Brief Definitive Report

Lyt-2 GLYCOPROTEIN IS SYNTHESIZED AS A SINGLE MOLECULAR SPECIES*

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The Lyt-2/Lyt-3 (Lyt-2/3) glycoprotein complex is only expressed on the surfaces of particular subpopulations of thymus-derived (T) lymphocytes in the mouse. Among the cells that express Lyt-2/3 are the vast majority of cytotoxic and suppressor T cells, but relatively few of the helpers (1, 2). Recently, functional studies with monoclonal antibodies against Lyt-2 have suggested that the Lyt-2/3 complex is associated with the antigen-binding structures on T cells (3–5). Therefore, we have asked whether the Lyt-2 polypeptides made in nonclonal cell populations are heterogeneous, as expected for diverse antigen receptors, or uniform. We report here that the Lyt-2 glycoproteins are subject to rapid post-translational processing, but that the newly synthesized molecules are unique.

Materials and Methods

Animals and Cells. Inbred C57BL/6(B6), C57BL/6-Lyt-2.1 (B6-Lyt-2.1), and C57BL/6-Lyt-2.1 Lyt-3.1 (B6-Lyt2.1/3.1) mice (6) were bred in our colony at the Salk Institute. Thymocyte suspensions were prepared as described previously (7–9) from animals 3–6 wk old.

Metabolic Labeling and Immune Precipitation. Thymocytes were incubated with [^{35}S]methionine at 37°C as described previously (7, 8). In most experiments, Lyt-2/3 complexes were immunoprecipitated from detergent lysates of the labeled cells using the monoclonal antibody 19/178 (3). Alternatively, the rat monoclonal antibody 53.6.72 (10) was used with a goat anti-rat Ig second-stage reagent. Conditions for preparation of lysates and immune precipitates, electrophoresis in sodium dodecyl sulfate (SDS) -containing gels, and fluorography were as described previously (7–9, 11). In all cases, samples were analyzed under reducing conditions.

Endoglycosidase H Digestion. After immunoprecipitation, samples were eluted from the *Staphylococcus aureus* immunoadsorbent (Pansorbin; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) and incubated with endo-β-*N*-acetylglucosaminidase H (endo H) overnight (7, 8), using 3–10 μg/ml of endo H for complete digestion. Dr. P. W. Robbins (Massachusetts Institute of Technology) donated the endo H.

Two-dimensional Nonequilibrium pH Gradient Electrophoresis (NEPHGE) and SDS Gel Electrophoresis. First-dimension NEPHGE electrophoresis (12) was for 1,800 V-h in 4% polyacrylamide tube gels with 1.6% pH 5–7 ampholines and 0.4% pH 3.5–10 ampholines (LKB Instruments, Inc., Rockville, MD). Second-dimension SDS gel electrophoresis was carried out in 10.5% polyacrylamide gels.

Results and Discussion

Sizes of Newly Synthesized Lyt-2/3 Glycoproteins. Antibodies against either Lyt-2 or Lyt-3 precipitate similar disulfide-linked complexes of glycoproteins (13–17). These

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can be dissociated into three types of components with apparent molecular weights of ≤30,000, 30,000–34,000, and 35,000–38,000 (15–17). This heterogeneity may result from differences in carbohydrate structure, and so we have attempted to define the forms that the Lyt-2/3 glycoproteins display before extensive carbohydrate processing has taken place.

To label newly synthesized proteins selectively, thymocytes were incubated with [^{35}S]methionine for 10 min. The Lyt-2/3 molecules labeled in these cells migrated in SDS-polyacrylamide gels as two sharply-defined species with apparent molecular weights of 31,000 (31 K) and 35,000 (35 K). To study the peptide moieties of these molecules, we digested pulse-labeled Lyt-2/3 immunoprecipitates with the glycosidase endo H. This enzyme selectively removes newly added carbohydrate from asparagine linkage sites, leaving only one sugar residue attached to the polypeptide (18, 19). Complete endo H digestion resulted in the replacement of the 31 K and 35 K components by molecules with apparent molecular weights of 20,000 (20 K) and 24,000–25,000 (24 K) (Fig. 1 a, lane 3), which represent the maximum sizes for their polypeptide backbones. The specificity of the enzyme treatment was confirmed by its failure to alter the mobility of immunoglobulin κ chains (cf. lanes 4 and 5), which are not glycosylated (20).

High-Mannose Carbohydrate Chains in the Lyt-2/3 Complex. To determine the number of carbohydrate chains associated with Lyt-2/3, we carried out partial digestions with limiting concentrations of endo H. The sizes of the resulting products are summarized in Table I. In this experiment, the Lyt-2/3 species showed slightly higher apparent sizes, relative to the markers, than they did in most cases, and the native 31 K and 35 K species migrated as molecules of 32,000 and 36,000 daltons. With limited exposure to the enzyme, discrete digestion intermediates were generated with apparent masses of 28,000–29,000 daltons and 24,000 daltons. These disappeared when digestion was complete, leaving major end products of ≃24 and 20 kilodaltons (Kd). This pattern indicated that a total of six carbohydrate chains could be removed from the two major glycoproteins, with a shift of 3,500–4,000 in apparent molecular weight as each one was cleaved.

Because the two major glycoproteins also differ in size by 4 Kd, it was difficult to separate their respective digestion intermediates. In several experiments, however, the relative labeling intensities suggested that the 24 K endo H product originated from the 35 K component, while the 20 K product was derived from the 31 K component (data not shown). This suggests that each component carried three carbohydrate chains.

Rapid Post-Translational Processing. To compare the newly made glycoproteins with components of cell-surface Lyt-2/3 complexes, we traced the fate of the pulse-labeled molecules during a nonradioactive chase. After a 10-min labeling period, thymocytes were cultured in excess cold methionine for intervals from 20 min to 4 h before they were lysed. As shown in Fig. 1 b, the molecules recognized by anti-Lyt-2 changed rapidly in the course of the chase.

Within the first 20 min, a new, heavily labeled component appeared in the size range of 38–40 Kd. This migrated as a heterogeneous mixture of species (Fig. 1 b, lane 2) and probably represents the 38 Kd cell-surface form of Lyt-2 reported by others (14–17). Once generated, this component was quantitatively stable throughout 4 h of chase (Fig. 1 b, lanes 2–5). In contrast, the pulse-labeled Lyt-2/3 species were all unstable. The intensity of the 35 K band dropped abruptly in the first 20 min and

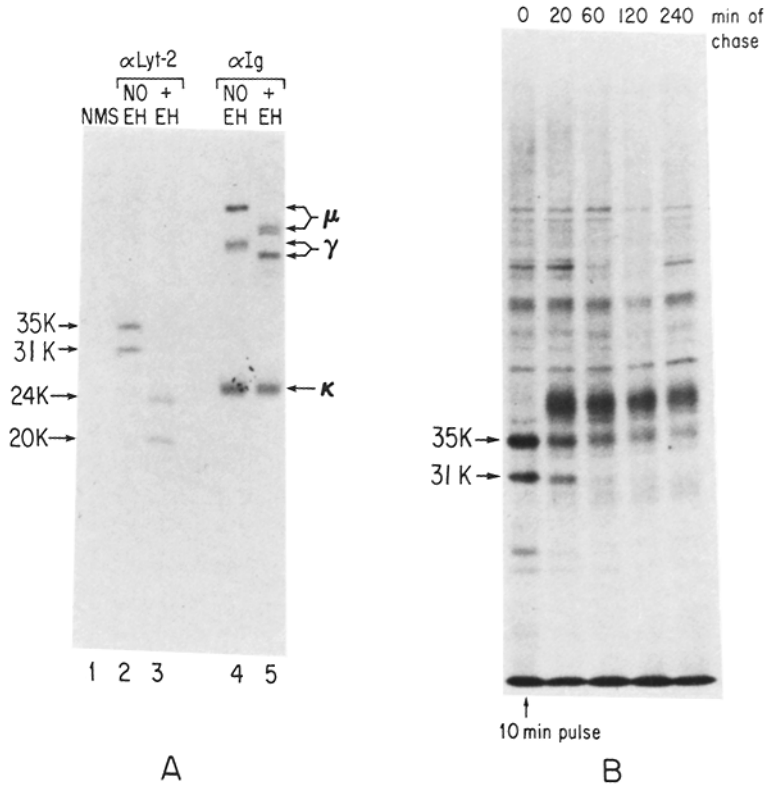

FIG. 1. Structural analysis and post-translational processing of biosynthetically labeled Lyt-2/3 complexes. (a) Newly synthesized Lyt-2/3 glycoproteins before and after glycosidase treatment. Lyt-2/3 complexes synthesized by pulse-labeled B6 thymocytes (lanes 2, 3). Immunoglobulin molecules made by pulse-labeled splenocytes were precipitated with a rabbit anti-mouse Ig serum (lanes 4, 5). The samples were mock digested (lanes 2, 4) or digested to completion with endo H (lanes 3, 5). Lane 1 is a normal mouse serum precipitate from the thymocyte lysate. Inputs of radioactive protein before precipitation were 10^6 cpm for lanes 1–3 and 5×10^5 cpm for lanes 4 and 5. Samples were fractionated in a 12.5% polyacrylamide gel. (b) Kinetics of processing of Lyt-2/3 complexes. Thymocytes pulse labeled as in panel (a) were incubated in nonradioactive medium (chase) for the lengths of time indicated, before lysis. Lyt-2/3 complexes were then precipitated from samples containing 9×10^6 cell equivalents of lysate. The inputs of radioactive protein decreased from 1.3×10^6 cpm in the pulse-labeled sample to 7.4×10^5 cpm at 240 min of chase. Samples were analysed by electrophoresis in a 10.5% polyacrylamide gel.

more slowly thereafter. The 31 K species lost radioactivity throughout the chase, perhaps giving rise to a series of discrete degradation products that appeared in the range of 28–30 kd.

The dramatic appearance of the 38 kd form could indicate that a new glycoprotein, itself unable to react with anti-Lyt-2 antibodies, became precipitable when it was added to the complex 20 min after it was made. However, the amount of radioactivity specifically precipitated did not increase during the first 20 min of chase, as determined by densitometric scanning of the 31–38-kd regions of the original autoradiogram (data not shown). Therefore, it seems more likely that all components of the complex were assembled within the first 10 min of synthesis, and that the 38-kd molecules were generated from the 35 K or 31 K forms by further elaboration of their

carbohydrate structures. This processing is notably more rapid than that observed for the other thymocyte glycoproteins TL (7) and Qa-1 (8).

The xenogeneic monoclonal antibody, 53.6.72 (10), which recognizes a different structure on the Lyt-2 antigen, precipitated molecular species that were identical in size and relative intensity to those observed using the allele-specific monoclonal

TABLE I

*Products of Partial Endo H Digestion of the Lyt-2/3 Complex**

	Endo H dose (μg/ml)				
	0.0	0.1	0.3	1.0	10.0
			× 10^{-3}		
Apparent molecular weights§	36	(36)‡			
	32	32	(32)	(32)	
		28–29	29		
			25	25	25
		24	24		
			20	20	20

* Lyt-2/3 complexes were immune-precipitated from a lysate of pulse-labeled B6 thymocytes.

‡ Faint bands are denoted by parentheses.

§ Apparent molecular weights were calibrated by the migration of ^{14}C-methyl labeled standards (New England Nuclear, Boston, MA) on the same 12.5% polyacrylamide gel: bovine serum albumin (69,000 mol wt), ovalbumin (46,000 mol wt), carbonic anhydrase (30,000 mol wt), and lactoglobulin A (18,300 mol wt).

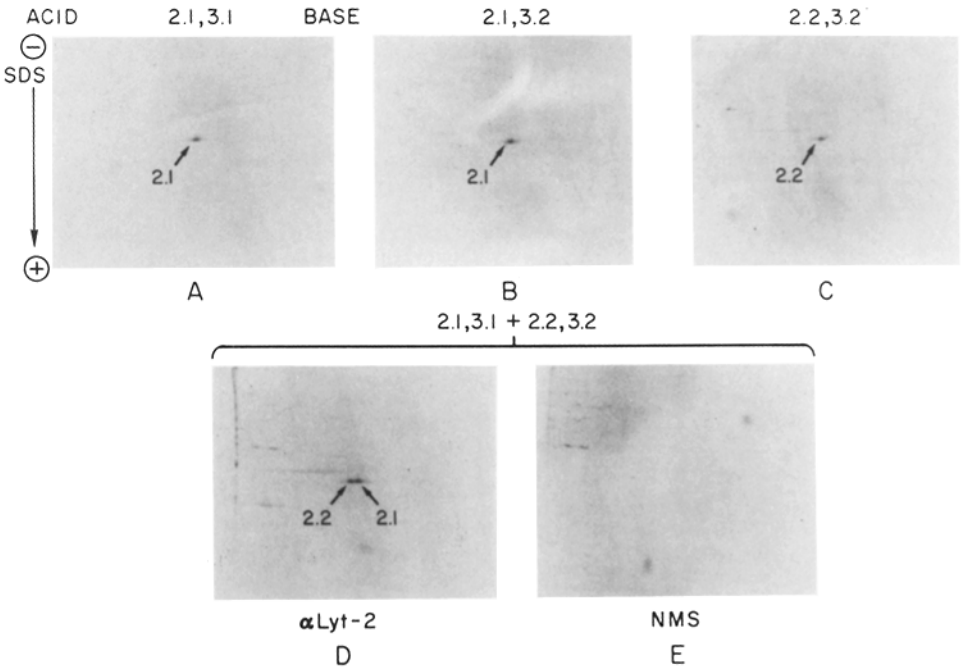

FIG. 2. Two-dimensional analysis of pulse-labeled Lyt-2. Lyt-2/3 complexes were precipitated from detergent lysates of thymocytes from B6-Lyt2.1/3.1 (A), B6-Lyt2.1 (B), and B6 (C) mice. In D and E, equal amounts of the lysates used in A and C were mixed before precipitation with anti-Lyt-2 (D) or normal mouse serum (E). Precipitates were eluted from the *S. aureus* immunoadsorbent and analyzed as described in Materials and Methods. The fluorographs are oriented so that the top of the NEPHGE gel (acidic end) is on the left. 2.1, presumptive *Lyt-2.1* gene product. 2.2, presumptive *Lyt-2.2* gene product. Both co-migrate with the 35 K band shown in Fig. 1.

antibody, 19/178 (data not shown). This makes it unlikely that conformational changes during processing either obscure or create the determinant recognized by the 19/178 antibody.

A Single Molecular Species Controlled by the Lyt-2 Locus. To study the product of *Lyt-2* gene selectively, we made use of the B6 mouse strains congenic at the *Lyt-2* locus (6). The molecule specified by *Lyt-2* was identified by comparison of the pulse-labeled products of B6 (Lyt 2.2; Lyt 3.2), B6-Lyt 2.1 (Lyt 3.2), and B6-Lyt 2.1:3.1 thymocytes, using the xenogeneic monoclonal antibody, 53.6.72, to precipitate Lyt-2/3 complexes. The samples were fractionated in the first dimension by NEPHGE and then by electrophoresis in SDS-polyacrylamide gels (12) (Fig. 2). In each case, the 35 K Lyt-2/3 component appeared as a single prominent spot. It was symmetrical in form and showed no evidence of heterogeneity. There was no size difference between the 35 K molecules made by thymocytes of *Lyt 2.1* and *Lyt 2.2* genotypes, but a charge difference was apparent. The 35 K spot from thymocytes of B6-Lyt 2.1:3.1 or B6-Lyt 2.1:3.2 mice (Fig. 2, panels A, B) was distinctly more basic than the 35 K spot from B6 (Lyt 2.2/3.2) thymocytes (Fig. 2, panel C). The separation was clear when the samples from panels A and C were mixed before immune precipitation and gel analysis (Fig. 2, panel D). Thus, the isoelectric point of the 35 K Lyt-2/3 component was determined by the allele at the *Lyt-2* locus.

We conclude that each *Lyt-2* allele encodes a polypeptide that is homogeneous but subject to rapid post-translational processing. Both the 35- and 38-Kd cell-surface molecules appear to be glycosylated derivatives of this initial product. Although separate T cell clones may express the 38- and 35-Kd forms of Lyt-2 in different ratios (17), this probably reflects differences in the extent of carbohydrate processing rather than the activation of different Lyt-2 structural genes.

Summary

We investigated the possibility that the Lyt-2 molecules made by uncloned mouse T lymphocytes would show variable primary structures like those of immunoglobulins. Newly synthesized Lyt-2/3 complexes were found to include only two major components, both discrete glycoproteins with apparent molecular weights of 31,000 (31 K) and 35,000 (35 K). When products of *Lyt-2.1* and *Lyt-2.2* thymocytes were compared by two-dimensional nonequilibrium pH gradient electrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis, the isoelectric points of the 35 K molecules were different; thus, the 35 K component was likely to be encoded by the *Lyt-2* locus itself. However, the 35 K molecules made by any one genotype were homogeneous in charge as well as in size. The homogeneity was obscured rapidly by post-translational modification. Most strikingly, within 30 min of initial synthesis, these processing events generated the conspicuous array of microheterogeneous products that form the "38 K" component of cell-surface Lyt-2/3.

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