We characterized the membrane-associated form of lymphotoxin (surface LT) on the activated II-23.D7 T cell hybridoma. Antibodies to rLT precipitated both surface LT and a distinct 33-kDa glycoprotein (p33). Because p33 and surface LT were antigenically unrelated, their coprecipitation suggested a physical association of p33 and surface LT on the membrane. Pulse-chase analysis indicated that LT and p33 associate with each other early in the LT biosynthetic pathway, precluding the possibility that LT is secreted and bound to p33 or a surface receptor. Furthermore, no p33 was associated with the secreted form of LT. Isoelectric focusing of surface LT and p33 under nondenaturing and denaturing conditions confirmed that surface LT and p33 existed as a complex. Treatment of cells with a high concentration of salt or with acid indicated that surface LT is a peripheral membrane protein. Although secreted LT is a homologous trimer, protein cross-linking studies revealed that surface LT existed as a monomer associated with a dimer of p33. Together the results demonstrate a novel mechanism for stable membrane expression of LT by activated T cells.

Several cytokines have been identified which exist in both membrane-bound and soluble forms. For example, precursors to epidermal growth factor, transforming growth factor-α, colony-stimulating factor-1, and mast cell growth factor/kit-ligand are synthesized with an internal hydrophobic domain that anchors it to the membrane (1–4). The soluble cytokine is then generated by proteolytic processing at the cell surface. Tumor necrosis factor (TNF-α/cachectin) also exists in membrane-bound and secreted forms; however the “leader” sequence serves as the membrane anchoring domain. Interleukin-1 (α and β) are found in membrane-associated forms by an uncharacterized mechanism (6, 7). The biological activities of membrane-bound and secreted forms of cytokines suggest that they can act in a localized or systemic manner (8–11).

Lymphotoxin (LT, TNF-β) is produced by activated T cells and possesses similar, although not identical, inflammatory and immunomodulatory functions as TNF-α (for reviews see Refs. 12–15). Lymphotoxin is structurally related to TNF-α (16) and exists in both membrane-associated (17, 18) and secreted forms (19–21), ranging in molecular mass from 21 to 25 kDa. Although secreted LT exists as a homotrimer, the quaternary structure of surface LT has not been determined.

We recently characterized a cell-surface form of LT expressed on human T cell hybridoma, II-23.D7, by fluorescence and precipitation using anti-rLT serum or monoclonal antibodies (mAb) (22). In addition to the 25-kDa form of LT, a unique 33-kDa glycoprotein was coprecipitated with LT from the surface of II-23.D7 cells. The coprecipitation of LT and p33 suggested that these proteins were either antigenically related or associated as a complex. The present studies were undertaken to distinguish between these two possibilities. The results indicate that surface LT and p33 are antigenically distinct and appear to form a noncovalently linked heteromeric complex on the cell surface, and it is through this physical association that surface LT is anchored to the membrane.

**MATERIALS AND METHODS**

**Cells and Reagents**—The origins and characteristics of the II-23.D7 cell line were described previously (23). It is a CD4+ T cell hybridoma constructed between lectin-activated peripheral blood lymphocytes and a variant of the CEM T lymphoblastoid cell line CEM-TET1. Cytokine production, cytotoxic activity, and TNF/LT receptor expression of the II-23.D7 line are well documented (22–24). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics (penicillin and streptomycin, 100 μg/ml each), and 2 mM glutamine. The rabbit anti-TLT serum was described previously (22), and the anti-rLT mAb clone 9B9 was purchased from Boehringer Mannheim.

**Cell Surface Iodination**—II-23.D7 cells were stimulated overnight (12–14 h) with 10 ng/ml phorbol 12-myristate acetate (PMA) or for 4 h with 50 ng/ml PMA. Cell concentration during the stimulation was 0.5–1×10^6/ml. The cells were harvested, washed 3 times with phosphate-buffered saline and iodinated using 50 μg of iodogen (Pierce Chemical Co.) and 1 μCi of Na^125I (15 μCi/μg, Amersham Corp.)/10^6 cells. The cells were then washed once in complete medium and twice in phosphate-buffered saline and then extracted in 1 ml of Tris-buffered saline (TBS), pH 8.0, 2% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 40 mM iodoacetamide per 10^6 cells. For cross-linking experiments phosphate-buffered saline was used instead of...
TBS during detergent extraction. The extracts were centrifuged for 5 min at 10,000 × g and then analyzed by immunoprecipitation. For isoelectric focusing (IEF) experiments the extracts were dialyzed for 2 h at 4 °C against 10 mM Tris, pH 8.0 (1 liter).

**Metabolic Labeling and Pulse-Chase Analysis—**II-23.D7 cells at a concentration of 10⁷/ml were stimulated for 2 h at 37 °C with 10 ng/ml of PMA. The cells were lysed and homogenized in a 1:1 mixture of 10 ng/ml of methionine (Met)-free RPMI 1640, 10% dialyzed fetal bovine serum, 20 mM Hepes, and 10 ng/ml PMA, and then [³⁵S]Met (1300 Ci/mmol, ICN) was added to a final concentration of 100 μCi/ml. Incubation in the label was carried out for 2 h at 37 °C, at which point the cells or supernatants were analyzed for labeled LT by immunoprecipitation. The supernatants were subjected to another round of immunoprecipitation to remove unassociated proteins and then incubated with [³⁵S]Met for 5 min (pulse), after which the medium containing [³⁵S]Met was removed and replaced with warm complete medium without label and incubated for various times at 37 °C (chase). At the indicated time points, 1-ml aliquots were centrifuged at 2000 × g, the supernatants collected, and each cell pellet washed once in 10 ml of complete medium and extracted in detergent.

**Immunoprecipitation—**Cell extracts or supernatants were preclarified of nonspecific binding proteins for 4 h at 4 °C with normal rabbit or mouse sera at 1:500 dilution, and 25 μl of protein G-Sepharose beads (Pharmacia LKB Biotechnology Inc.)/ml of extract. The samples were centrifuged for 2 min at 5000 × g and the supernatants were subjected to IEF under nondenaturing condition for 12-16 h using anti-rLT serum at 1:500 or anti-rLT mAb at 2 μg/ml, and 25 μl of protein G beads. The immune complexes adsorbed to the protein G beads were washed three times with 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, and 0.05% sodium dodecyl sulfate (SDS). The beads were heated in Laemmli sample buffer containing 2% 2-mercaptoethanol and analyzed by polyacrylamide gel electrophoresis (PAGE). Unless otherwise noted the concentration of the resolving gel was 12%. The gels were dried and subjected to autoradiography using X-Omat AR film (Eastman Kodak) and a Cronex intensifying screen (Du Pont) at -70 °C for various lengths of time. Gels containing [³⁵S]Met-labeled proteins were incubated with 1 m sodium dodecyl sulfate (SDS) or ethylene glycol. Cross-linking and control experiments were performed and analyzed by SDS-PAGE. The pH gradients for both the denatured and nondenatured proteins were eluted by heating at 100 °C on a shaker. The appropriate slices were then neutralized by incubating in control buffer for 10 min. The intact gel slices were rehydrated and then analyzed by SDS-PAGE under nondenaturing condition.

**Chemical Cross-linking—**Cellular extracts from [³⁵S]Met-labeled cells or supernatants containing [³⁵S]Met-labeled proteins were cross-linked by the addition of bis[2-(succinimidoxy-carbonyloxyethyl)sulfone (BSOCOES) or ethylene glycol (succinimidylsuccinate) (EGS)]. The S-succinimidylsuccinate (EGS) (Pierce) at final concentrations of 0.1 and 1 mM, respectively, for 30 min at 4 °C with rotation. The reaction was then stopped by the addition of glycine (5 mM final concentration). Cross-linked and control samples were immunoprecipitated and analyzed by SDS-PAGE. The choice of these cross-linkers was based on the observation that EGS is more efficient than BSOCOES for soluble LT, whereas BSOCOES and EGS yielded similar results with surface LT. Cleavage of BSOCOES cross-linked adducts was performed by excising the gel band of interest, rehydrating, and with 1 ml of cleavage buffer (50 mM Tris, 100 mM phosphoric acid, pH 11.6, 0.5% SDS) or control buffer (100 mM Tris, pH 6.8, 0.5% SDS) for 1 h at 37 °C on a shaker. The appropriate slices were then neutralized by incubating in control buffer for 10 min. The intact gel slices were then loaded directly into the wells of a stacking gel and analyzed by SDS-PAGE.

**Isoelectric Focusing—**Two-dimensional IEF was performed essentially as described by O’Farrell (25). [³⁵S]Labeled antigens were immunoprecipitated from II-23.D7 cell extracts, and the precipitated proteins were then eluted at constant voltage (400 V). The second dimension was run to resolve proteins by molecular weight using 12% SDS-PAGE.

For IEF under nondenaturing conditions, [³⁵S]Met-labeled cell extracts were subjected to IEF directly on tube gels identical to those above except without urea. The labeled extract (200 μl volume) was centrifuged at 100,000 × g (30 p.s.i., Beckman airfuge) for 10 min before focusing. IEF was performed at 4 °C under the same conditions as described above. The tube gel was then removed and sliced into 1-cm sections and the proteins eluted by incubating each slice in 1 ml of TBS. The supernatants containing the eluted proteins were then immunoprecipitated and analyzed by SDS-PAGE. The pH gradients for both the denatured and nondenatured tube gels were determined by measuring the pH of the ampholines eluted from individual slices of gels run in parallel.

**RESULTS**

**LT and p33 Are Antigenically Distinct—**Because p33 and LT coprecipitate with antibodies to rLT (22), we examined whether these proteins are antigenically related or physically associated with one another (22). To determine if these proteins are antigenically related, [³⁵S]Met-labeled LT and p33 were first partially purified by immunoprecipitation and resolved by SDS-PAGE, as described under “Materials and Methods.” Isolated LT and p33 proteins were then subjected to a second round of immunoprecipitation using either polyclonal or monoclonal anti-rLT antibodies (Fig. 1). Rabbit anti-rLT reimmunoprecipitated [³⁵S]Met-labeled LT (lane 2) but not p33 (lane 3). The anti-rLT mAb precipitated [³⁵S]Met-labeled LT (lane 5) and a 21-kDa protein (lane 4), which as shown below, is a precursor of LT; however, it did not precipitate p33 (lane 6). These results show that after SDS-PAGE purification of LT and p33, both polyclonal and monoclonal anti-rLT antibodies only react with LT and not p33. Furthermore, identical results were observed when the initial immunoprecipitation was performed under mildly denaturing conditions created by the addition of deoxycholate into the Nonidet P-40 cell extracts (1:1 ratio/data not shown). These data provide evidence that p33 is not antigenically related to LT and most likely coprecipitates due to an association with LT. LT and p33 Biosynthesis—**To determine if p33 followed a parallel time course of expression as surface LT, shown previously by immunofluorescence (22), immunoprecipitation was performed from surface radiiodinated II-23.D7 cells at various times following stimulation with PMA (Fig. 2). Both LT and p33 were detected after 1-2 h of stimulation, and the relative ratio of p33/LT was constant. The proportional in-

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**FIG. 1.** LT and p33 exhibit distinct epitopes. LT and p33 from 1²⁵I- or [³⁵S]Met-labeled II-23.D7 cells were immunoprecipitated and isolated by SDS-PAGE as described under “Materials and Methods.” The isolated 1²⁵I-LT (p25) (lane 2) or p33 (lane 3) was reimmunoprecipitated with rabbit anti-rLT serum. Lane 1 serves as a reference for the positions of p33 and LT. Anti-rLT mAb was used to reimmunoprecipitate isolated [³⁵S]Met-labeled p21 (lane 4), LT (lane 5), or p33 (lane 6). Immunoprecipitated antigens were then analyzed by SDS-PAGE and autoradiography.
Surface LT Is Expressed as a Complex with p33

FIG. 2. Time course of LT and p33 surface induction on PMA-activated II-23.D7 cells. Cells (5 x 10^6) were stimulated with 10 ng/ml PMA for various times (lane 1, nonstimulated cells; lanes 2–6, post-PMA stimulation at times indicated). At each time-point the cells were surface-iodinated, extracted in detergent, and the extracts immunoprecipitated using anti-rLT serum as described under "Materials and Methods." The precipitated antigens were analyzed by SDS-PAGE (10% resolving gel) and autoradiography.

crease in p33 and surface LT expression following induction with PMA was consistent with them being complexed together.

To determine at what point in the biosynthesis of LT the association with p33 occurs, a pulse-chase analysis was performed. After the initial pulse, two bands of 21 (p21) and 33 kDa were immunoprecipitated from the cell-associated fraction (time 0, Fig. 3A). During the chase period the band density of p21 decreased as the density of the LT (p25) increased, in the cell-associated and supernatant fractions (Fig. 3B). This pattern suggests that a precursor-product relationship exists between p21 and LT. Furthermore, digestion of the p25 and p21 bands with both N- and O-glycosidases produced an 18-kDa band for both LT forms, indicating that they possessed a common polypeptide backbone (data not shown). Another report has shown the existence of two forms of LT in a T cell line (MT-2) and a B cell line (RPMI-1788) of 21–26 kDa; however, these two forms did not appear to possess a precursor-product relationship (26).

The band density of p33 remained relatively constant during the chase, in contrast to the changing levels of p21 and p25. Although p33 is immunoprecipitated with the LT precursor (p21), it does not undergo further processing during the chase and is not associated with secreted LT. These results indicate that p33 associates with LT early in the LT biosynthetic pathway, but does not determine whether p33 synthesis is inducible or constitutive in II-23.D7 cells.

Evidence That LT and p33 Are Physically Associated—To further investigate the hypothesis that surface LT and p33 form a complex, isoelectric focusing was performed based on the rationale that LT and p33 should cofocus under non-denaturing conditions. The pI for LT and p33 under denaturing conditions were determined by two-dimensional gel analysis (Fig. 4A). LT possessed five charge isomers ranging in pI from 6.5 to 7.3, whereas p33 possessed four charge isomers ranging in pI from 5.5 to 6.0. When IEF was performed under

FIG. 3. Pulse-chase analysis of LT and p33 biosynthesis in II-23.D7 cells. A, Cells were activated for 2 h with 10 ng/ml PMA and then labeled for 5 min with [35S]Met (time 0) and chased in complete medium at the times indicated. The labeled cellular extracts or supernatants were then immunoprecipitated using anti-rLT serum and analyzed by SDS-PAGE and autoradiography. B, densitometric analysis of the autoradiogram shown in A. All species on the plot are cell-associated, except for p25 designated as secreted.

FIG. 4. Isoelectric focusing analysis of surface LT/p33. A, two-dimensional gel analysis was carried out as described under "Materials and Methods" on [35S]-labeled LT (p25) and p33 that were immunoprecipitated from II-23.D7 cell extracts. The analysis was performed under denaturing conditions in the presence of urea. B, IEF of Nonidet P-40 extracts from [35S]-labeled II-23.D7 cells was performed in the absence of urea. Proteins were eluted from 1-cm sections of the focusing gel and subjected to immunoprecipitation with anti-rLT serum and analyzed by SDS-PAGE and autoradiography. Precipitated antigens from the gel in lanes 1–12 correspond to tube gel slices 2–13. The pH gradients generated for both the denatured gel (A) and non-denatured gel (B), based on 1-cm gel increments, are shown below each autoradiogram.
Surface LT Is Expressed as a Complex with p33

non-denaturing conditions LT and p33 cofocused with a pI range from 6.3 to 7.2 (Fig. 4B, lanes 6–8). The migration of p33 was significantly retarded under these conditions, suggesting that p33 focused as a complex with surface LT. In addition, the IEF positions of HLA class I and LFA-1 (CD11a/18), and total protein profile were monitored and showed that the proteins in the non-denaturing IEF were not nonspecifically aggregated (data not shown).

Surface LT Is Peripherally Associated with the Membrane—
The existence of surface LT/p33 suggested that one or both of these proteins may serve as a membrane anchor. Previous studies indicated that LT was not anchored either by its leader sequence or by a phosphatidylinositol glycan linkage (22). It was therefore plausible that p33 could serve as the membrane anchor, in which case LT would display the characteristics of a peripheral membrane protein. To test whether LT is peripherally associated with the membrane we extracted 125I-surface-labeled II-23.D7 cells with a high concentration of salt or with acid (Fig. 5). After treatment, both the supernatant and the cell fraction were fractionated by immunoprecipitation with anti-rLT serum. The results revealed that treatment with salt (lane 4) or acid (lane 6) extracted ~50% of the labeled LT from the cells; whereas, no p33 could be detected in the supernatants, but remained associated with LT in the cell pellet. These results are consistent with LT being a peripheral membrane protein and suggest that p33 is stably associated with the membrane. However, we cannot rule out the possibility that p33 is extracted from the membrane because anti-rLT does not react with p33.

Protein Cross-linking Analysis—To further define the subunit interactions between LT and p33, chemical cross-linking with the homobifunctional cross-linking reagents, BSOCOES and EGS, was performed on secreted and membrane forms of LT produced by the activated II-23.D7 hybridoma (Fig. 6). Supernatants from [35S]Met-labeled II-23.D7 cells (lanes 1 and 2) and detergent extracts from 125I-labeled II-23.D7 cells (lanes 3 and 4), each from 10⁶ cells, were subjected to cross-linking by the addition of 1.0 mM EGS or 0.1 mM BSOCOES, respectively, as described under ‘‘Materials and Methods.’’ The cross-linked species were immunoprecipitated with anti-rLT serum and analyzed by SDS-PAGE. The supernatant material was run on a 12% resolving gel, whereas surface material was run on a 5–15% gradient resolving gel. Cleavage of the cross-linked p60 adduct was accomplished by treating a rehydrated gel slice containing the adduct in buffer at pH 11.6 (lane 6) or as control buffer at pH 6.8 (lane 5) and then reanalyzed by SDS-PAGE (12% resolving gel).

The data presented in this paper provide evidence that LT and p33 form a complex on the surface of the II-23.D7 T cell hybridoma, with p33 serving as the membrane anchor. We previously established the distinct nature of p33 and presented evidence that the surface form of LT was very similar to the secreted form of LT (22). Recently, N-terminal sequence analyses of surface LT has confirmed that it is identical to LT as defined previously by molecular cloning.²

The present results indicate that the coimmunoprecipitation of LT and p33 is due to their physical association, rather than antigenic cross-reactivity (Fig. 1). This conclusion is strengthened by isoelectric focusing experiments performed under non-denaturing conditions where LT and p33 cofocused, apparently as a molecular complex; whereas, under denaturing conditions LT and p33 focused separately possessing distinct isoelectric points (Fig. 4). Consistent with this model of a physical complex was the finding that LT and p33 followed a similar time course of membrane expression (Fig. 2). Analysis of surface LT and p33 by chemical cross-linking showed a single adduct of 60 kDa that was composed of a dimer of p33; surface LT was not cross-linked to p33 or to itself (Fig. 6). It was unexpected that cross-links were not formed between LT and p33; however, this could be due to a lack of the necessary protein conformation to allow efficient cross-linking to occur. Cross-linking of secreted LT from the II-23.D7 cells revealed that soluble LT was secreted in homotrimeric form; whereas, surface LT appeared to be in a monomeric state. Thus, the

² J. Browning and P. Chow, unpublished observation.

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**Fig. 5.** Effect of acid and salt extraction on surface LT and p33. Surface-iodinated PMA-activated II-23.D7 cells (5 x 10⁶ cells) were treated with either TBS (lanes 1 and 2), 2 M KCl in 10 mM Tris, pH 7.5 (lanes 3 and 4), or 50 mM glycine, pH 3.0 (lanes 5 and 6) for 10 min at 4°C (total volume: 1 ml). After extraction, the cell pellets were lysed in detergent and the supernatants and cell lysates were immunoprecipitated with anti-rLT serum. C, cell-associated; S, supernatant.

**Fig. 6.** Protein cross-linking analysis of surface LT/p33. Supernatants from [35S]Met-labeled II-23.D7 cells (lanes 1 and 2) and detergent extracts from 125I-labeled II-23.D7 cells (lanes 3 and 4), each from 10⁶ cells, were subjected to cross-linking by the addition of 1.0 mM EGS or 0.1 mM BSOCOES, respectively, as described under “Materials and Methods.” The cross-linked species were immunoprecipitated with anti-rLT serum and analyzed by SDS-PAGE. The supernatant material was run on a 12% resolving gel, whereas surface material was run on a 5–15% gradient resolving gel. Cleavage of the cross-linked p60 adduct was accomplished by treating a rehydrated gel slice containing the adduct in buffer at pH 11.6 (lane 6) or as control buffer at pH 6.8 (lane 5) and then reanalyzed by SDS-PAGE (12% resolving gel).
two forms of LT appear to differ in their quaternary structure. Together these data suggest a tentative model in which a dimer of p33 is associated noncovalently with a monomer of LT.

Several experiments point to the hypothesis that LT is anchored to the membrane via its association with p33. We have shown previously that the leader sequence is processed normally in the surface form of LT, making it unlikely that LT is anchored in a mechanism similar to TNF-α. We were also unable to detect a phosphatidylinositol glycan-type membrane anchor associated with surface LT (22). Pulse-chase analysis of LT biosynthesis showed that p33 is associated early in LT biosynthesis (Fig. 3A), ruling out the possibility that secretion of LT and subsequent binding to p33 or a surface receptor provides a mechanism for membrane association. In addition, cross-linking of LT to receptors expressed on the surface of the II-23.D7 cell line showed the adduct to be 92 kDa (24), clearly distinct from the results presented here (Fig. 6). Furthermore, the molecular and biochemical characteristics of the two known receptors for TNF/LT (27-29), including their Met/Cys ratio, and antigenic and molecular size properties make it unlikely that the TNF receptors are involved in anchoring LT to the surface of activated T cells. Conditions which are known to elute peripheral membrane proteins released LT from the surface of II-23.D7 cells, suggesting that at least a portion of surface LT is peripherally associated with the membrane, whereas it appeared that p33 was stably associated with the membrane (Fig. 5). This result is consistent with the notion that p33 provides a membrane anchoring function for LT.

Up to now, surface forms of cytokines have been shown to attach to membranes through hydrophobic domains encoded within their gene sequence. Here we present evidence for an additional mechanism of membrane association whereby the cytokine is attached via another membrane protein. It remains unclear as to whether p33 itself possesses cytokine-like activity or whether it simply serves to target LT to the surface. Perhaps p33 serves as an escort/carrier protein for surface LT by virtue of its being stably associated with the membrane. Such an escort function for p33 would be unique to T cells, because neither the B cell line RPMI 1788 nor Chinese hamster ovary (CHO) cells transfected with the LT gene express surface LT.

A working model which incorporates the present findings is illustrated in Fig. 7. LT appears to be assembled into two distinct forms during biosynthesis in II-23.D7 cells. Nascent LT polypeptides within the lumen of the endoplasmic reticulum can either associate with themselves to form trimers, or they can associate with p33 dimers to form a heteromeric complex. That these oligomerization events occur early in the biosynthetic pathway for LT is consistent with the well-established principle that oligomerization of multimeric proteins occurs prior to their exit from the endoplasmic reticulum (30, 31). After assembly, the two LT forms then progress through the secretory pathway together. The processing observed for the p21 precursor of LT into its mature 25-kDa form would presumably occur in the Golgi. Upon fusion of the secretory vesicle with the plasma membrane the LT/p33 complex remains on the cell surface due to the membrane anchoring properties of p33, whereas trimeric LT is secreted. This allows for the expression of both surface and soluble forms of LT. Investigation into the structure and function of surface LT/p33 should provide new insights into the mechanisms of LT expression and the role of LT in T cell-mediated immune responses.

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