (δ chain) and 21 kDa (γ chain), and two endoglycosaminidase-F-insensitive proteins of 25 kDa (ε chain) and 16 kDa (ζ chain). The 16-kDa ζ chains exist in the complex as a homodimer. We have shown (31) that a chain, termed p21, also associates with this receptor complex. All of these components can be identified in receptors of normal murine peripheral T cells and T-cell clones.

To elucidate the stoichiometric relationship between the chains on the surface of 2B4 cells, cross-immunodepletion experiments using anti-α and anti-δ antibodies were carried out (16). These results show that all α-β chains on the cell surface are associated with δ chains and vice versa. Thus the T cell effectively assembles complete complexes for expression in the plasma membrane. Finally, steady-state metabolic labeling of cells with [^{3}H]leucine followed by immunoprecipitation of the complex was consistent with a stoichiometry of $\alpha\beta\delta\gamma\varepsilon\zeta_2$ as determined by densitometry and correction for the core peptide molecular weights (data not shown).

There Is a Hierarchy of Assembly and Catabolism of Receptor Subunits. 2B4 cells were labeled with [^{35}S]methionine for 30 min in methionine-free medium. The cells were then washed and reincubated for various times in complete medium before the receptor complex was isolated with either an anti-α-β (A2B4-2) or anti-δ (R9) antibody. Analysis on NaDodSO$_4$/PAGE (Fig. 1) reveals the following two striking facts: (*i*) Most of the pulse-labeled components directly recognized by the antibodies is rapidly degraded. (*ii*) At the end of the pulse period the target antigens of these two

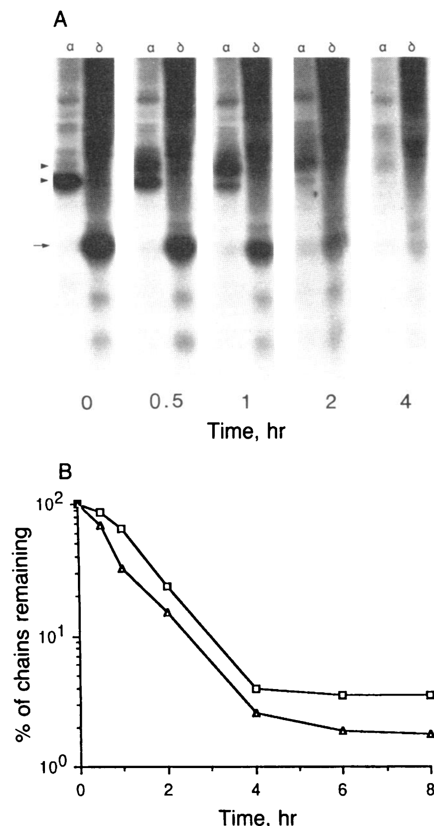

FIG. 1. (*A*) NaDodSO$_4$/PAGE analysis of immunoprecipitates. 2B4 cells were pulse labeled for 30 min with [^{35}S]methionine, and cell lysates were immunoprecipitated with either A2B4-2 (lanes α) or R9 (lanes δ). The times shown are the hour of chase in unlabeled methionine-containing medium. Incompletely and completely processed α-β chains (arrowheads) and δ chains (arrow) are indicated. (*B*) Survival of δ (△) and α-β (□) pulse-labeled chains. The experiment was performed as in *A*.

antibodies are detected in great excess over the other chains. This is in contrast to the pattern observed in the mature receptor complex as assessed by either extrinsic labeling or by steady-state metabolic labeling (data not shown). Quantitation of the amount of α-β or δ recognized by the anti-α or anti-δ reagents demonstrates that each decays linearly (i.e., as a single exponential function) over 4 hr with a clear break at that time. Approximately 5–10% of the α-β complex present at the end of the pulse survives with a long half-life (15–20 hr) and 3–5% of the δ chain similarly has a long survival. Thus 90–95% of α-β and 95–97% of δ are rapidly destroyed with half-lives of between 30 and 50 min (Fig. 1*B*).

The fates of individual chains after they assemble with either α-β or δ were assessed by examining the coprecipitated subunits with either the A2B4-2 or R9 antibodies (Fig. 2). The fate of coprecipitated chains contrasts markedly with the fate of the target antigens. When the amount of δ, ε, or ζ coprecipitated by A2B4-2 is quantitated, there is a small increase over the first 1–2 hr of chase. The most striking aspect of the chase is that there is no significant degradation of the coimmunoprecipitated chains. Likewise the amount of α-β chain precipitated by the anti-δ antibody remains constant through the chase period. The α and β chains are quantitated by two-dimensional diagonal gels due to the nonspecific precipitation seen in this region of the one-dimensional gel using the R9 serum. Thus no α-β chain that is assembled with δ at the end of the pulse is degraded over the time course of this chase period. In contrast to α-β, only 15–25% of the γ chain initially coprecipitated with δ remains as a long-lived species, whereas 30–40% of the initially

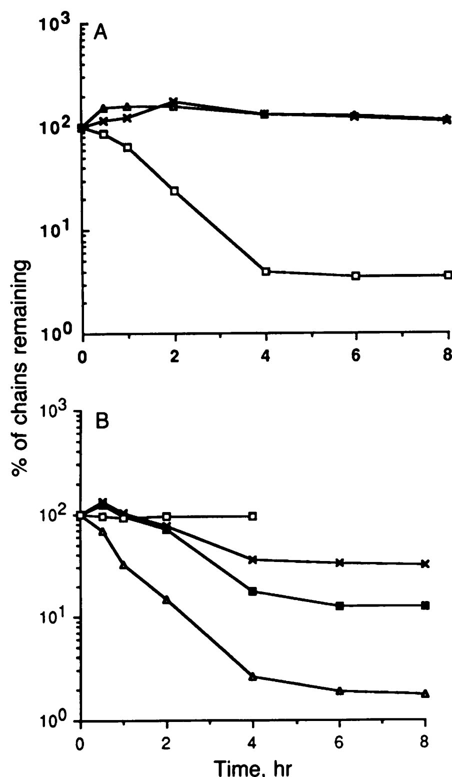

FIG. 2. Survival of antigen receptor chains. 2B4 cells were labeled for 30 min with [^{35}S]methionine and chased in unlabeled medium for the times shown. (A) Anti-α A2B4-2 was used. α-β (□), δ (△), and ζ (×) were quantitated as described in Fig. 1B. (B) Anti-δ R9 antiserum was used. δ (△), α-β (□), γ (■), and ζ (×) were quantitated.

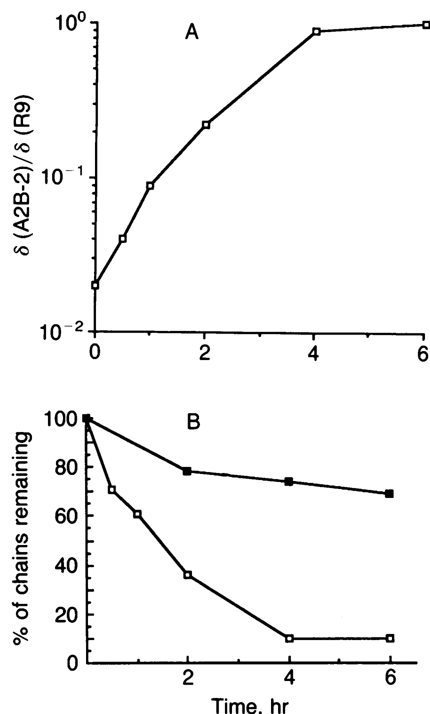

FIG. 3. (A) Verification of the coimmunoprecipitation. 2B4 cells were labeled for 30 min with [^{35}S]methionine and, at the times of chase shown, subjected to immunoprecipitation with either A2B4-2 (anti-α) or R9 (anti-δ). The data shown are the ratio of the amount δ chain coimmunoprecipitated with anti-α over the amount of δ chain directly precipitated with anti-δ. (B) Degradation of ε and ζ chains. 2B4 cells were pulse labeled for 30 min and chased for the times shown. Lysates were subjected to immunoprecipitation with either an anti-ζ (■) or an anti-ε (□) rabbit antiserum. The ζ represents the average of two experiments using two anti-ζ sera each demonstrating different epitope specificities.

coprecipitated ζ chain survives. Using one-dimensional reducing gels the resolution of δ and ε chains is difficult and varies from gel to gel. In those experiments where ε was distinguished from δ, the fractional degradation of the ε chain, assessed with an anti-δ antibody was about 90%. This number is confirmed directly with anti-ε antibodies (see below).

We can calculate the percentage of δ linked to each of the other chains by dividing the fraction of δ that survives long term by the long-term fractional survival of each of the coprecipitated chains. Such a calculation predicts that about 50% of δ is complexed with ε, that 15–25% of δ is complexed with γ, and that 10–20% of δ is complexed with ζ. Using antibodies alone, we cannot rule out the existence, soon after biosynthesis, of δ–ε–ζ (without γ) complexes that do not survive.

The lack of any degradation of α-β when precipitation is performed with anti-δ predicts that the percentage of δ that survives long term is exactly equal to the percentage of δ that assembles with the α-β heterodimer. We checked whether the dramatic excesses in the biosynthesis of α-β and δ chains observed in these pulse–chase metabolic labeling experiments were reconcilable with the stoichiometry determined in the plasma membrane. To accomplish this, we examined the absolute amount of δ chains detectable during the pulse–chase points when saturating amounts of either A2B4-2 or R9 reagents were used. The results (Fig. 3A) demonstrate that the molar ratio of δ chain precipitated by the two antibodies approaches a value of 1 by 4 hr of chase. The molar ratio at the end of the pulse is $\approx$50:1 (R9:A2B4-2). The absolute amount of δ chain coprecipitated by A2B4-2 remains constant (Fig. 2). These results are consistent with the survival, in this experiment, of only 2–3% of the synthesized δ chain and the rapid assembly with α-β heterodimers leading to the stabilization of that 2–3% by the end of the chase. This experiment demonstrates that no artifact due to the exchange of unlabeled for labeled chains is occurring.

Are All Chains Made in Excess of the Amount Assembled? The much greater fractional degradation of δ chains than of coassembled γ or ζ chains indicates that a large amount of δ chain does not assemble with either of the two other subunits. To assess what percentage of γ, ζ, and ε chains assemble with δ, we employed polyclonal antibodies raised in our lab against each of these subunits. Cells were pulse labeled, and lysates were depleted of all δ chain by sequential immunoprecipitation with the R9 antiserum. The remaining lysate was then assayed for ε, γ, and ζ chains by immunoprecipitation with appropriate antibodies. The results show that $\approx$100% of the pulse-labeled ε chains were associated with δ by the end of the chase, that at least 80% of γ, and that at least 70% of ζ are also assembled with δ. Fractional degradation of coprecipitated γ and ζ chains allowed us to calculate the percentage of δ chains associated with γ and ζ (see above). One of our anti-ε antibodies leads to dissociation of ε from the other chains of the complex when metabolically labeled complexes are solubilized in detergent. Thus, precipitating ε with this reagent obviates the problem of being able to distinguish ε from δ on one-dimensional NaDodSO$_4$/PAGE. These results, shown in Fig. 3B, demonstrate that about 90% of ε is rapidly degraded. This fractional degradation and the observations that 100% of ε is assembled with δ and that about 95% of δ is destroyed allow us to predict that $\approx$50% of δ is complexed with ε at the start of the chase period. Also shown in Fig. 3B is the degradation of newly synthesized ζ chains when ζ chains are directly immunoprecipitated with an anti-ζ serum. In this case only 30–35% of ζ is degraded after

a 6-hr chase period. This is somewhat less than that seen in the coimmunoprecipitation experiments in which 40–60% of ζ was degraded after a 6-hr chase when assayed with the anti-δ antibody. However, the conclusion that little ζ is degraded is clear. Thus the predictions of the coprecipitation experiment when coupled with the information derived from the anti-δ immunodepletion are corroborated.

Degradation of the Unassembled Components. We next asked whether we could determine the site of degradation of the unassembled and incompletely assembled chains. When short pulses of methionine are used (15 min or less), there is a clear lag before any degradation is seen (see Fig. 4*A*). These results, however, suggested that the proteins were somehow processed in the cell before being degraded. Numerous pharmacologic inhibitors have been used to mark various cellular proteolytic pathways. Perhaps most diagnostic are the amines such as NH_4Cl that inhibit lysosomal proteolysis by raising the acidic pH of these organelles but should have no effect on any of the other described cellular proteolytic systems. When cells were treated with NH_4Cl, there was a dramatic inhibition of degradation. The inhibition was somewhat more striking for α-β than for δ chains (Fig. 4 *A* and *B*) but both are markedly protected from destruction by this amine. NH_4Cl did not alter the efficiency of assembly or the rate of carbohydrate processing. To more directly test whether the excess α-β chains are actually transported to lysosomes, we attempted to examine the transport of the heterodimer to these organelles in the presence of NH_4Cl (to block rapid degradation). This proved to be difficult in that the 2B4 hybridoma cells were quite resistant to breakage using a wide variety of techniques, under conditions that allowed the recovery of intact lysosomes. However, when the recovery of intact lysosomes (less than 10%) was accurately assessed to correct for the amount of unassembled chains transported to the high-density lysosomal fraction on Percol density gradients (21), we found that in different experiments after a 2-hr chase in the presence of NH_4Cl >60–100% of the unassembled α-β chains were in lysosomes (compared to 2–8% at time 0 of chase and 30–40% after 1 hr of chase). No associated δ chains were observed in the lysosomal fraction using the anti-α monoclonal consistent with the transport of unassembled chains to this organelle for degradation.

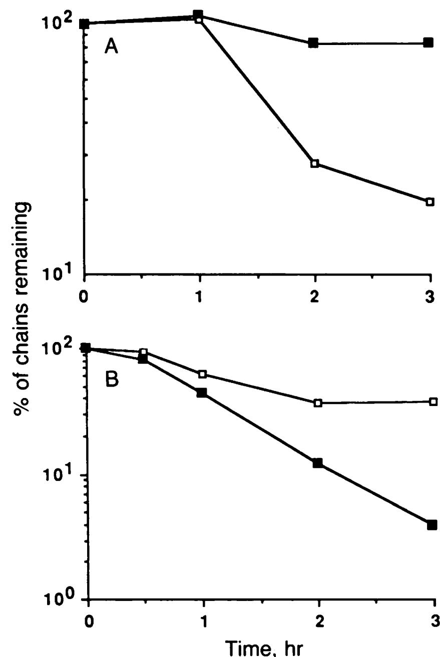

FIG. 4. Degradation of excess chains. 2B4 cells were pretreated for 30 min either with (□) or without (■) 10 mM NH_4Cl before being labeled for 15 min with [^{35}S]methionine. The NH_4Cl was maintained during the chase, and the amount of α-β (*A*) or δ (*B*) was quantitated using A2B4-2 or R9, respectively. The NH_4Cl had no effect on the level of protein synthesis or viability of the cells. The processing of α-β and level of coassembly was also unchanged by the NH_4Cl treatment.

DISCUSSION

That the T-cell antigen receptor is a multichain complex is now well established in both murine and human cells. Data derived from pulse–chase metabolic labeling experiments have allowed us to model a hierarchy of assembly of partially and completely assembled complexes. A number of the chains appear to be synthesized in great excess over the amount that will be incorporated into the final complex. This excess is rapidly destroyed by the cell. Two classes of components exist; those that are rapidly degraded and those that are long lived. It is clear that assembly is a prerequisite for long-term survival. The approximate fractions of each subunit in the rapidly degraded pool are 0.95 for δ, 0.9 for ε, 0.8 for γ, 0.6 for ζ, and 0.9 for α-β. The hierarchy of these values was reproducible from experiment to experiment. The fractional degradation of the isolated and partially assembled chains supports a hierarchical model of assembly. The validity of interpreting the coprecipitation experiments was corroborated in several ways. First was the observation of the ratio of directly to indirectly precipitated chains as shown in Fig. 3. That the ratio approached unity at long times of chase assured us that there was no exchange or assembly of labeled with unlabeled subunits. In addition this confirmed that the assembled subunits entirely accounted for the long-term survivors seen with direct precipitation studies. Second, the immunodepletion studies with anti-δ predicted that the fractional degradation of coassembled subunits should be reproduced by direct precipitation of those subunits. This was indeed confirmed by the studies performed with anti-ε and anti-ζ antisera.

The lack of coassembly of labeled with unlabeled chains suggests that the complex is formed from a biosynthetic cohort of subunits. This is consistent with the observation that assembly takes place relatively rapidly after biosynthesis (data not shown). The lack of exchange supports the stability of the complex once formed in that subunits do not exchange between assembled receptor complexes. The hierarchical assembly of the newly synthesized cohort of subunits is shown schematically in Fig. 5. The numbers over each arrow represent approximate percentage distributions, derived from several experiments, of each subunit and of each partial complex. Our data do not allow us to determine with certainty whether γ exists in excess of ζ. The question of where and how the excess chains are degraded has been addressed. We favor the lysosome as the most likely site for the reasons outlined above.

The assembly model proposed here has a variety of implications for the T-cell antigen receptor. Elegant studies by Weiss and colleagues (22) have demonstrated that the expression of the clonotypic receptor subunits are required for the surface expression of the invariant subunits. This is explained by our work that demonstrates that assembly with α-β is required for survival of the complex. Studies using MOLT-4 T-cell leukemia cells reveal the presence of intracellular δ, γ, and ε chains but no cell surface expression (23, 24). These cells have β-chain transcripts but lack mRNA for the α chain (25). The reports of the existence of surface δ chain in the absence of α-β heterodimer in a subset of human T cells are of particular interest (26). In these cases another chain encoded by a rearranged T-cell-specific gene replaces the α-β heterodimer and presumably provides the assembly

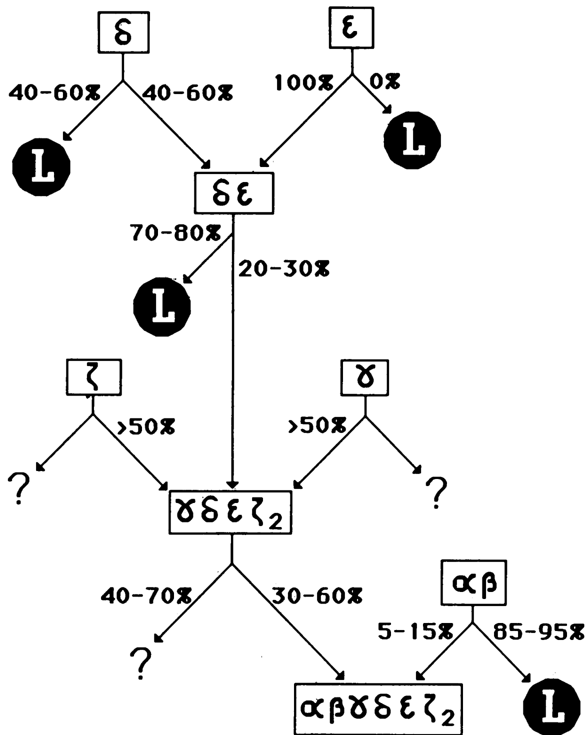

FIG. 5. Schematic diagram of the hierarchy of T-cell receptor assembly. Flow sheet for the hierarchy of subunit assembly. Each subunit can either assemble or be degraded (L), and each partially assembled complex can do likewise. The percentages give the probabilities of degradation and assembly for each stage of assembly. The range of numbers reflects data derived from multiple experiments.

signal necessary for survival and expression on the plasma membrane.

The data presented here are consistent with the ζ chain being the limiting chain for the assembly of the full T-cell receptor complex. Since <100% of the ζ chains survives long term, the presence of this chain does not absolutely guarantee survival. Whether a small amount of ζ fails to assemble or whether some percentage of ζ forms incomplete or incorrect complexes cannot be determined in these studies. Nevertheless, the huge excess of α, β, δ, and ε strongly suggests that the rate of synthesis of these chains does not govern the amount of mature receptor. It remains to be tested whether assembly, and thus the number of mature receptors, can be increased by increasing the rate of synthesis of the ζ chain.

The relationship between assembly of multichain complexes and intracellular transport may prove to be a general one. Surface expression of the acetylcholine receptor (1), surface immunoglobulin (3), class I (27) and class II (28) major histocompatibility complex molecules, and viral glycoproteins (29) all involve multichain assembly. In some situations, like the transport of influenza hemagglutinin, assembly is required for transport out of the endoplasmic reticulum (30). This is in contrast to the T-cell receptor where correct assembly determines not whether transport will occur but to where. The signals inherent in multisubunit membrane protein transport may provide valuable insights into the mechanisms of intracellular sorting.

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