Analysis of the Structure of the Human T Cell Surface Antigen T8 by Limited Proteolysis and Chemical Cleavage*

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The structure of the human T lymphocyte surface antigen T8 (Leu 2) has been explored utilizing limited proteolysis on viable cells and cellular lysates. The positions of the cleavage sites of trypsin and papain were placed relative to the single CNBr cleavage point. Additional data allowed the location of the amino and carboxyl termini relative to the enzymatic and chemical cleavage sites. This information, together with earlier evidence concerning the position of a membrane binding site, allowed the construction of a model illustrating the vectorial orientation of the molecule on the cell. Within this model, the approximate positions of disulfide linkages were indicated based on the results of nonreduced/reduced two-dimensional sodium sulfate-polyacrylamide gel electrophoresis. Carbohydrate moieties were localized using cleavage with trifluoromethanesulfonic acid, a reagent which cleaves both N-linked and O-linked oligosaccharides. Finally, the implications of the proteolysis experiments in relation to the function of T8 were discussed.

The T8 molecule is a human T lymphocyte-specific surface antigen which has been defined by monoclonal antibodies (1-3). During differentiation of T cells in the thymus, the T8 molecule is found on mature thymocytes and immunocompetent T lymphocytes, which are exported to the periphery. With regard to these peripheral T lymphocytes, T8 is found on a specific subset, which includes some cytotoxic T cells and suppressor T cells (4). The possible functional role of the molecule on these mature cells has been examined by several investigators (5, 6). Inhibition of cytolysis by cytotoxic T cells has been observed when the effector cells are incubated in the presence of anti-T8 monoclonal antibodies (5, 6). Further investigations have shown that the T8 molecule appears to be important in the initial adhesion stage of cytolysis, rather than at some subsequent event of the lytic process (7).

We have been interested in examining the structure of this cell surface antigen. It has been shown by us and others that the T8 protein on thymocytes is different from that on peripheral T lymphocytes (2, 8). On both these cell types, the molecule exists as a homodimer of two 34-kDa moieties which are disulfide-linked into two different conformational forms. These migrate as molecules of 67 and 76 kDa on SDS-PAGE under nonreducing conditions and are thought to differ only in intrachain disulfide linking, since no third peptide could be found to account for their differential migration (5). The molecule present on mature T lymphocytes is also found as high molecular mass, disulfide-bonded homomultimers of this 34-kDa glycoprotein. On thymocytes, multimers of molecular mass greater than 100 kDa are found too, but in addition to the 34-kDa polypeptide the multimers contain a 46-kDa protein covalently attached by disulfide bridges. This molecule has no simple relationship to the 34-kDa moiety as characterized by pI, peptide maps, and carbohydrate content (8).

Earlier, we have been able to show that treatment of the 34-kDa molecule with CNBr yields fragments of 9 and 24 kDa. Furthermore, we have demonstrated that the larger fragment contains a hydrophobic region which presumably anchors the molecule in the membrane. The 24-kDa portion of T8 also appears to be the site of the interchain disulfide bridges in the multimers (6).

Here we describe a series of experiments which aid in the delineation of the in situ vectorial organization of the 34-kDa T8 surface antigen. We have utilized limited proteolysis on viable cells and on detergent-solubilized membrane proteins, combined with cleavage of the peptides by CNBr, to further probe the membrane orientation of the molecule. These techniques have allowed deductions to be made concerning the orientation of the N terminus of the protein with respect to the membrane. Furthermore, the general positions of carbohydrate moieties and disulfide bridges were localized within the molecule. The proteolysis experiments are interpreted in relation to the function of the T8 molecule in CTL-target cell adhesion.

**MATERIALS AND METHODS**

**Cells**—Studies described in this paper utilized the HPB-ALL cell line (9) and phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes. The HPB-ALL cells were cultured in RPMI 1640, supplemented with 5% fetal calf serum, at 37°C in a 5% CO2 atmosphere. Peripheral blood lymphocytes were obtained from normal healthy donors and were purified on Ficoll-Hypaque density gradients (10). They were used for cell surface labeling after activation with PHA. Before incubation with PHA, the peripheral blood lymphocytes were washed four times with RPMI 1640 and then cultured for 72 h in the presence of 5% fetal calf serum and 0.25 μg/ml PHA (HA 16, Burroughs Wellcome) at a density of 3 x 10^6/ml.

**Monoclonal Antibodies**—The following monoclonal antibodies were used: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CTL, cytotoxic T lymphocyte; NP40, Nonidet P-40; TFMS, trifluoromethanesulfonic acid; PHA, phytohemagglutinin; TEA, triethanolamine; PBS, phosphate-buffered saline, NMS, normal mouse serum.

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T lymphocytes from peripheral blood lymphocytes were washed four times in RPMI medium without amino acids and acid. Lyophilized amino acids which were redissolved in RPMI without amino acids were then added to the cells, and incubation proceeded for 10 h at 37 °C in an atmosphere of 5% CO2. The cells were collected by centrifugation at 300 g for 10 h at 37 °C in an atmosphere of 5% CO2. The cells were centrifuged at 300 g for 10 min and prepared for immunoprecipitation.

**Immunoprecipitation**—After labeling, the cells were lysed with 1% Nonidet P-40 (NP40; Particle Data Laboratories, Elmhurst, IL) in 0.7 M trichloracetic acid (TCA), pH 7.8, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.02 mM MgCl2, and 0.01% SDS trypsin inhibitor (Sigma). The lysates were centrifuged at 13,000 g for 15 min to remove nuclei. The supernatant fluid was centrifuged for 30 min at 12,000 g for 10 min. The precipitate was washed two times with PBS before solubilization and immunoprecipitation.

**Radiosequencing and Sample Preparation**—Samples which were radiolabeled with 3H-amino-acids as described by Coligan et al. (13). HPB-ALL PHA-stimulated peripheral blood lymphocytes were washed four times in RPMI medium without amino acids and were resuspended at a concentration of 5 × 106/ml in the same medium containing 10% fetal calf serum and 1 mM (aminoxy)acetic acid. Lyophilized amino acids which were redissolved in RPMI without amino acids were then added to the cells, and incubation proceeded for 10 h at 37 °C in an atmosphere of 5% CO2. The cells were collected by centrifugation at 300 g for 10 min and prepared for immunoprecipitation.

**Treatment with CNBr**—Immunoprecipitates or eluted proteins were dissolved in 150 μl of 70% formic acid which was saturated with CNBr. The reaction chamber was flushed with N2, and cleavage was allowed to proceed for 24 h at 20 °C in the dark at which time the reaction was stopped by drying under vacuum. The residue was then lyophilized three times from water before analysis on SDS-PAGE.

**Electrophoresis and Autoradiography**—SDS-polyacrylamide electrophoresis was carried out on discontinuous vertical slab gels according to the method of Laemmli (16). The gel was made with 5–10% or 5–12% acrylamide for two-dimensional nondenatured or electrophoresed, the procedure was as follows. Gel slices from the nonreduced dimension were excised and soaked for 15 min in a buffer of 0.125 M Tris/HCl, pH 6.8, 3% SDS, 10% glycerol, 5% 2-mercaptoethanol. The slices were then layered onto the stacking gel of the second gel and run as described above. Molecular weight markers used were immunoglobulin G (150,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (29,000), and cytochrome c (14,000). For autoradiography of 125I-labeled materials, Kodak XAR-5 film was used in combination with intensifier screens (Cronex Lightning Plus, DuPont Chemical Co., Newtown, CT).

**Limited Proteolysis with Trypsin**—Earlier studies had shown that the T8 antigen could be removed from the cell surface of human cytotoxic T cell clones by limited proteolysis with trypsin (17). Treatment of T8+ CTL clones with trypsin resulted in a rapid disappearance of the ability of these CTL to adhere to target cells. In contrast, identical treatment of T4+ CTL clones did not affect their function. Upon overnight culture, both the expression of T8, as measured by indirect immunofluorescence, and the cytolytic activity of the CTLs disappeared (17). To study the structure of T8 on the cell surface, a series of experiments employing limited treatment with trypsin were conducted.

The leukemic cell line HPB-ALL was labeled by surface radiiodination and incubated with varying concentrations of trypsin. The fragments released into the supernatant and the molecules retained on the cell were isolated by immunoprecipitation from the supernatants or from NP40 lysates of the cells. The proteolytic fragments which could be recognized by the OKT8 monoclonal antibody were then analyzed by SDS-PAGE (Fig. 1). Under reducing conditions, the material which remained bound to the cell consisted of uncleaved 34-kDa T8 and fragments of approximately 31 and 3 kDa (Fig. 1A). Fragments of 25, 26, and 3 kDa were released in the supernatant fluid (Fig. 1B). When radiiodinated T lymphocytes were first solubilized in the detergent NP40 and the detergent lysate was treated with trypsin, major fragments of 26, 23, and 3 kDa were found under reducing conditions after anti-OKT8 immunoprecipitation (Fig. 2). In this regard, it should be noted that several other monoclonals, such as anti-Leu 2A (2), anti-T6A (1), and WT 92 (5), precipitated no tryptic fragments of T8 from either T cells or cells, deleting the data not shown. A series of experiments was conducted to determine...
FIG. 1. Analysis of the products of the T8 molecule from cells treated with trypsin. HPB-ALL cells were surface labeled with $^{131}$I and lactoperoxidase and resuspended in PBS at $25 \times 10^6$/ml. A, cells were treated with trypsin at a concentration of $0 \mu$g/ml (lane 2), $8 \mu$g/ml (lane 3), $15 \mu$g/ml (lane 4), $30 \mu$g/ml (lane 5), and $60 \mu$g/ml (lane 6) for 10 min at $20^\circ$C. The reaction was stopped by the addition of soybean trypsin inhibitor. An NP40 lysate was prepared and immunoprecipitated with OKT8 monoclonal antibody. Lane 1 represents a normal mouse serum control immunoprecipitation. B, supernatants from cells treated with trypsin at concentrations of $8 \mu$g/ml (lane 2), $15 \mu$g/ml (lane 3), $30 \mu$g/ml (lane 4), and $60 \mu$g/ml (lane 5) were clarified by centrifugation at $100,000 \times g$ for 15 min and subjected to immunoprecipitation with the OKT8 monoclonal antibody. Lanes 1 and 6 are control NMS immunoprecipitations. All samples were analyzed on a 10-15% polyacrylamide gradient gel under reducing conditions. The open arrowheads indicate the specific cleavage products referred to in the text.

FIG. 2. Immunoprecipitation of T8 molecules after digestion in a cellular detergent lysate. HPB-ALL cells were surface labeled with $^{131}$I and lactoperoxidase, and a NP40 lysate was prepared. The lysate was diluted to $25 \times 10^6$ cell equivalents/ml and treated with trypsin at a concentration of $60 \mu$g/ml (lane 1), $30 \mu$g/ml (lane 2), $15 \mu$g/ml (lane 3), $3 \mu$g/ml (lanes 4 and 5), and no trypsin (lane 5). The products of the digestion were immunoprecipitated with the OKT8 monoclonal antibody. Lanes 1 and 6 are control NMS immunoprecipitations. The samples were analyzed on a 10-15% polyacrylamide gradient gel under reducing conditions. Open arrowheads indicate specific cleavage products.

the relation between the tryptic fragments of T8 and the T8 molecule.

CNBr Cleavage of the Tryptic Fragments—We showed previously that treatment of the T8 antigen with CNBr resulted in the production of two fragments with $M_r$ 24,000 and 9,000 (8). Furthermore, the 24-kDa peptide could be shown to contain a site which could be labeled with the hydrophobic reagent $[^{125}]$iodonaphthylazide (2, 8). Moreover, the 24-kDa CNBr fragment (and not the 9-kDa fragment) participated in the formation of interchain disulfide bridges (8). Based on these observations and our finding that T8 exists primarily as a homodimer (2, 8), a model of T8 was made as shown in Fig. 3A.

In order to map the tryptic cleavage sites within the T8 model, tryptic fragments were cleaved with CNBr and analyzed on SDS-PAGE. As shown in Fig. 4 (lanes 1-3), the 31-kDa tryptic fragment which remained on the cell contained the 24-kDa CNBr fragment and lacked the complete 9-kDa fragment of native T8. The 3-kDa tryptic fragment was not cleaved by CNBr. CNBr cleavage of the three main products found in the supernatant after trypsin treatment yields the patterns depicted in Fig. 4 (lanes 4-6). In this instance, it appears as though the largest tryptic fragment (29 kDa) arises from a cleavage site present within the 24-kDa CNBr fragment. The intermediate tryptic 26-kDa peptide is derived from the larger product by a second cleavage within the 9-kDa CNBr peptide. The third and smallest fragment (3 kDa) is not cleaved by CNBr treatment. The combined CNBr and trypsin fragmentation studies demonstrate that two major tryptic cleavage sites exist. One is near one end of the 9-kDa CNBr fragment, and the other is close to the putative transmembrane region of T8. The fact that the predicted 8-kDa tryptic fragment which contains the transmembrane region was never detected could be due to its inability to be labeled by surface radioiodination, but is most likely due to its inability to be recognized by the OKT8 antibody.

Partial Amino Acid Sequence Analysis of the Major Tryptic and CNBr Fragments—In order to more precisely define the relationships of the fragments to each other and to the intact
molecule, limited radiosequencing was performed. First, T8 was immunoprecipitated from HPB-ALL cells which had been metabolically labeled with $[^3H]$Phe, $[^3H]$Val, $[^3H]$His, $[^3H]$Ile, and $[^35S]$Cys. The immunoprecipitates were treated with CNBr, and the 9- and 24-kDa fragments were isolated on preparative SDS-PAGE. The peptides were subjected to amino acid sequencing on a Beckman model 890C protein sequencer, and the $[^3H]$phenylthiohydantoin derivatives were analyzed by high pressure liquid chromatography (13). Fig. 5A illustrates that the 9-kDa fragment contains the N terminus, whereas the sequence of the 24-kDa fragment is unrelated.

Tryptic fragments were isolated by immunoprecipitation from HPB-ALL cells which had been metabolically labeled with $[^3H]$Arg and $[^35S]$Cys. Protein sequence analysis was performed on these fragments as described above. As shown in Fig. 5C, the 3-kDa peptide contained the N terminus. These data offered direct confirmation of the sequence data of the CNBr fragments and strengthened the findings obtained utilizing CNBr treatment of the tryptic fragments. These findings are summarized in Fig. 3B.

Limited Proteolysis of T8 Using Papain—The major cleavage sites of papain in T8 were studied in a similar fashion as had been done with trypsin. Cleavage products which remained on the cell were isolated by immunoprecipitation with anti-T8 (anti-Leu 2a monoclonal antibody) after detergent solubilization. SDS-PAGE under reducing conditions showed the original 34-kDa T8 and a 26-kDa fragment (Fig. 6A). The supernatant contained three fragments of 26, 24, and 22 kDa (Fig. 6B). When papain was added to a radiolabeled detergent lysate prepared from HPB-ALL, two major fragments of 24 and 22 kDa were obtained after anti-T8 immunoprecipitation (Fig. 6C).

CNBr cleavage of the papain fragments which remained bound to the cells demonstrated that papain cleaves in the 24-kDa CNBr portion of T8, as this fragment decreases in size relative to T8 which had not been digested with papain. The 9-kDa fragment from this papain peptide remains intact (Fig. 7A). Since, according to the tryptic model, cleavage within the 24-kDa CNBr fragment would be expected to result in release of the fragment into the supernatant, this result must be explained by cleavage of only one member of a pair (or multimer) of 34-kDa T8 molecules. This fragment remains disulfide-bonded to the uncleaved member, therefore remaining cell-associated. CNBr digestion of the papain fragments from the supernatant also showed a consistent pattern of progressively decreasing size within the 24-kDa CNBr peptide accounting for the difference within the fragments, since the 9-kDa peptide remains constant (Fig. 7A). Finally, CNBr cleavage of the papain products found after treatment of the cell lysate yielded similar results as the supernatant products (Fig. 7B). Here too, the decrease in size of the entire T8 molecule after papain cleavage was accounted for by successive cleavages within the region corresponding to the 24-kDa CNBr peptide. The 9-kDa peptide remained untouched.

These data suggest that cleavage within the 24-kDa CNBr peptide leads to release into the supernatant. This is consistent with the findings made with trypsin (Fig. 3C).

Localization of Inter- and Intrachain Disulfide Bonds—We have previously shown that T8 exists as disulfide-linked multimers on the cell surface, with two dimers of 76 and 67 kDa being most prominent (2, 8). Utilizing two-dimensional nonreducing/reducing electrophoresis, we attempted to localize the positions of the disulfides.

When tryptic digests of $^{125}$I-labeled HPB-ALL cells were analyzed under nonreducing conditions, four species were

![Fig. 3. Graphic representations of the T8 molecule.](image-url)
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Fig. 5. Limited protein sequence analysis of the CNBr and tryptic fragments of T8. HPB-ALL cells were labeled with [3H]-amino acids and [35S]Cys as described under "Materials and Methods." A, summary of multiple protein sequencing analyses of T8 using combinations of [3H]-amino acids. Protein sequencing was performed on a Beckman model 890C protein sequenator. Phenylthiohydantoin derivatives amino acids were detected as described under "Materials and Methods." B, immunoprecipitates of T8 biosynthetically labeled with [3H]Val, [3H]Phe, [3H]Ile, [3H]His, and [35S]Cys were treated with CNBr (see "Materials and Methods"). The cleavage products were separated on a 10-15% polyacrylamide gradient gel and visualized by autoradiography. The 24- and 9-kDa fragments were excised, eluted, and subjected to protein sequence analysis on a Beckman 890C protein sequenator. C, HPB-ALL cells (25 x 10⁶/ml) biosynthetically labeled with [3H]Arg and [35S]Cys were treated with trypsin (30 μg/ml) for 10 min at 20°C. The cells were lysed in 1% NP40, and immunoprecipitates were prepared with the OKT8 monoclonal antibody. The products were separated on a 10-15% polyacrylamide gradient gel, and specific products were visualized by autoradiography. After excision and elution from the gel, the peptides were subjected to sequence analysis on an Beckman model 890C protein sequenator.

Fig. 6. Analysis of the papain digestion products of T8. HPB-ALL cells were surface-labeled with ¹²⁵I and lactoperoxidase. The cells were resuspended at 25 x 10⁶/ml in PBS and treated with activated papain at 75 μg/ml for 10 min at 20°C. A, anti-T8 (anti-Leu 2a monoclonal antibody) immunoprecipitates were prepared from a 1% NP40 lysate of the cells. Lane 1, anti-T8 immunoprecipitate; lane 2, anti-T8 immunoprecipitate. B, supernatant from the papain treatment was clarified by centrifugation and immunoprecipitated with anti-T8 (anti-Leu 2a) monoclonal antibody. Lane 1, NMS control immunoprecipitate; lane 2, anti-T8 immunoprecipitate. C, HPB-ALL cells surface-labeled with ¹²⁵I and lactoperoxidase were lysed in 1% NP40. The cell lysate (25 x 10⁶ cell equivalents/ml) was treated with activated papain at 75 μg/ml for 10 min at 20°C. An anti-T8 (anti-Leu 2a) immunoprecipitate was then prepared. Lane 1, NMS control immunoprecipitate; lane 2, anti-T8 immunoprecipitate. All samples were analyzed on 10-15% polyacrylamide gradient gels under reducing conditions. Open arrowheads indicate specific cleavage products (see text).

material, as well as the 31- and 3-kDa tryptic fragments (Fig. 8B). This suggested that the 76- and 67-kDa species observed under nonreducing conditions were cleaved in a similar fashion but remain associated due to disulfide bridging. The 40- and 34-kDa forms found under nonreducing conditions would seem to be structural isomers which represent half-molecules corresponding to the 76- and 67-kDa dimers, respectively. These species must therefore differ in intrachain disulfide...
under nonreducing conditions on a 10-15% polyacrylamide gradient gel. Immunoprecipitates were made from the cellular lysate and analyzed on a 10-15% polyacrylamide gradient gel under reducing and reduced conditions. The products labeled a-d were eluted from the gel and analyzed on a 10-15% polyacrylamide gradient gel under reducing conditions.

Similar experiments were performed on material retained on the cell and released into the supernatant after papain treatment (Fig. 9, A and B, respectively). In the case of the cellular material, the three major nonreduced bands with molecular masses of 72, 65, and 55 kDa (Fig. 9A, lane 1) all reduced to two bands of 34 and 26 kDa (lanes a-c). The two small fragments from the supernatant (24 and 22 kDa) migrated identically under both reduced and nonreduced conditions (Fig. 9B, lanes 1 and b-c). The larger peptide dimer which migrated at 50 kDa under nonreducing conditions is composed of two peptides (or two of a series of closely related peptides) migrating in the range of 26-29 kDa (Fig. 9B, lanes 1 and a). Based upon these findings, the disulfide bond cross-linking the two polypeptides must be N-terminal to the 26-kDa cleavage site and C-terminal to the 24-kDa cleavage site.

Products from papain cleavage upon a detergent lysate were examined using the same methodology with similar results (Fig. 9C). The fragments which were found in the lysate have apparent molecular masses of 65, 45, 24, and 22 kDa under nonreducing conditions (Fig. 9C, lane 1). When these fragments were analyzed under reducing conditions (Fig. 9C, a-d), a familiar pattern emerges. The 45- and 24-kDa species migrated as 24 kDa and the 22-kDa moiety migrated as 22 kDa. Only the 65-kDa moiety appeared to produce a novel product, of 31 kDa (compare Fig. 2). Since this fragment has not been characterized with CNBr, it cannot be ascertained where the cleavage has occurred. However, the finding that the 24-kDa fragment exists both as disulfide-bonded and nonbonded forms while the 22-kDa peptide is only found as a non-disulfide-bridged form suggests that a disulfide bridge must be located N-terminal to the 24-kDa cleavage site and C-terminal to the 22-kDa cleavage point (Fig. 3D and “Discussion”).

**Placement of the Carbohydrate Moieties of T8**—That T8 is a glycoprotein has been established by procedures which selectively label carbohydrate moieties (2). It seemed possible to locate the positions of these sugar residues within the CNBr map of the T8 molecule.

The sites of glycosylation on the T8 molecule relative to the CNBr cleavage site were examined utilizing the reagent TFMS. This agent has been shown to hydrolyze all glycosidic linkages of glycoproteins (15). In order to ascertain the approximate positions of oligosaccharides on T8, SDS-PAGE-purified T8 labeled with [3H]Leu, [3H]Tyr, and [35S]Cys was first cleaved with CNBr. This digest was subsequently treated with TFMS, and products were analyzed on SDS-PAGE in comparison with untreated CNBr-cleaved T8 (Fig. 10). It is evident that the 24-kDa CNBr peptide contains all of the sugar moieties which are susceptible to cleavage with this reagent, since the mobility of this fragment, and not the 9-kDa peptide, is altered by TFMS treatment. The decrease in molecular mass corresponding to approximately 2 kDa in both the uncleaved 34-kDa T8 molecule and the 24-kDa CNBr peptide confirms previous findings obtained on native T8 (18).

**DISCUSSION**

The experiments described provide further insight into the structure of the human T lymphocyte antigen T8. Understanding the structure of this molecule as expressed on the cell surface will aid in the elucidation of its functional role in the recognition phase of cytolysis by human CTL. A summary of the present findings is presented in Fig. 3D.

In the model we have placed the membrane binding portion of the molecule within the carboxyl terminal 5 kDa of the molecule. This is based on several observations, from this and previous work. We had shown earlier that the hydrophobic label iodonaphthylazide binds to a region within the 24-kDa CNBr fragment of T8, and assumed this to be the only membrane binding region (8). The largest fragment (29 kDa) which was released into the supernatant by proteolytic cleavage contained a CNBr fragment shorter than the 24-kDa peptide of native T8 and an unchanged 9-kDa fragment. The finding that the 9-kDa peptide is the N-terminal portion of T8 indicated that the carboxyl terminus is inserted into the membrane. This is in agreement with data from several other membrane proteins, where a similar orientation has been established (19, 20).

The positions of the tryptic and papain cleavage points were placed based on the CNBr maps of the fragments from the cells, supernatants, and lysates. This model takes into account the experimental data and explains some of the findings which at first appear discrepant. For example, the papain product which is associated with the cell has the same CNBr map as the largest fragment which is found in the supernatant. According to the model this is due to a proteolytic cleavage point on only one member of a given dimer. This interpretation is strengthened by the results of the two-dimensional nonreduced/reduced analysis of papain cleavage products.

While the fragments generated from trypsin treatment of a cellular lysate were not tested for their susceptibility to CNBr cleavage, a prediction may be made based on the findings from the cell and the supernatant. Thus, it would be expected that the 26-kDa tryptic fragment results from cleavage at a
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FIG. 9. Analysis of papain digests of T8 under nonreduced and reduced conditions. HPB-ALL cells labeled with $^{125}$I and lactoperoxidase were treated with papain (75 μg/ml) as described either on intact cells or in a NP40 detergent lysate. A, in lane 1, the products which remained associated with the cell were separated on a 5–15% polyacrylamide gradient gel under nonreducing conditions. The bands a–c were excised from the gel and directly analyzed under reducing conditions on a 10–15% polyacrylamide gel. B, lane 1 represents the products which are immunoprecipitated from the supernatant with anti-T8a monoclonal antibody and analyzed under nonreducing conditions as in A. The products indicated as a–c were excised and separated under reducing conditions on a 10–15% polyacrylamide gel. C, the cleavage products from the cellular lysate were analyzed under nonreducing conditions on a 5–15% polyacrylamide gradient gel (lane 1). The products a–d were excised and directly separated on a 10–15% polyacrylamide gradient gel under reducing conditions.

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site exposed only after cellular lysis and concomitant membrane disruption (Fig. 3B). The 23-kDa fragment is generated by additional tryptic cleavage 3 kDa from the N terminus, as is seen in both cell and supernatant.

Carbohydrate moieties are located within the larger CNBr fragment of T8. Earlier experiments have demonstrated that it is unlikely that these are of the common N-linked variety, since they are resistant to cleavage with endoglycosidase F (8). This enzyme has been found to have a specificity for both simple and complex-type N-linked sugar groups (21). Thus, the conclusion is that the carbohydrate moieties on T8 are of the O-linked type. This also explains our finding that the addition of oligosaccharides to the T8 molecule is not affected by tunicamycin, which inhibits N-linked glycosylation.2

The exact location of sulfhydryl cross-links has not been indicated on our model. However, estimates may be made as to their general placement. The data from the papain fragmentation studies as well as the CNBr maps of the fragments place some constraints on the positions of disulfide bridges. For example, the finding that the 22-kDa form exists solely in a monomeric form indicates that all interchain disulfides must be carboxyl-terminal to this cleavage site. That the 24-kDa papain fragment from the lysate is found in both monomeric and dimeric states suggests that two forms of disulfides are present. These may represent bridges between monomers to form dimers and bridges between the resultant dimers to form multimers. This is consistent with previous findings (8), where the T8 molecule has been shown to exist on the cell surface as disulfide-linked homomultimers. If this is the case, at least one of these sets must be carboxyl-terminal to the 24-kDa lysate cleavage point and one set N-terminal to this site in order to explain the data.

The sulfhydryl group located within the N-terminal 9-kDa CNBr fragment is known to exist on the basis of limited sequence analysis (Fig. 5). While it seems unlikely, based on evidence cited above, that this residue contributes to interchain bridging, there is some preliminary evidence that it may be involved in intrachain sulfhydryl bridging.2 The possibility that this could be at least one of the factors affecting the differing mobilities of the 67- and 76-kDa dimers is appealing. Furthermore, the evidence presented regarding nonreduced/reduced analysis of trypsin digests suggests the presence of at least one intrachain disulfide in each chain involving this fragment (Fig. 8).

The data presented here also allow some tentative conclusions about the folding of the molecule. The finding that both

2 P. Snow, unpublished observations.
enzymes seem to have cleavage sites within a very similar region would suggest that this portion of the molecule possesses an open structure, rendering it more susceptible to proteolytic digestion. There would also seem to be a region which is more open approximately 3 kDa from the N terminus, where trypsin has a cleavage site. The finding that a major portion of the molecule shows no cleavage has at least two possible explanations. One is that this portion of the protein is in a tightly packed globular domain(s), which is not accessible to proteolytic cleavage. The other explanation is that when cleavages do occur in this region, the determinant recognized by the monoclonal antibody is destroyed, such that the fragments are no longer recognized. Several observations suggest that the latter is a possibility.

The finding that OKT8 is unique in its ability to recognize the tryptic cellular fragments when compared with several monoclonals suggests that the determinants seen by these reagents are destroyed by the treatment. This is in contrast to the papain cleavage products, which are recognized by all monoclonal antibodies tested. Furthermore, reduction and alkylation experiments have shown that even the OKT8 determinant is more labile after trypsinization. When the native T8 molecule is reduced and alkylated, the 34-kDa monomer is precipitated. However, when the trypsinized cellular products are treated similarly, only uncleaved material is seen. Therefore, the trypsin treatment seems to remove a fragment from T8 which stabilizes the determinant seen by the antibody. The destruction of the determinant need not imply that the determinant itself is cleaved by the enzyme, but only that a region of the molecule which is necessary for the conformation of the determinant is destroyed.

Recently, Biddison et al. (22) have examined the susceptibility of a number of epitopes on the T8 molecule to trypsin treatment. Their data, based on analysis of trypsinized cells with a fluorescence-activated cell sorter, showed that epitopes differed considerably in their rate of disappearance upon trypsinization. The epitope defined by OKT8, which was utilized here, was one of the most resistant. Furthermore, their work as well as our earlier studies (17) have shown that the ability of CTL to lyse target cells decreases concomitantly with the loss of T8 when these cells are treated with trypsin.

It is indeed interesting to speculate that the action of trypsin at a point 3 kDa from the N terminus of T8 causes a conformational change in the molecule, resulting in both loss of determinants seen by several monoclonal antibodies and functional inactivation of the protein. Further work should be directed at addressing this question at the molecular level. These studies, coupled with additional functional data, will elucidate the role of this protein on the cell surface of T lymphocytes.

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