with BHBN and amino acids than in the group treated with BHBN alone, and the amino acids exhibited dose-dependent effects (Table 2). However, the incidences of simple hyperplasia, PN hyperplasia, and papilloma in groups 1 through 4 were not significantly different from those in group 5. Groups 6, 7, and 8 did not show any abnormalities of the bladder epithelium. These results confirm that L-isoleucine and L-leucine have tumor-promoting effects on bladder carcinogenesis in rats.

The amounts of free L-isoleucine in the urine of rats given CE-2 diet or CE-2 diet supplemented with 2.0 percent or 4.0 percent L-isoleucine were 0, 149, and 1008 μg/ml, respectively (*11*); the amount of free L-leucine in the urine of rats given CE-2 diet without or with 2.0 percent or 4.0 percent L-leucine were 0, 309, and 1141 μg/ml, respectively. The concentrations of L-isoleucine and L-leucine as protein in the CE-2 diet were 1.03 and 1.80 percent, respectively. Animals given the CE-2 diet alone excreted neither L-isoleucine nor L-leucine in their urine. When we added 2.0 or 4.0 percent free L-isoleucine or 2.0 or 4.0 percent free L-leucine to the diet, the amounts of the free forms of both amino acids detected in the urine were less than 0.5 percent of those given orally. There may be a relation between the tumor-promoting effects of these two amino acids and the high incidence of human bladder cancer in western countries, where the diet is rich in protein (*12*).

REFERENCES AND NOTES

1. I. Berenblum and P. Shubik, *Br. J. Cancer* **1**, 383 (1947).
2. S. M. Cohen, M. Arai, J. A. Jacob, G. H. Friedell, *Cancer Res.* **39**, 1207 (1979).
3. R. M. Hicks and J. Chowaniec, *ibid.* **37**, 2943 (1977).
4. S. Fukushima *et al.*, *ibid.* **43**, 4454 (1983).
5. M. M. Melicow, *J. Urol.* **68**, 261 (1952).
6. T. Kakizoe, T. Kawachi, T. Sugimura, *Cancer Res.* **39**, 3353 (1979).
7. T. Kakizoe, H. Komatsu, T. Niijima, T. Kawachi, T. Sugimura, *ibid.* **40**, 2006 (1980).
8. ———, *ibid.* **41**, 4702 (1981).
9. T. Kakizoe, H. Komatsu, Y. Honma, T. Niijima, T. Sugimura, *Gann* **73**, 870 (1982).
10. N. Ito, Y. Hiasa, A. Tamai, E. Okajima, H. Kitamura, *ibid.* **60**, 401 (1969).
11. Urine samples were collected for 24 hours from rats in metabolic cages that were given CE-2 diet alone or CE-2 diet supplemented with 2.0 or 4.0 percent L-isoleucine or L-leucine. Samples were treated with an equal volume of 10 percent trichloroacetic acid and then centrifuged for 10 minutes at 12,000 rev/min. The supernatant was diluted with citrate buffer and subjected to high-performance liquid chromatography (Toyo Soda Company, Tokyo), and fractions were treated with ninhydrin.
12. E. L. Wynder and G. B. Gori, *J. Natl. Cancer Inst.* **58**, 825 (1977).

13. This work was supported by a grant-in-aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control and grants-in-aid from the Ministry of Health and Welfare (58-5) and the Ministry of Science, Education, and Culture (58010062) of Japan. We thank N. Ito for helpful discussions.

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Expression Cloning of a Lymphocyte Homing Receptor cDNA: Ubiquitin Is the Reactive Species

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The lymphocyte cell surface receptor for the high endothelial venules (HEV's) of peripheral lymph nodes is specifically recognized by the monoclonal antibody MEL-14. Three independent complementary DNA (cDNA) clones, each of which encodes the protein ubiquitin, were detected by virtue of the expression of the MEL-14 antigenic determinant on cDNA–β-galactosidase bacterial fusion proteins. The antigenic determinant defined by MEL-14 resides in the carboxyl terminal 13-amino-acid proteolytic peptide of ubiquitin, but is undetected in intact undenatured ubiquitin and other cellular ubiquitinated proteins. Antisera and monoclonal antibodies to ubiquitin determinants bind to the surface of both HEV-receptor positive and negative cell lines. The MEL-14–identified cDNA clones hydridize to RNA transcripts that encode tandemly repeated ubiquitins. Sequence analysis of these polyubiquitin cDNA's does not identify a leader sequence for export to the cell surface. The expression of the MEL-14 epitope of ubiquitin depends upon its local environment. The steady-state levels of expression of the ubiquitin messenger RNA's do not correlate with either the tissue derivation of the RNA or the expression of the lymphocyte HEV receptor. Regulation of the expression of the HEV receptor is not likely to reflect the transcriptional control of ubiquitin genes, but rather to reflect control of the expression of the HEV core polypeptide or its level or form of ubiquitination.

MATURE LYMPHOCYTES CIRCULATE throughout the body, passing through the lymphoid organs between the blood vasculature and lymphatic systems (*1*). The mobility of these cells allows them to encounter the various microenvironments necessary for maturation, to interact with the various other lymphocyte subsets, and to interact specifically with antigen. A major event in the migration pathway occurs when lymphocytes specifically recognize and adhere to specialized high endothelial cells of the postcapillary venules (called HEV's) of the peripheral lymph nodes and the gut-associated Peyer's patches. These bound lymphocytes transmigrate into the parenchyma of the lymphoid organ, and may eventually return by way of the lymphatic system to the bloodstream (*1*).

Most normal murine small lymphocytes express receptors for HEV cells in peripheral nodes and Peyer's patches (*2, 3*). The organ distribution of lymphocyte subsets may be determined in part by the HEV adherence properties of individual lymphocytes. These adherence properties may be measured both in vivo and in vitro. Lymphocytes, incubated on a tissue section of peripheral lymph nodes or Peyer's patches, adhere avidly and specifically to the exposed HEV's (*4, 5*). Some murine lymphomas express a virtually unispecific preference for one or the other type of HEV (*2, 6, 7*). These data suggest that there are at least two types of lymphocyte HEV receptors, one for the HEV of peripheral lymph nodes and one for the HEV of Peyer's patches.

A monoclonal antibody, MEL-14, appears to recognize the lymphocyte receptor for peripheral lymph node HEV (*7*). The expression of the cell surface MEL-14 epitope correlates without known exception to the peripheral lymph node adhesion phenotype of both normal cells and neoplastic lymphoid cell populations (*7, 8*). All peripheral node HEV binding lymphomas express a MEL-14 reactive antigen, while Peyer's patch HEV unispecific or nonbinding tumors lack MEL-14 antigen. In addition, clonal variants of lymphoid tumors, selected for the expression of high or low levels of the MEL-14 antigen (*9*), express the expected adhesion phenotype. Treatment of both MEL-14 antigen-positive lymphoid tumors and normal mesenteric node lymphocytes

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with MEL-14 antibodies specifically inhibits their adhesion in vitro and homing in vivo to peripheral lymph node HEV's (*7*). In contrast, treatment with MEL-14 antibodies has no effect on adhesion of either a Peyer's patch–specific lymphoma or normal lymphocytes to Peyer's patch HEV (*7*). This specificity strongly suggests that the MEL-14 epitope is a component of, or is tightly associated with, the lymphocyte cell surface receptor for peripheral lymph node HEV.

MEL-14 recognizes a cell surface glycoprotein designated gp90^{MEL-14} with an apparent molecular size of 90 ± 10 kD, differing slightly between cell lines. This molecule is not disulfide-linked to any other polypeptide chain, although there is evidence for internal disulfide bonding (*7*).

We report here that the MEL-14 monoclonal antibody detects hybrid β-galactosidase complementary DNA (cDNA) fusion proteins (*10, 11*) expressing the MEL-14 epitope from a bacteriophage λ cDNA-lacZ gene fusion library. This cDNA library was constructed from messenger RNA (mRNA) isolated from the B-cell lymphoma 38C-13, which is the cell line used (*7*) for the immunization that led to the production of the MEL-14 monoclonal antibody. Using this antibody screening technique, we detected three independent cDNA clones, each of which encodes a well-studied protein, ubiquitin.

Ubiquitin is a highly conserved 76-amino-acid protein (8.5 kD) (*12*). The sequence of ubiquitin is identical between species as diverse as human, cow, Mediterranean fruit fly, *Xenopus* (*13*), and chicken (*14*). Ubiquitin is found within cells as free monomer, and as a part of branched chain polypeptides. It may be exceptional in its ability to form branched chain conjugates with a number of cellular proteins by means of an "isopeptide" bond between its COOH-terminal carboxyl group and the ε-amino groups of lysine residues of selected cytoplasmic and nuclear proteins (*15–17*). The best studied such isopeptide structure is uH2A (ubiquitinated histone 2A), the A24 chromosomal protein (*15*). Functions suggested for ubiquitin include roles in specific proteolysis (*18–20*) and chromatin structure and gene regulation (*21, 22*). An absolute cellular requirement for an intact ubiquitin conjugation system is strongly supported by genetic evidence (*23*). A mammalian cell cycle mutant, ts85, has been shown to have a temperature-sensitive defect in the ubiquitin-activating enzyme E1. Recently, the gene structures encoding the precursor polyubiquitin in human (*24*), *Saccharomyces* (*25*), *Xenopus* (*13*), and chicken (*14*) have been described.

At present, ubiquitin has only been detected as a component of the cytoplasm and nucleus. We now show that the cell surface MEL-14 epitope is located in the ubiquitin polypeptide, and that antisera to β-galactosidase–ubiquitin fusion proteins bind to the surfaces of both gp90^{MEL-14}-positive and gp90^{MEL-14}-negative lymphoid tumors. We have also demonstrated (*26*), by amino acid

A

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    glu phe arg ala asn Met Gln Ile Phe Val Lys Thr Leu Thr
  1 GAA TTC CGC GCC AAC ATG CAG ATC TTC GTG AAG ACC CTG ACG  42

    Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile
 43 GGC AAG ACC ATC ACT CTT GAG GTC GAG CCC AGT GAC ACC ATC  84

    Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro
 85 GAG AAT GTC AAG GCC AAG ATC CAA GAC AAG GAA GGC ATC CCA 126

    Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu
127 CCT GAC CAG CAG AGG CTG ATA TTC GCG GGC AAA CAG CTG GAG 168

    Asp Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser
169 GAT GGC CGC ACC CTG TCC GAC TAC AAC ATC CAG AAA GAG TCC 210

    Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly ile ile glu
211 ACC TTG CAC CTG GTG CTG CGT CTG CGC GGT GGC ATC ATT GAG 252

    pro ser leu arg gln leu ala gln lys tyr asn cys asp lys
253 CCA TCC CTT CGT CAG CTT GCC CAG AAG TAC AAC TGT GAC AAG 294

    met arg gly gly gly gly gly gly
295 ATG AGG GGG GGG GGG GGG GGG G                         319
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B

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    glu phe arg Arg Leu Arg Gly Gly Met Gln Ile Phe Val Lys
  1 GAA TTC CGT CGC CTC AGA GGT GGC ATG CAG ATC TTT GTG AAG  42

    Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser
 43 ACC CTG ACA GGC AAG ACC ATC ACC CTG GAG GTC GAG CCC AGT  84

    Asp Thr Ile Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu
 85 GAC ACC ATA GAG AAT GTC AAG GCA AAG ATC CAG GAC AAG GAG 126

    Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys
127 GGC ATC CCC CCT GAC CAG CAG AGG CTG ATC TTT GCA GGC AAG 168

    Gln Leu Glu Asp Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln
169 CAG CTG GAA GAT GGC CGC ACC CTG TCA GAC TAC AAC ATC CAG 210

    Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly
211 AAA GAG TCC ACC CTG CAC CTG GTC CTT CGC CTC AGA GGT GGC 252

    Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr
253 ATG CAG ATC TTT GTG AAG ACC CTG ACA GGC AAG ACC ATC ACC 294

    Leu asp Val Glu Pro Ser val Thr thr lys lys gly gly gly
295 TTG GAC GTC GAG CCC AGT GTT ACC ACC AAG AAG GGG GGG GGG 336

    gly gly gly
337 GGG GGG GGG GG                                        347
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C

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ARF2        f r a n - - - - - - - - - - - - - - - - - -
UB      L V R L R G G M Q I F V K T L T G K T I T L E V E
ARF3    f r - - - - - - - - - - - - - - - - - - - - - - -

ARF2    - - - - - - - - - - - - - - - - - - - - - - - - -
UB      P S D T I E N V K A K I Q D K E G I P P D Q Q R L
ARF3    - - - - - - - - - - - - - - - - - - - - - - - - -

ARF2    - - - - - - - - - - - - - - - - - - - - - - - - -
UB      I F A G K Q L E D G R T L S D Y N I Q K E S T L H
ARF3    - - - - - - - - - - - - - - - - - - - - - - - - -

ARF2    - - - - - - - - i i e p s l r q l a q k y n c d k
UB      L V L R L R G G M Q I F V K T L T G K T I T L E V
ARF3    - - - - - - - - - - - - - - - - - - - - - - - d -

ARF2    m r g g g g g g
UB      E P S D T I E N V K A K I Q D K E G I P P D Q ...
ARF3    - - - v - t k k g g g g g g
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Fig. 1. Ubiquitin is encoded by the cDNA sequences detected with MEL-14. The MEL-14 monoclonal antibody was used to isolate λgt11 cDNA–β-galactosidase gene fusion clones expressing the epitope detected on the cell surface protein gp90^{MEL-14} as described below. The complete DNA sequences of the cDNA inserts of two of these clones, arf2 (A) and arf3 (B), and their protein coding sequence in frame with that of the lacZ gene is shown. These sequences begin with the Eco RI linker installed during cDNA synthesis. The first ubiquitin amino acid in arf2 is a ubiquitin NH_2-terminal methionine, located at position 6. In arf3 the COOH-terminal ubiquitin sequence RLRGG (Arg, Leu, Arg, Gly, Gly) precedes a complete 76-amino-acid ubiquitin coding sequence and a second, truncated, ubiquitin coding unit beginning with the Met residue (underlined) located at nucleotide 253. The amino acids corresponding to those of the ubiquitin sequence are capitalized. (C) Comparison of these sequences with the ubiquitin sequence. Ubiquitin amino acids are shown as dashes in arf2 and arf3. Arf2 contains seven nucleotides of nonubiquitin sequence between the linker sequence and the first ubiquitin amino acid, and encodes a single ubiquitin coding unit, terminated with nonubiquitin amino acids. Arf3 encodes about 1.25 ubiquitin coding units with a single COOH-terminal to NH_2-terminal ubiquitin junction. Single-letter abbreviations for the amino acid residues are: A, alanine; B, aspartic acid or asparagine (ASX); C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; Tyr, tyrosine; V, valine; and W, tryptophan.

sequencing, that the purified MEL-14 reactive cell surface protein has two NH_2 termini, one of which is ubiquitin, the other a novel sequence to which ubiquitin is presumably conjugated. We here demonstrate that independent monoclonal antibodies to ubiquitin (*27*), distinct from MEL-14, also stain the surface of lymphoid cells. Taken together, these data suggest that ubiquitin is an important component of the lymphocyte receptor for peripheral lymph node HEV, and that ubiquitin may be a component of other cell surface structures.

Antibody screening of fusion proteins has been used to isolate both yeast (*10*) and mouse (*11*) genes encoding the β-galactosidase fusion partner. We have screened λgt11 cDNA libraries with the MEL-14 antibody to isolate cDNA clones encoding the homing receptor antigen. The C3H mouse B lymphoma cell line 38C13 was chosen as the source of mRNA for cDNA library construction. This lymphoma expresses high levels of the MEL-14 cell surface antigens, and was the immunizing cell line used to develop the MEL-14 monoclonal antibody (*7*). Complementary DNA clones expressing the MEL-14 determinant were rare, being identified and successfully plaque-purified at a frequency of 1×10^{-5}. The three cDNA clones arf2, arf3, and arf4 were ultimately examined. These three cDNA's cross-hybridized to each other, yet represented independent isolations of related DNA sequences.

The complete DNA sequences of arf2 and arf3 are shown in Fig. 1, A and B. The arf2 cDNA encodes a single open reading frame in each direction extending the entire length of the clone. However, arf3 encodes only one long reading frame. The reading frame of arf3 and one of the reading frames of arf2 are in frame with the upstream β-galactosidase coding sequences. These reading frames encode the entire sequence of the 76-amino-acid protein ubiquitin.

The ubiquitin portion of the protein sequence encoded by arf2 and arf3 is identical to the sequence of human ubiquitin (Fig. 1C). In arf2, the ubiquitin coding sequence begins with the ubiquitin NH_2-terminal methionine residue which is four amino acids from the point of fusion with β-galactosidase and proceeds through the entire 76 amino acids of ubiquitin before diverging from the ubiquitin sequence. In arf3, the 76-amino-acid ubiquitin sequence is flanked on the NH_2-terminal side with seven amino acids, five of which are derived from the ubiquitin COOH-terminal sequence; it is flanked on the COOH-terminal side with 25 amino acids derived from a ubiquitin-related sequence (Fig. 1, B and C). The arf2 and arf3 cDNA clones both encode nonubiquitin-derived amino acids located downstream from a ubiquitin COOH-terminus. The downstream nucleotide and amino acid sequences in arf2 are unrelated to ubiquitin. In arf3, the downstream DNA sequence completely diverges from that of the ubiquitin coding region after several nucleotide differences that result in amino acid substitutions. The sequence of these cDNA clones compared to additional full-length cDNA clones indicates that the arf2 and arf3 cDNA clones are derived from sequences near the 3′ ends of two different ubiquitin mRNA's. The arf3 encodes a novel ubiquitin head-to-tail tandem repeat junction, with NH_2-terminal ubiquitin sequences adjacent to COOH-terminal ubiquitin sequences. A feature common to these two clones is the exact ubiquitin coding unit, suggesting that the MEL-14 antigenic determinant is encoded within the ubiquitin portion of these clones.

The asymmetrically located Bgl II site in the arf2 cDNA clone allows the orientation of this insert relative to the lacZ gene to be verified. The arf2 cDNA encodes a fusion of β-galactosidase and ubiquitin coding sequences. The partial DNA sequence analysis of the Pvu II fragment that spans the lacZ-cDNA junction (beginning 358 nucleotides 5′ to the Eco RI insertion site of lacZ and ending at the Pvu II site 160 nucleotides into the arf2 insert) confirms the fusion of β-galactosidase with ubiquitin coding sequences.

While the antibody-reactive arf cDNA clones each encode a ubiquitin unit, the MEL-14 determinant is present on only a rarely occurring subset of β-galactosidase–ubiquitin fusion proteins. In these experiments, three cDNA's expressed the MEL-14 determinant. These clones were present in the cDNA library at a frequency of approximately 10^{-5}. Screening the library with a nucleic acid probe encoding ubiquitin indicated that 0.5 percent of all cDNA clones contained ubiquitin sequences. Since one-sixth of these cDNA's can be expected to be in-frame ubiquitin fusions to β-galactosidase, it appears that MEL-14 reactivity is unusual, occurring in about 1 percent of the in-frame fusions. Thus, only a specific subset of β-galactosidase–ubiquitin structures can mimic the conformation of the cell surface antigen.

To examine whether other ubiquitin epitopes might be accessible on lymphocyte cell surfaces, rat antisera to a β-galactosidase ubiquitin fusion protein were prepared. These sera bind to MEL-14 determinant positive (38C13), low (EL-4), and negative (P815) cell lines. Fluorescence-activated cell sorter (FACS) analysis of these cell lines by indirect immunofluorescence with nonimmune sera and antisera to the ubiquitin fusion protein is shown in Fig. 2. The sera bind at high levels to these cells. Thus ubiquitin–β-galactosidase antigenic determinants are present on the cell surface. This antibody binding may be due to the detection of ubiquitin determinants, to determinants generated by the linear fusion of the two dissimilar polypeptides in the fusion protein, or to determinants of β-galactosidase. The last possibility seems unlikely; we have not observed cell surface binding with bona fide monoclonal or polyclonal antibodies to β-galactosidase. These antisera must contain antibodies with epitope specificities in addition to that of MEL-14 in that they also stain MEL-14 negative cells (RAW112, P815) (Fig. 2). The presence of

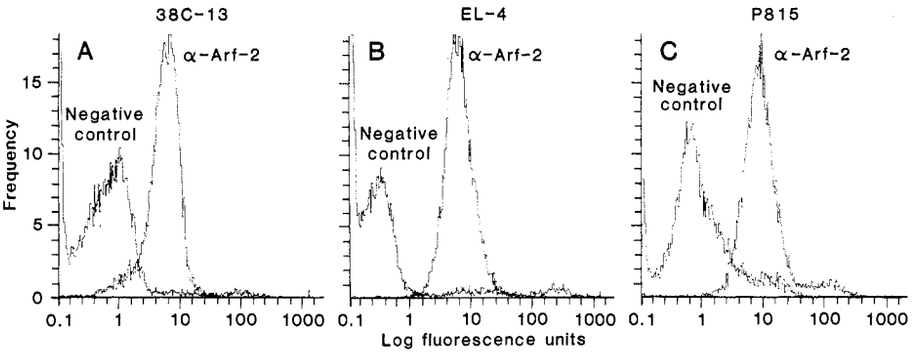

Fig. 2. Rat antisera to the β-galactosidase–ubiquitin fusion protein detects antigenic determinants on the cell surface. The cell surface staining of (A) the C3H B-cell lymphoma 38C-13 (MEL-14–positive), (B) the C57Bl/Ka T-cell lymphoma EL-4 (MEL-14 low), and (C) the DBA/2 mastocytoma P815 (MEL-14–negative) was performed as described in the legend to Fig. 3. The β-galactosidase–ubiquitin fusion protein was purified from induced SG1041 (lacU169, proA$^+$, lon$^-$, araD139, StrA) lysogens of the arf2 cDNA phage by either SDS–acrylamide gel electrophoresis or by immunoprecipitation with MEL-14–Sepharose. In some cases, the cleared lysate was used as a source of soluble fusion protein, although the bulk of the fusion protein material was located in the insoluble sedimented fraction. Rats were immunized (by intraperitoneal injection) with either crushed acrylamide gel slice, or with material eluted from MEL-14–coupled Sepharose 4B.

these epitopes on MEL-14 positive and negative cells led us to test for the existence of other ubiquitin determinants on the cell surface using authentic monoclonal antibodies to ubiquitin. At the same time we found that the putative lymph node homing receptor glycoprotein contains at least one ubiquitin peptide (*26*). Mouse monoclonal antibodies to human ubiquitin have been prepared and characterized (*27*); the specificity of these monoclonal antibodies to ubiquitin was determined by solid-phase immunoassays [radioimmunoassay (RIA) and Western blots] as these reagents have a relatively low affinity for free ubiquitin in solution. Of the ten monoclonal antibodies to ubiquitin tested, 12H11 and 43E12 unequivocally reacted with structures expressed at the cell surface. The FACS analysis of the indirect immunofluorescence data obtained with these reagents of an EL-4 variant (EL-4/MEL-14^{hi}), which was selected by flow cytometry for high-level expression of the MEL-14 antigen (*9, 26*), is shown in Fig. 3A. Although neither antibody exhibited binding at levels comparable to MEL-14, both reagents bound to the cell surface at levels significantly higher than that observed with the isotype-matched negative control antibodies. These two monoclonal antibodies also bind the surfaces of MEL-14 negative cell lines. The median fluorescence with these antibodies differs between cell lines. These differences suggest that the monoclonal antibodies to ubiquitin are not recognizing the same ubiquitin epitopes or have different affinities. The fact that binding above background was not observed with the other eight antibodies may simply be due to lower affinities, or perhaps, more significantly, it may reflect the location of the reactive epitopes within ubiquitin and their availability in the context of ubiquitin presentation on the cell surface. In any case, the fluorescence of MEL-14 antigen–negative cells observed with the antisera to the arf2 fusion protein and with monoclonal antibodies to ubiquitin suggests that cell surface structures other than the lymphocyte receptor for HEV may contain ubiquitin, a finding confirmed by immunoprecipitation analysis (*26*).

Radioimmunoassay (RIA) of ubiquitin and its enzymatically prepared peptides reveals the epitope specificity of MEL-14 and other ubiquitin monoclonal antibodies (Table 1). The mouse monoclonal antibodies 42D8 and 12H11 to ubiquitin bind to intact ubiquitin, but not to the NH_2- and COOH-terminal peptides tested (*27*). MEL-14 binds specifically to the COOH-terminal peptide of ubiquitin, but not intact monomeric ubiquitin, unless the ubiquitin had been denatured in sodium dodecyl sulfate (SDS) prior to placement in the RIA plastic microwell. Thus by two independent assays MEL-14, the lymph node homing receptor antibody, is an antibody to ubiquitin.

Table 1. MEL-14 recognizes a carboxyl terminal epitope on ubiquitin. The binding of monoclonal antibodies to ubiquitin epitopes was determined by radioimmunoassay. Wells were coated with ^{125}I-labeled peptide. The coated wells were incubated with antibody and washed, and developing reagents were added. Binding of 42D8, 12 H11, and 61C8 mouse antibodies to the wells was assayed with ^{125}I-labeled goat antibody to mouse Ig as a second-stage reagent. MEL-14 is a rat antibody and was identified in a three-stage assay; the developing reagents were a second-stage rabbit antibody to mouse Ig, followed by ^{125}I-labeled protein A. The three-stage reaction gave higher backgrounds than the two-stage reaction.

Antigens*	Monoclonal antibodies† (count/min, bound)			
	4-2D8	1-2H11	61C8	MEL-14
None	250	369	300	1350
Ub 1-11	249	364	379	1215
Ub 64-76	241	400	ND	**8135**
Ub	**6870**	**2424**	328	1800‡

*Ub, ubiquitin; Ub 1-11, the NH_2-terminal 11-amino-acid fragment of ubiquitin. Ub 64-76, the COOH-terminal 13-amino-acid fragment of ubiquitin. All peptides were solubilized in phosphate-buffered saline before application to plates. Peptides were prepared as described in (*27*). †4-2D8 and 61C8 are mouse IgG_1 antibodies; 1-2H11 is a mouse IgM antibody, and MEL-14 is a rat IgG_{2a} antibody; 61C8 is an isotype control, and its antigen is an anion transport protein (*27*). ‡If ubiquitin is first denatured in SDS-containing buffer, this value approaches that of the Ub 64-76 peptide.

Our findings suggest that ubiquitin is a component of the lymph node HEV receptor and of other cell surface proteins. We have used MEL-14 antibodies to isolate the gp90^{MEL-14} for amino acid sequence analysis and have evidence that the murine lymphocyte lymph node HEV receptor is a cell surface protein modified by the attachment of the highly conserved 76-amino-acid protein, ubiquitin (*26*).

MEL-14 antibody is directed against an unusual conformational determinant of the 8.5-kD protein ubiquitin. This determinant is not revealed in undenatured monomeric ubiquitin, or other ubiquitin conjugates, as determined by direct examination of purified ubiquitin and as evidenced by the lack of significant cytoplasmic or cell surface staining on most cells (*8, 28*). These experiments provide evidence that this amino acid sequence–specified antigenic determinant may depend on its microenvironment for its expression, as proposed for other peptide antigens (*29*). The impressive selectivity of expression of a particular antigenic determinant on a highly conserved, ubiquitous protein suggests a specific effect on the tertiary structure of the ubiquitin polypeptide by the associated protein. The portion of ubiquitin most likely to be affected is the COOH-terminal region, the portion nearest the carrier sequences. Indeed, by analysis of the reactivity of MEL-14 with proteolytic fragments of ubiquitin, the MEL-14 determinant is located within the COOH-terminal 13 amino acids (numbers 64 to 76) of ubiquitin (Table 1) (*27*). Examination of the Dayhoff Protein Sequence Data Base indicates that residue sequence 64 through 76 is unique to ubiquitin, as are the eight possible six-amino-acid residue subfragments. Consistent with these observations, the three-dimensional structure of ubiquitin, as determined by x-ray diffraction methods (*30*), indicates that the COOH-terminus of ubiquitin projects outward from the rest of the molecular structure and may be either unordered, or highly flexible. As such, it appears to be accessible for covalent interactions with other proteins.

The absence of immunohistochemical staining of all cells with MEL-14 in mouse or human tissue sections, despite the omnipresent nature of the ubiquitin polypeptide (present, at least, as free ubiquitin, and the well-characterized nuclear protein uH2A) suggests that other specific conformations of ubiquitin may exist which do not display the MEL-14 determinant, while one specific structure, the lymphocyte receptor for peripheral lymph node HEV, does (*8, 28*).

In view of the conformation-dependent aspects of the expression of the MEL-14 epitope, many traditional approaches to the isolation of the genes encoding the branched chain polypeptide expressing the epitope would not have been successful. Our immunological detection techniques described here and determination of the NH_2-terminal amino acid sequences by Siegelman *et al.* (*26*) are complementary routes to the isolation of the HEV receptor gene sequences.

The developmentally regulated expression on lymphocytes of the MEL-14 epitope (*28, 31*) may result solely from the regulation of the expression of the carrier polypeptide (*26*), or by regulation of the ubiquitination of this protein. Because the MEL-14 epitope is located within ubiquitin, and not within the associated core polypeptide, we are unable to detect nonubiquitinated forms of the HEV receptor structure and cannot yet assess independently the level of expression of the HEV receptor core polypeptide.

We have been able to show (*32*) that the arf2 cDNA clone hybridizes to several independent polyadenylated RNA transcripts in all cell types tested. The sequence of the three largest transcripts are mainly made up of head-to-tail polyubiquitin multimers; none of these polyubiquitin transcripts con-

tain 5′ sequences encoding the type of hydrophobic amino acid stretches characteristic of membrane-targeting signal sequences (*32*). The levels of these ubiquitin mRNA transcripts do not differ in any obvious fashion between MEL-14 antigen–positive and MEL-14 antigen–negative lymphoid tumors. Therefore, differences in the level of expression of the cell surface HEV receptor are probably not a consequence of the regulation of ubiquitin gene expression at the transcriptional level. If, however, there is regulation of the level or form of ubiquitination, or the rate at which ubiquitination and ubiquitin removal occurs, such regulation may allow rapid alteration of HEV receptor activity. Antigen-activated mouse lymphocytes, for example, lose the MEL-14 epitope from their surfaces very rapidly after stimulation (*31*). Maintenance of the MEL-14 epitope on the cell surface could involve mechanisms to remove and restore ubiquitin to the carrier polypeptide (*26*), perhaps by endocytosis and receptor recycling, in addition to de novo synthesis.

The extreme amino acid sequence conservation of ubiquitin suggests that its cellular function is critical. However, the function of ubiquitin is not strictly associated with the mammalian immune system. Both genetic and biochemical analyses demonstrate that ubiquitin is a cofactor in a cellular proteolysis system (*18–20*), required for cellular function (*23*), may play a role in chromosome organization and gene expression (*21, 22*), and is a heat shock protein in chickens (*14*). We present evidence that ubiquitin probably plays a role in the recognition of HEV cell surface molecules by circulating lymphocytes. The sequence conservation of ubiquitin may be the result of the requirement that it interact with many different proteins. Such a requirement would reduce the acceptability of mutational events that significantly altered its ability to interact with its various companion sequences. The strict conservation of the tandemly repeated ubiquitin coding unit gene structure (*32*) suggests that the primary product, polyubiquitin, may also have a critical function in cellular metabolism, perhaps distinct from the function of monomeric ubiquitin. For example, polyubiquitin may be an important polypeptide-specific conjugation substrate in vivo. Because these ubiquitin transcripts contain no obvious signal sequences (*32*), the means by which polyubiquitin gains access to the cell surface is yet to be discovered. The functional significance of the association of ubiquitin with the lymphocyte HEV receptor is, at present, unclear. Our data, here and in (*26*), indicate that other cell surface proteins also contain ubiquitin epitopes, and therefore may be conjugated to ubiquitin. Rat antiserum to the β-galactosidase–ubiquitin fusion protein and monoclonal antibodies to ubiquitin recognize epitopes on one or more cell surface structures. A priori, it is possible that the lymphocyte endothelial cell receptor is the only ubiquitinated cell surface protein and is expressed on non-HEV binding cells in an inactive form. A concealed MEL-14 ubiquitin epitope could then be revealed by further modification of the preexisting ubiquitinated structure. We consider it more likely that the detection of ubiquitin epitopes on the surfaces of MEL-14 antigen negative cells suggests the presence of other ubiquitinated cell surface proteins (*26*). The biology of this and other cell surface ubiquitinated proteins may suggest functions for ubiquitin in those structures.

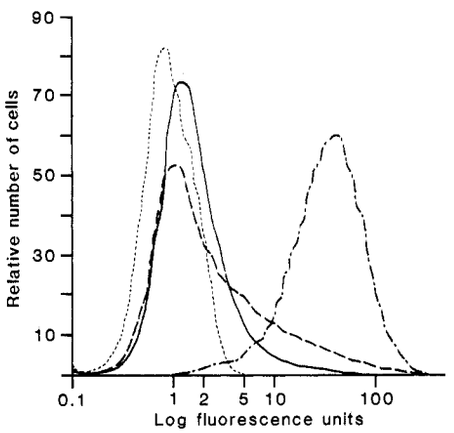

Fig. 3. Monoclonal antibodies to ubiquitin detect antigenic determinants present on the cell surface. EL-4-11/MEL-14^{hi} cells (10^6) or, as in Fig. 2, 38C-13, El-4, and P815 cells were incubated at 0°C for 30 minutes with each rat antiserum (Fig. 2) or monoclonal antibody (Fig. 3), EL-4-11/MEL-14^{hi} is an EL-4 variant selected via single-cell cloning on the basis of FACS for high-level expression of the MEL-14 determinant (*9*). The cell samples were then washed, resuspended, and incubated with 2 μg of either a fluoresceinated goat antiserum to rat or mouse immunoglobulin (Ig) or, in the case of mouse IgM monoclonal reagents, with a fluoresceinated goat antibody to mouse IgM (second stage). Samples were then incubated at 0°C for 5 minutes, washed, resuspended, and analyzed immediately with a Becton-Dickinson FACS-II. All incubations and washings were carried out in the presence of 10 m*M* sodium azide to prevent capping. Propidium iodide (1 m*M*) was added to the second-stage incubations to facilitate identification of dead cells on the FACS and to exclude them from analysis. The MEL-14 antibody was purified from serum-free culture supernatant and used at 1 μg per 10^6 cells. For each analysis, 5 ml of each dilution of monoclonal antibody to ubiquitin was concentrated fivefold by filtration. The 38C-13 immunoglobulin was used at 50 μg/ml, 0.5 ml per 10^6 cells, as an isotype-matched (IgM), negative control for binding with 1-2H11 and 43E12 (Fig. 3). Normal nonimmune rat serum was used as a negative control for the rat antiserum to the fusion protein (Fig. 2). The background fluorescence obtained with a (MEL-14 isotype-matched) rat IgG_{2a} negative control antibody was essentially identical to that observed with 38C-13 immunoglobulin and is excluded from the histograms. Dots and dashes, binding with MEL-14; dotted line, staining with 38C-13 Ig (negative control); dashed line, binding with 1-2H11; solid line, binding with 4-3E12.

A working model for the HEV receptor is suggested by analogy to the chromosomal protein uH2A (A-24). This molecule is a branched chain polypeptide consisting of one molecule of histone 2A and one molecule of ubiquitin (*15*). The example of uH2A suggests that another polypeptide is involved in the structure of the peripheral lymph node HEV receptor, and the findings of Siegelman *et al.* confirm that hypothesis (*26*). The ubiquitination of the lymphocyte HEV receptor raises the following questions. Is the other major lymphocyte HEV receptor, that for the venule endothelium of Peyer's patches, also ubiquitinated? Is the structure of its carrier polypeptide related to that of the peripheral lymph node HEV receptor? Is ubiquitination a general property of cell adhesion receptors? Are other cell surface receptors ubiquitinated? We have identified one cell surface ubiquitinated protein. That there is only one cell surface, lymphoid-specific, ubiquitinated protein seems unlikely—we expect that numerous other cell surface ubiquitinated proteins specific to various cell types will be discovered.

REFERENCES AND NOTES

1. J. L. Gowans, E. J. Knight, *Proc. R. Soc. London Ser. A.* **159**, 257 (1964).
2. E. C. Butcher, R. G. Scollay, I. L. Weissman, *Eur. J. Immunol.* **10**, 556 (1980).
3. R. A. Rasmussen, Y. Chin, J. J. Woodruff, T. G. Easton, *J. Immunol.* **135**, 19 (1985).
4. H. B. Stamper, Jr., and J. J. Woodruff, *J. Exp. Med.* **144**, 828 (1976).
5. E. C. Butcher, R. G. Scollay, I. L. Weissman, *J. Immunol.* **123**, 1996 (1979).
6. E. C. Butcher, G. Kraal, S. K. Stevens, I. L. Weissman, *Adv. Exp. Med. Biol.* **149**, 199 (1982).
7. W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Nature (London)* **304**, 30 (1983).
8. R. A. Reichert, W. M. Gallatin, I. L. Weissman, E. C. Butcher, *J. Exp. Med.* **157**, 813 (1983).
9. W. M. Gallatin, personal observations and in preparation.
10. R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983).
11. N. R. Landau *et al.*, *ibid.* **81**, 5836 (1984).
12. D. H. Schlesinger, G. Goldstein, H. D. Niall, *Biochemistry* **14**, 2214 (1975).
13. E. Dworkin-Rastl, A. Shrutkowski, M. B. Dworkin, *Cell* **39**, 321 (1984).
14. U. Bond and M. J. Schlesinger, *Mol. Cell. Biol.* **5**, 949 (1985).
15. I. L. Goldknopf and H. Busch, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 864 (1977).
16. A. L. Haas, J. V. Warms, I. A. Rose, *Biochemistry* **22**, 4388 (1983).
17. C. M. Pickart and I. A. Rose, *J. Biol. Chem.* **260**, 1573 (1985).
18. R. L. Seale, *Nucleic Acids Res.* **9**, 3151 (1981).
19. A. Ciechanover, D. Finley, A. Varshavsky, *J. Cell. Biochem.* **24**, 27 (1984).
20. A. Hershko, *Cell* **34**, 11 (1983).
21. L. Levinger and A. Varshavsky, *ibid.* **28**, 375 (1982).
22. I. L. Goldknopf, G. Wilson, N. R. Ballal, H. Busch, *J. Biol. Chem.* **255**, 10555 (1980).
23. R. S. Wu, K. W. Kohn, W. M. Bonner, *ibid.* **256**, 5916 (1981).

24. O. Wiborg, M. S. Pederson, A. Wind, L. E. Berglund, K. A. Marcker, J. Vuust, *EMBO J.* **4**, 755 (1985).
25. E. Ozkaynak, D. Finley, A. Varshavsky, *Nature (London)* **312**, 663 (1984).
26. M. Siegelman, M. W. Bond, W. M. Gallatin, T. St. John, H. T. Smith, V. A. Fried, I. L. Weissman, *Science* **231**, 823 (1986).
27. V. A. Fried, H. T. Smith, M. Morrison, in preparation.
28. R. A. Reichert, W. M. Gallatin, E. C. Butcher, I. L. Weissman, *Cell* **38**, 89 (1984).
29. R. I. Fox and I. L. Weissman, *J. Immunol.* **122**, 1697 (1979); I. A. Wilson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
30. S. Vijay-Kumar, C. E. Bugg, K. D. Wilkinson, W. J. Cook, *Cell* **82**, 3582 (1985).
31. M. O. Dailey, C. G. Fathman, E. C. Butcher, E. Pillemer, I. Weissman, *J. Immunol.* **128**, 2134 (1982); P. J. Fink, W. M. Gallatin, R. A. Reichert, E. C. Butcher, I. L. Weissman, *Nature (London)* **313**, 233 (1985).
32. T. St. John *et al.*, in preparation.
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Long-Term Cultures of HTLV-III–Infected T Cells: A Model of Cytopathology of T-Cell Depletion in AIDS

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Long-term cultures were established of HTLV-III–infected T4 cells from patients with the acquired immune deficiency syndrome (AIDS) and of T4 cells from normal donors after infection of the cells in vitro. By initially reducing the number of cells per milliliter of culture medium it was possible to grow the infected cells for 50 to 60 days. As with uninfected T cells, immunologic activation of the HTLV-III–infected cells with phytohemagglutinin led to patterns of gene expression typical of T-cell differentiation, such as production of interleukin-2 and expression of interleukin-2 receptors, but in the infected cells immunologic activation also led to expression of HTLV-III, which was followed by cell death. The results revealed a cytopathogenic mechanism that may account for T4 cell depletion in AIDS patients and suggest how repeated antigenic stimulation by infectious agents, such as malaria in Africa, or by allogeneic blood or semen, may be important determinants of the latency period in AIDS.

THE PATHOGENESIS OF THE ACquired immune deficiency syndrome (AIDS) involves a decrease in the number and function of mature T4 lymphocytes. When cultured in vitro, these cells are the main target of infection by the human T-lymphotropic retrovirus designated HTLV-III/LAV (*1–3*), and it is from these cells that HTLV-III/LAV is usually isolated (*4*). T4 lymphocytes from normal donors infected by HTLV-III in vitro, as well as HTLV-III–infected primary T4 cells from AIDS patients, have been difficult to maintain in culture for longer than 2 weeks (*4, 5*), and it has often been assumed that the virus has a direct cytolytic effect on these cells (*4, 5*). However, in this report we describe culture conditions that permit the long-term growth of HTLV-III–infected T cells derived from AIDS patients and of normal donor T cells infected with HTLV-III in vitro. In the long-term cultures, the expression of HTLV-III was always preceded by the initiation of interleukin-2 secretion, both of which occurred only when T cells were immunologically activated. Thus, the immunologic stimulation that was required for IL-2 secretion also induced viral expression, which led to cell death.

For these experiments we obtained heparinized peripheral blood lymphocytes (PBL) from six patients with AIDS and from three normal donors. The T cells were separated from mononuclear cells by Ficoll-Hypaque gradients. The sera of the six AIDS patients were positive for antibodies to HTLV-III, whereas sera from the normal donors were negative. The PBL (6×10^5 per milliliter) were activated by 0.1 percent (PHAp; Difco) in round-bottom tissue culture tubes (white caps RBTC; Falcon) containing 2 ml of medium composed of RPMI 1640, 20 percent fetal calf serum (FCS), sheep antiserum to α-interferon (neutralizing titer 6 IU at 10^{-5} dilution), and goat antiserum to human γ-interferon (neutralizing titer 1 IU at 10^{-5} dilution). After 2 to 3 days, IL-2 was added to the cultures. The medium was changed twice each week.

The primary PBL cultures from AIDS patients proliferated for about 12 to 15 days and consisted of adherent macrophages, T cells, and a few B cells. After this period they exhibited abundant cell lysis. However, when these cultures were activated with phytohemagglutinin (PHA), they first secreted IL-2 (days 1 and 2 after activation) and then transiently produced virus (days 6 to 12), as indicated by reverse transcriptase (RT) activity in the culture supernatants (*3–5*) and by the presence of HTLV-III p15 and p24 antigens on the surface of a few acetone-fixed cells detected by specific monoclonal antibodies (*6*). These observations suggested that agents that inhibit IL-2 production might enhance virus expression [for example, hydrocortisone (*6, 7*)] and favor cell death, whereas inhibitors of virus production might be associated with increased IL-2 secretion (for example, γ interferon) (*8*).

To obtain cultures of T cells from AIDS patients, we used the conditions described previously for the long-term growth of normal T-cell clones, with certain modifications (*9*). The modifications consisted of dilution of the cell number from 10^5 to 10^6 cells per milliliter (usual culture conditions) to 10^5 to 10^4 cells per milliliter and the addition of a feeder cell layer of 10^5 to 10^6 irradiated (4000 rads) PBL pooled from 10 to 20 normal human donors (Fig. 1). With these conditions, the HTLV-III–infected T cells could be maintained in the presence of exogenous IL-2 for 50 to 60 days. The initial reduction of the cell concentration was sufficient to select out those cells that could survive; the lack of further antigenic stimulation and, presumably, the reduced concentrations of toxic substances released by the mature cells, permitted cell survival at concentrations ranging from 5×10^5 to 1×10^6 per milliliter. However, after this period, cell degeneration occurred in a manner similar to that of normal T cells grown under the same conditions (*9*).

The cultures of infected T cells showed other similarities to cultures of normal T cells. When T-cell surface antigens were measured by the rosette technique in the presence of specific monoclonal antibodies (*10*), the proportion of infected cell cultures exhibiting T4, T8, and Tac antigen were in the same range as normal T cells. No cytogenetic changes occurred in the T-cell cultures from three AIDS patients when the cultures were stained by the Giemsa banding tech-

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