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Molecular characterization of a major nephritogenic domain in the autoantigen of anti-tubular basement membrane disease

(autoimmunity/immunologic epitopes/protein domains/renal pathology)

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ABSTRACT **Anti-tubular basement membrane (αTBM) disease is a form of primary interstitial nephritis mediated by autoimmune T cells and αTBM antibodies. In mice and humans the nephritogenic immune response is directed to a glycoprotein (3M-1) found along the proximal tubule of the kidney. We have isolated cDNAs from an expression library that encodes for the common framework domain of the 3M-1 antigen. This common domain was once related evolutionarily to a family of intermediate filament-associated proteins. Northern hybridization revealed that all isoforms of 3M-1 range between 1700 and 1900 base pairs and *in situ* hybridization studies indicate that transcripts are found in tubular epithelium. Candidate peptide fragments were deduced and synthesized from the sequence encoding this common framework domain, and one of the peptide residues was able to bind a monoclonal 3M-1-reactive αTBM antibody, stimulate the growth of 3M-1-reactive helper T cells, and induce nephritogenic effector T cells capable of producing interstitial nephritis. Our results indicate that a unique, immunodominant region of the 3M-1 antigen is an informative participant in the emergence of autoimmune injury to certain basement membranes.**

Inflammatory interstitial nephritis, either as a primary or secondary process, plays a central role in the progressive development of nearly all forms of chronic renal failure (1). Very little, however, is known about the target antigens that focus injury toward parenchymal structures residing in the tubulointerstitium. Our laboratory has been studying this problem using a rodent model of human interstitial nephritis called anti-tubular basement membrane (αTBM) disease. αTBM disease develops in mice or rats following their immunization with renal tubular basement membranes in adjuvant (2, 3). αTBM antibodies (αTBM-Abs/α3M-1-Abs) develop over 7–10 days, and extensive mononuclear infiltrates with tubular atrophy and fibrosis appear within several weeks.

The target antigen of this disease, the 3M-1 glycoprotein (4), is expressed in mice on nearly all cortical tubular basement membranes and by cultured proximal tubular epithelium (MCT cells). It can be found intracellularly, on their cell surface, or in the culture supernatant as a secreted product of ≈30,000 M_r (5). Eventually some isoforms of 3M-1 are translocated to the lateral border of the proximal tubular basement membrane *in vivo* (4). The genes for 3M-1 map to the first (6) and fourth (7) linkage groups in rats and probably to chromosome VII in mice (6). The 3M-1 protein has been purified immunochemically from rabbits (4), mice (5), rats (8), and humans (9).

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The nephritogenic autoimmune response has also been characterized in mice, rats, and humans with polyclonal antisera (2, 8–11), by constructing monoclonal αTBM-Abs reactive to 3M-1 (Mα3M-1-Abs; refs 4, 10), and by establishing tubular antigen (3M-1)-specific $CD4^+$ helper (12, 13) and $CD8^+$ effector T clones (14) that recognize 3M-1 on the surface of MCT cells (5, 15) or as a soluble moiety processed by traditional splenic antigen-presenting cells (12). The nephritogenic epitopes on 3M-1, as well as their paratypic recognition, seem to be highly conserved among mammalian species. By competitive inhibition radioimmunoassay, for example, using 3M-1 as the target antigen, we observed that 21 of 22 Mα3M-1-Abs from five separate fusions inhibited each other (10). Such serologic findings would seem to indicate that our Mα3M-1-Abs recognize similar or nearly identical epitopes on 3M-1.

To better understand the protein structure of this interstitial autoantigen as well as to further analyze its epitopic domains (10), we have isolated cDNAs from MCT cell expression libraries using monospecific polyclonal α3M-1-Abs. In this, our initial report, we indicate that 3M-1 is a protein of complex structure built around a framework region that encodes for a nephritogenic domain immunologically visible to Mα3M-1-Ab and cloned helper T cells.†

METHODS

Cell Culture. Proximal tubular cells (MCT cells) from the kidneys of SJL mice were isolated and carried in culture as a differentiated cell line (5). Tubulointerstitial fibroblasts (TFB) were isolated from the renal cortex, cloned by limiting dilution using cloning rings (15). They all were passaged in culture using Dulbecco's modified Eagle's medium (DMEM) with 100 units of penicillin per ml, 100 mg of streptomycin per ml, and 10% heat-inactivated fetal calf serum (GIBCO) at 37°C in 5% CO_2 in air every 48–72 hr.

Polyclonal α3M-1 Antibody. LEW rats were hyperimmunized with rabbit renal tubular antigen in complete Freund's adjuvant (4, 5). The specificity of this polyclonal antibody was confirmed by immunofluorescence (4) and by

Abbreviations: TBM, tubular basement membrane; αTBM, anti-TBM; 3M-1, nephritogenic target antigen of αTBM disease; α3M-1-Ab, antibody to 3M-1; Mα3M-1-Ab, monoclonal α3M-1-Ab; αTBM-Ab, antibody to the TBM; CSRTA, soluble TBM containing 3M-1; MCT, murine proximal tubular cell line expressing 3M-1; M30.2 cells, 3M-1-reactive helper T-cell line from SJL mice; TFB, tubulointerstitial fibroblasts; IPTG, isopropyl β-D-thiogalactopyranoside.

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†The sequence reported in this paper has been deposited in the Protein Identification Resource/National Biomedical Research Foundation data base (accession no. A33150).

immunoprecipitation of biosynthetically labeled ([^{3}H]leucine) 3M-1 from MCT cells using biotinylated mouse anti-rat IgG and streptavidin/agarose (5). Precipitates were disrupted with Laemmli sample buffer containing 5% 2-mercaptoethanol and run through an 8% SDS/PAGE gel (4, 5, 16). Gels were fixed in methanol/acetic acid, soaked in En^{3}Hance, dried, and exposed to X-Omat AR film at −70°C for 2 weeks.

Polyclonal Antibody Screening of Mouse Proximal Tubular cDNA Expression Libraries. Libraries were constructed and screened following the practices of Huynh *et al.* (17). mRNA from MCT cells (5) was used for reverse transcription with oligo(dT) or *Xho* I-oligo(dT) (300 ng/ml) and Moloney murine leukemia virus (200 units/μg of mRNA). *Eco*RI linkers or adaptors were ligated to blunt-end cDNAs polished with T4 DNA polymerase (10 units) following protection either with *Eco*RI methylase or by 5-methyl-deoxycytidine 5′-triphosphate incorporation during first-strand synthesis. These cDNAs were then cut with *Eco*RI or *Eco*RI/*Xho* I, separated on Sepharose CL-4B, ligated either into λgt11 or unidirectional λZAPII phages, and allowed to form plaques under nitrocellulose filters presoaked in 10 mM isopropyl β-D-thiogalactopyranoside (IPTG). Subsequent antibody screening of these filters was performed with polyclonal LEW α3M-1-Abs (preadsorbed against Sepharose 4B-immobilized lysates of BNN97 containing wild-type λgt11) followed by ^{125}I-labeled mouse anti-rat IgG. Informative phage isolates were subcloned, and their ^{32}P-labeled cDNA inserts were further used to rescreen the phage libraries for additional clones containing extended sequence.

DNA Sequence Analysis. cDNA inserts of plaque-purified phage were either subcloned into M13mp18 or excised with R408 helper phage (Stratagene) to form pBS phagemids. All reported clones were sequenced on both strands with universal or internal primers and Sequenase (United States Biochemical) using dideoxynucleotide chain-termination methods (18, 19). Sequences were stored and analyzed by MacVector, and data base searches (20, 21) were run on a VAX computer using modified software from Intelligenetics.

Northern Hybridizations. mRNA (20 μg) was electrophoresed through 1.2% agarose/formaldehyde gels and transferred to Zetabind (22). Filters were probed with a 120-base-pair (bp) *Eco*RI fragment of pM-1 labeled to high specific activity with hexamer primers in the presence of [^{32}P]dCTP (>3000 Ci/mmol; 1 Ci = 37 GBq). Hybridizations were performed at 62°C in 7% SDS/0.5 M sodium hydrogen phosphate/1 mM EDTA/50 μg of polyadenylic acid per ml with 50 μg of salmon sperm per ml. Filters were washed at 65°C in 0.1% standard saline citrate (SSC) with 0.5% SDS and exposed to Kodak XAR film for 18 hr.

Peptide Synthesis. Peptide sequences for P1 and P2 were deduced from an open-reading frame cDNA clone, pM-1, as described in the text. Peptides were then synthesized by the simultaneous multiple peptide method of Houghten (23) by the Protein Chemistry Core Laboratory, Department of Pathology, University of Pennsylvania.

***In Situ* Hybridizations.** Kidney sections of 5 μm were cut from paraffin blocks after fixation in ethanol/formaldehyde and then applied to chromalum-treated slides (22). Sections were deparaffinized in 100% xylene, incubated in 1% Triton X-100 for 10 min at 22°C, and permeabilized in 10 μg of proteinase K per ml in 20 mM Tris·HCl/2 mM $CaCl_2$, pH 7.4, for 20 min at 37°C. Hybridization fluid containing 50% formamide, 5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 10% dextran sulfate, 500 μg of single-stranded DNA per ml, 0.1% Triton X-100, and 0.5 μg of ^{35}S-labeled pM-2 or pBR322 cDNA probes per ml (0.5 to 1.0 × 10^8 cpm/μg) was heated to 100°C for 2 min, iced, and admixed with dithiothreitol to a final concentration of 10 mM. An aliquot of 40 μl of fluid was applied to each slide, enclosed with a glass coverslip, and hybridized at 40°C for 4–18 hr. Sections were then washed 30 min with 2× SSC at 22°C, 30 min each with 0.1× SSC and 0.1× SSC at 65°C, and 15 min with 0.1× SSC at 37°C. Slides were dipped in Kodak NTB-2 and developed for 2–5 days at room temperature, and the reaction product was developed with D19 and Kodak fixer. Slides were then counterstained with hematoxylin and examined under the microscope. Silver grains were counted in tubular cells expressing visible brush border from 10 random high-power fields (800×).

T-Cell Proliferation and Radioimmunoassays for 3M-1. Peptide recognition by 3M-1-reactive MαTBM-Ab was evaluated by solid-phase radioimmunoassay where serial dilutions of 56R-6 antibody (10) were incubated for 2 hr in polyvinyl chloride wells of a microtiter plate lined with various concentrations of P1 and P2 peptides blocked with 4% bovine serum albumin. The reactions were developed with affinity-purified ^{125}I-labeled goat anti-rat IgG, and individual replicate wells were counted for γ emissions. Helper T-cell recognition of peptides was measured by [^{3}H]thymidine incorporation (12, 15). M30.2 helper cells were grown under standard culture conditions on irradiated syngeneic splenocytes in RPMI medium with 10% fetal calf serum containing 10% interleukin 2 and various concentrations of P1 antigen, P2 antigen, or 3M-1-containing soluble tubular antigen (CSRTA). After 72 hr of coincubation at 37°C in 5% CO_2 in air, quadruplicate 100-μl samples of cell suspension were removed from each well and placed in a new 96-well plate along with 1 μCi of [^{3}H]thymidine. The cells were pulsed for 8 hr, after which they were harvested for scintillation spectroscopy. Values were expressed as the mean ± SEM.

Adoptive Transfers of Induced Effector T Cells. CD8$^+$-reactive effector T cells were induced from multiple cultures containing 30 × 10^6 nonirradiated splenocytes grown in standard medium in 25-cm^3 flasks at 37°C in 5% CO_2 in air for 5–6 days in the presence of 10 μg of P1 or P2 antigen per ml, interleukin 2, and T-cell helper factor (13, 16). Lymphocytes (4 × 10^7) harvested from these effector cell induction flasks were washed and injected under the kidney capsule in 75 μl of phosphate-buffered saline (15, 16). After 7 days the kidneys were harvested, fixed in 10% buffered formalin, and stained with hematoxylin/eosin (2).

RESULTS AND DISCUSSION

Isolation of cDNA Encoding 3M-1. A monospecific, polyclonal α3M-1-Ab was first prepared by hyperimmunizing a LEW rat (4, 5) with renal tubular antigen containing 3M-1 (4). This antibody produced a classical pattern of immunofluorescent staining by binding only to the proximal tubular basement membranes of BN/3M-1$^+$ and F344/3M-1$^+$ rats, but not to LEW/3M-1$^-$ or W/F/3M-1$^-$ (data not shown).

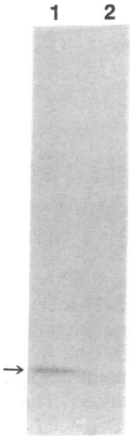


FIG. 1. Immunoprecipitation of 3M-1 secreted by MCT cells. MCT cells were biosynthetically labeled with [^{3}H]leucine, and their lysates were immunoprecipitated with LEW α3M-1-Ab (lane 1) or control antibody (lane 2) using biotinylated secondary antibody and streptavidin/agarose (5). The results on autoradiography revealed a single band of ≈30,000 M_r (indicated by arrow).

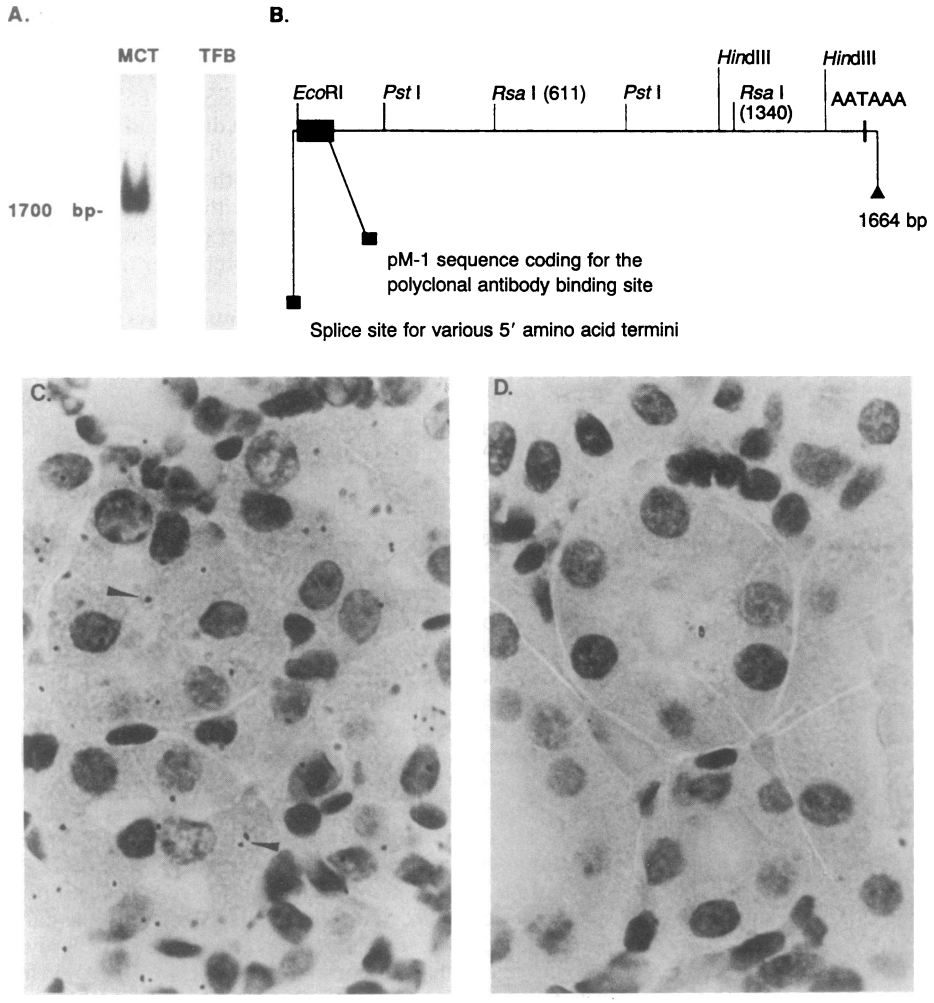


FIG. 2. Partial isolation of cDNA encoding 3M-1. (*A*) Northern hybridization of pM-1 with 20 μg of total RNA from MCT cells and TFB fibroblasts isolated from the same interstitial microenvironment (15) revealed a broad band hybridizing between 1700 and 1900 bp from MCT cells only. (*B*) Restriction map of cDNA clones representing pM-1 and pM-2 sequences from the framework region of the 3M-1 family of proteins. pM-1, isolated first by screening with monospecific, polyclonal antibody, contains a 5′ framework domain encoding the relevant peptide sequence (see Fig. 3*A*). pM-2 is the 3′ extension of this framework domain containing a poly(A) signal. The putative splice site for the various 5′ regions encoding the different amino termini is near the *Eco*RI site. (*C*) *In situ* hybridization of paraffin-embeded cortical mouse kidney with ^{35}S-labeled pM-2 cDNA (22) demonstrated multiple reaction products (arrowheads) localized to cortical tubular cells. (*D*) Control *in situ* hybridization of paraffin-embeded cortical mouse kidney with ^{35}S-labeled pBR322. No reaction product was observed in these cortical sections (350× original magnification).

Biosynthetically labeled MCT cell lysates immunoprecipitated with this antibody and run on reducing SDS/PAGE gels produced a single protein band of ≈30,000 M_r not observed with control antibodies (Fig. 1). This band is identical to the 3M-1 band immunoprecipitated by Mα3M-1-Ab and is of the same size as 3M-1 isolated from naive mouse kidney (5).

Candidate cDNA was initially isolated from λ phage libraries made from MCT epithelium by screening IPTG-induced filters with this monospecific, polyclonal α3M-1-Ab recognizing murine 3M-1 (17). Clones in the correct reading frame and orientation were identified at a rate of ≈1 per 200,000 plaques using our antibody detection system. Northern hybridizations (Fig. 2*A*) with pM-1, one of the initially selected clones as a probe, demonstrated the presence of a tight smear of transcripts between 1700 and 1900 bp in MCT cells but not in genetically identical TFB (15). Since the Northern blot suggested that a family of mRNAs was detectable, clone pM-1 was also used to isolate additional cDNAs by hybridization screening (17). DNA sequencing (18, 19) revealed that clone pM-2 extended the pM-1 sequence to a downstream poly(A) signal (Fig. 2*B*). From primer extension experiments (data not shown) the pM-1 and pM-2 sequences provide a structural framework, or common mRNA region, that extends in the 5′ direction into several different translational start sites confirmed by partially sequencing five different clones that diverge just proximal to the pM-1 region (unpub-

A. pM-1 open reading frame:

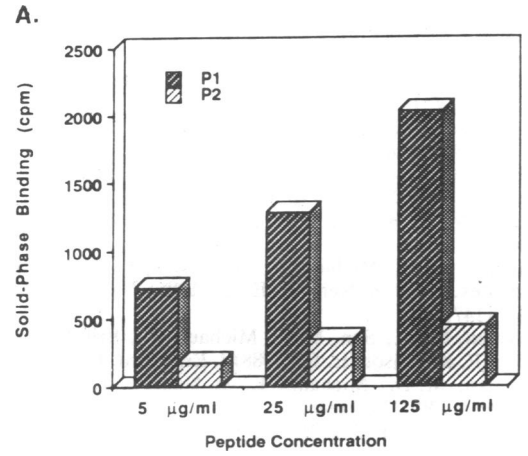
L L R R R H G D R R S T M S A E V P E A A S A E E Q K E M E D K V T S P E K A E E A

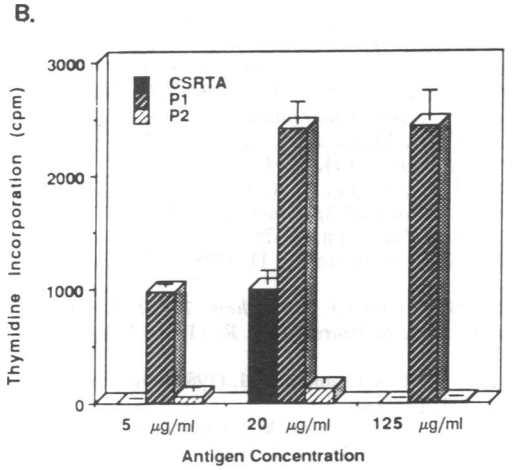
P1 P2

B. Database search:

PM-1	L L R R R H G D R R S T M S A E V P E A A S A E E Q K E M E D K V T S P E K A E E A		
MMP	I E E T K V E D E K S E M E E T L T V I A E E L A A S A K E E K E E A E E K E E E P E A	V K S P V K S P E A	**POSIT (457-510)**
RMP	I E E T K V E D E K S E M E D A L T A I A E E L A A S A K E E K E E A E E K E E E P E	E K S P V K S P E A	**POSIT (457-509)**

FIG. 3. Amino acid analysis of the pM-1 peptide. (*A*) Deduced single-letter amino acid sequence of pM-1 containing an immunodominant epitope (see Fig. 4). Two synthetic peptides P1 (18-mer) and P2 (22-mer) were synthesized using the underlined regions of the pM-1 sequence so that each reflected approximately half of the region (23). Abbreviations for the amino acid residues are A, Ala; D, Asp; E, Glu; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val. (*B*) Amino acid sequence comparisons of pM-1 and mouse (MMP) and rat (RMP) intermediate filament-associated proteins (24, 25). The analyses demonstrate a nonrepetitive sequence similarity involving 20 matches and 2 conserved substitutions. The gaps were inserted to optimize the alignment. The positions of the MMP and RMP peptides are indicated at the right.





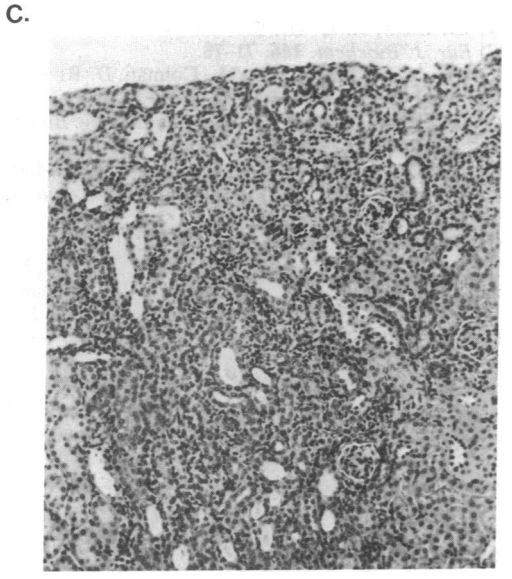


FIG. 4. Characterization of an immunodominant epitope. (*A*) Monoclonal antibody 56R-6, with specificity for an immunodominant epitope expressed by 3M-1 (10), was also observed to preferentially react with the P1 peptide when compared with the P2 peptide. The assay was performed by solid-phase radioimmunoassay and the values are reported as cpm. There was <5% variability between replicates. (*B*) M30.2 helper T cells (12, 13, 16) were originally established in culture as a 3M-1-reactive cell line. These cells were grown in the presence of collagenase-solubilized renal tubular antigen (CSRTA) containing 3M-1 or with various concentrations of P1 and P2 peptides. After 72 hr in culture the experimental wells were labeled for 6 hr with [^{3}H]thymidine. Incorporations were expressed as mean cpm ± SEM reflecting cell growth. This helper T-cell line expressed preferred reactivity with peptide P1. (*C*) P1 peptide was added to a nephritogenic effector T-cell induction culture (13, 16). After 5 days in culture 4×10^7 harvested lymphocytes were injected under the kidney capsule of a naive mouse. Seven days later the kidney was removed and processed for light microscopy. The interstitial inflammation initiated by infiltrating cells extends down into the cortical interstitium (107× original magnification). This infiltration and injury only occurs with 3M-1-reactive CD8$^+$ effector T cells. T cells of other phenotype or antigenic specificity remain beneath the capsule but out of the kidney (refs. 12, 16; unpublished observations).

lished results). The structural characterization of these additional family members is beyond the scope of the current report. Since the 3M-1 antigen is expressed widely along most murine cortical TBMs by serological criteria (2), it was reassuring that ^{35}S-labeled pM-2 detected *in situ* transcripts (22) within the majority of cortical tubular cells of the naive adult mouse kidney (Fig. 2 *C* and *D*). The *in situ* grain counts for the pM-2 reaction product were ≈13.5 ± 3.3 grains per high-power field with pBR322 control grain counts near zero.

Characterization of a Major Nephritogenic Domain. The pM-1 cDNA encoding the αTBM-Ab binding sequence contains an open reading frame that was used to search the National Biomedical Research Foundation (Protein Identification Resource) and the University of Geneva protein sequence (Swiss-Prot) data bases (Fig. 3*A*). This open-reading frame shares a 37% nonrepetitive structural similarity (Fig. 3*B*) with two intermediate filament-associated proteins (MMP and RMP; refs. 24, 25). Intermediate filament-associated proteins constitute a rather large and heterogeneous protein family that contributes to the organization of the intracellular infrastructure as well as provides specialized support for cell–cell surface interactions at desmosomes or with extracellular matrix at basal hemidesmosomes (26, 27).

Two short peptides, P1 and P2, deduced from the pM-1 framework sequence, were analyzed by a software algorithm (28) integrating a variety of determinants of protein conformation to assist in the prediction of peptide immunogenicity. They were then manually synthesized by the simultaneous multiple peptide method of Houghten (ref. 23; Fig. 3*A*) and subsequently employed in a series of immunologic studies to determine whether they could be recognized by 3M-1-reactive antibodies and T cells derived from animals with αTBM disease. Monoclonal αTBM-Ab (56R-6), which binds an immunodominant epitope of 3M-1 (10), recognizes P1 in a dose-dependent fashion but does not recognize the P2 peptide (Fig. 4*A*). M30.2 cells (17), a 3M-1-reactive helper T-cell clone, also preferentially recognizes and proliferates in the presence of P1 but not P2 peptide (Fig. 4*B*).

Antibodies, to the extent a general statement is possible, tend to recognize conformational determinants, whereas helper T cells tend to recognize small fragments of processed, linear peptide that are bound and presented by major histocompatibility complex class II molecules on the surface of antigen-presenting cells (29, 30). One prediction of these general observations is that T cells and antibodies might tend to recognize different epitopes (30). Some investigators have even suggested that relevant T-cell epitopes, on occasion, preferably express amphipathic α-helices (31) or contain certain tetrameric or pentameric patterns of amino acid sequence (32). Such antigenic regions, when utilized by helper T cells, can influence the specificity of subsequent antibody responses (33). In our case, P1 and P2 peptides were found to have an equal lack of amphipathicity but a similar degree of hydrophilicity and surface probability. P1 peptide, however, has a higher antigenic index (34), suggesting more epitopic visibility in spite of the fact that P2 had greater predicted helicity. P1, furthermore, did not contain any

preferred patterns of T-cell recognition sequence (32) and seems to be a curious example of an autoepitopic domain that is recognized by antibodies and T cells.

Although the T helper clone and monoclonal antibody described above were initially isolated by virtue of their reactivity to 3M-1, and then randomly selected for analysis, they each preferentially recognize the P1 region of 3M-1. In preliminary experiments, however, mice immunized with P1 peptide conjugates in adjuvant did not develop interstitial nephritis. These unexpected results are open to several interpretations. One is that normal, protective mechanisms of tolerance to 3M-1 (35) are also P1 specific. Their enhancement, by immunization with informative peptides mimicking important regions of self, might obviate the early induction of disease (unpublished results). Another explanation is that, although P1 is of sufficient size to be recognized by an established anti-3M-1 helper cell response, the induction of such a response may require a more complex structure than the 18-amino acid peptide. We, therefore, next designed an *in vitro* experiment that bypassed the early induction of helper cells but still used the P1 or P2 antigen peptide, in conjunction with premade 3M-1-binding helper factor (16), to help engage the development of nephritogenic effector T cells from naive lymphocytes in culture (13, 16). Effector T cells, induced with P1 antigen and harvested after 7 days of culture, were capable of producing interstitial lesions on adoptive transfer (Fig. 4*C*). No such lesions were produced with T cells harvested from cultures induced with P2 antigen (data not shown). Such a finding is consistent with previous studies where P1-reactive nephritogenic effector T cells could be harvested from mice with interstitial nephritis and established as cultured lines (14).

The 3M-1 antigen appears to be a unique, complex protein family of unknown functional significance to the tubular epithelium. All of the family members share an immunologically visible framework domain. Only very few target antigens of autoimmune disease have been sufficiently described at the molecular level to be analyzed for target epitopes (36, 37). Further study of the P1 region, and perhaps other peptide fragments comprising these molecules, may contribute a better understanding of the mechanisms for selecting autoimmune-relevant T- and B-cell repertoires during the development of nephritogenic immune responses. Identification of immune products that recognize dominant, pathogenic epitopes may also permit the further refinement of therapeutic strategies that may favorably alter the course of autoimmune injury (1, 10).

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