

Cell Surface Molecule Associated with Lymphocyte Homing Is a Ubiquitinated Branched-Chain Glycoprotein

MARK SIEGELMAN, MARTHA W. BOND, W. MICHAEL GALLATIN, TOM ST. JOHN, HARRY T. SMITH, VICTOR A. FRIED, IRVING L. WEISSMAN

Partial amino acid sequence analysis of a purified lymphocyte homing receptor demonstrates the presence of two amino termini, one of which corresponds precisely to the amino terminus of ubiquitin. This observation extends the province of this conserved polypeptide to the cell surface and leads to a proposed model of the receptor complex as a core polypeptide modified by glycosylation and ubiquitination. Independent antibodies to ubiquitin serve to identify additional cell surface species, an indication that ubiquitination of cell surface proteins may be more general. It is proposed that functional binding of lymphocytes to lymph node high endothelial venules might involve the ubiquitinated region of the receptor; if true, cell surface ubiquitin could play a more general role in cell-cell interaction and adhesion.

THE DYNAMISM OF THE CIRCULATING LYMPHOID SYSTEM IS relieved by scattered solid collections of lymphoid elements, such as thymus, lymph nodes, Peyer's patches, and spleen, which together constitute the lymphoid organs. These organs are architecturally organized so that proper inductive microenvironments ensure appropriate lymphocyte differentiation and maturation. The portal of entry of lymphocytes from the bloodstream into peripheral lymphoid organs has been identified as specialized lymphoid organ vessels, called postcapillary high endothelial venules (HEV) (1-3). Lymphocytes specifically recognize, adhere to, and migrate through this highly specialized endothelium.

The fundamental role of HEV-lymphocyte interaction in lymphocyte trafficking has been demonstrated (4-5). Peripheral node lymphocytes exhibit binding preference for peripheral node HEV's, while Peyer's patch lymphocytes favor binding to homologous Peyer's patch HEV (6, 7). Some clonal murine T and B lymphoma lines display exclusive specificity for either peripheral node or Peyer's patch-type HEV, while others recognize neither venule type (8). Together these data are consistent with a model involving complementary cell surface recognition structures mediating organ-specific lymphocyte HEV-interactions.

Evidence for the existence of such cell surface recognition structures was provided by the development of a rat monoclonal antibody against murine lymphocytes MEL-14 (8), that detects a cell surface determinant present only on those cells which bind peripheral lymph node HEV and is absent on those which either bind Peyer's patch HEV only or have no HEV binding activity at all (8), and blocks specific *in vitro* binding and *in vivo* homing of lymphocytes to these HEV's. MEL-14 specifically precipitates from

the cell surface of lymph node HEV-binding lymphocytes a protein band of 85,000 to 95,000 daltons, the size varying slightly with the cellular source of antigen. Monoclonal reagents that detect functionally similar structures in another species have also been described (9).

Screening of bacteriophage λ gt11 libraries by MEL-14 results in the isolation of independent, expressed complementary DNA (cDNA) clones encoding ubiquitin (10). This result suggested that ubiquitin might in fact be present as all or a portion of this cell surface interactive molecule. Ubiquitin is a small 8451-dalton polypeptide first isolated from bovine thymus (11). Primary structural analysis of various ubiquitins and their encoding genes has revealed remarkable evolutionary conservation (12-15). Independent investigations of the protein structure of the nucleosomal protein A24 revealed that it is a branched chain complex with ubiquitin in isopeptide linkage to histone 2A (H2A) (16). Subsequently, covalent conjugation of ubiquitin to cytoplasmic proteins was demonstrated, further generalizing the principle (17, 18). Functional roles attributed to ubiquitin have included participation in regulation of intracellular protein degradation (17-20), gene transcription and organization of chromatin structure (21-23), and mitosis (24). To test whether ubiquitin contributed to the MEL-14 antibody-defined cell surface molecule, we sought to isolate and obtain amino acid sequence of this structure.

We have now isolated by antibody affinity and sodium dodecyl sulfate (SDS) gel purification the cell surface species recognized by MEL-14, which we designate gp90^{MEL-14}, for structural analysis. By partial NH₂-terminal amino acid sequence analysis with intrinsic labeling techniques, we demonstrate the presence of two NH₂-termini, one of which corresponds precisely with the NH₂-terminus of ubiquitin; the other is a second distinct glycosylated polypeptide. Independent monoclonal antibodies to ubiquitin identify other cell surface species that bear ubiquitin epitopes, strongly suggesting that ubiquitination of cell surface proteins is a more general phenomenon. These findings extend the location of this highly conserved polypeptide to the third major cellular compartment, the cell surface, and invite investigations of its role at this site.

Isolation and purification of the MEL-14 antibody-defined cell-surface glycoprotein gp90^{MEL-14}. The cell line utilized for these studies was EL-4/MEL-14^{hi}, a variant of the murine T-cell lymphoma cell line, EL-4, selected by fluorescence flow cytometry

M. Siegelman, W. M. Gallatin, T. St. John, and I. L. Weissman are with the Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305. M. W. Bond is on the staff of DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304. H. T. Smith and V. A. Fried are in the Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38101. The present address of W. M. Gallatin and T. St. John is Division of Basic Science, Fred Hutchinson Cancer Research Institute, Seattle, WA 98104.

for high-level expression of the MEL-14 antigen, a property that cosegregated with the capacity to bind peripheral node venules (25). The molecular species specifically recognized by MEL-14 antibody was isolated by immune complex formation between MEL-14 (8), and affinity-purified rabbit or goat antibody to rat immunoglobulin G (IgG). The profile of an immunoprecipitation from cell surface ^{125}I -labeled cell lysates of EL-4/MEL-14^{hi}, similar to that reported previously (8), is shown in Fig. 1A.

To isolate the MEL-14 reactive antigen directly from whole cell lysates made from 2×10^8 cells labeled internally with [^3H]- or ^{35}S -labeled amino acids, we interposed a lentil-lectin enrichment step to remove 90 to 95 percent of total radioactivity (counts per minute) in the lysate. Typical gel profiles of a MEL-14 antibody immunoprecipitation from a sample (1.5×10^6 count/min) from a lectin

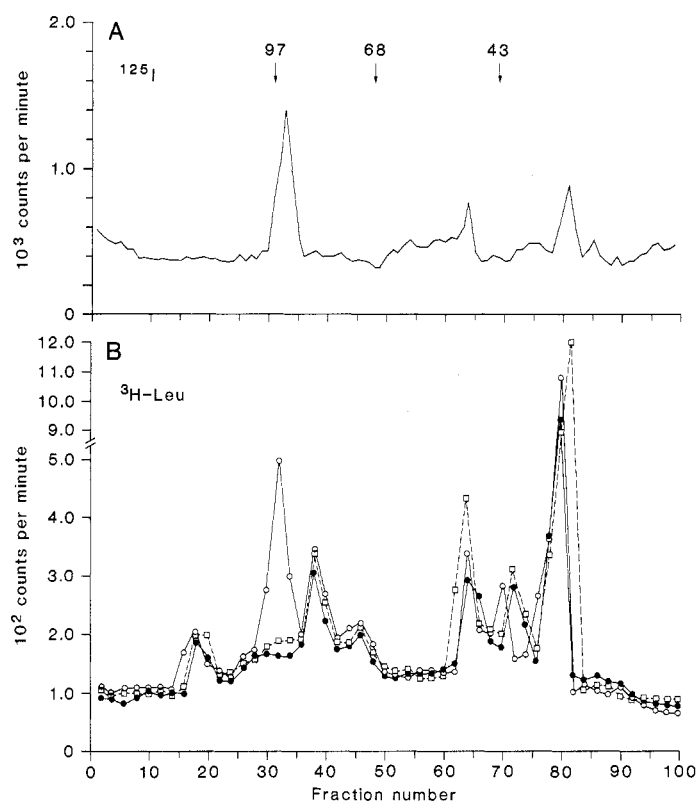


Fig. 1. Immunoprecipitation and SDS-PAGE analysis of the putative lymphocyte homing receptor, gp90^{MEL-14}. (A) Immunoprecipitation of cell surface ^{125}I -labeled EL-4/MEL-14^{hi} by MEL-14 antibody. Cells (2×10^7) were ^{125}I -labeled (lactoperoxidase catalysis at the cell surface), and then solubilized, and clarified by ultracentrifugation (45). The lysate was incubated with a $20\times$ concentrated MEL-14 hybridoma supernatant (8), equivalent to 10 to 20 μg of monoclonal antibody, for 3 to 4 hours at 4°C ; purified goat antiserum to rat IgG was then added to effect formation of a solid precipitate. Complexes were analyzed on 10 percent SDS-polyacrylamide tube gels in the Laemmli discontinuous gel system (46), as modified by Cullen (47). The profile was obtained by gel fractionation at 1-mm intervals and subsequent counting. Gel fraction number is plotted against the number of counts per minute in each fraction. (B) Immunoprecipitation by MEL-14 antibody of EL-4/MEL-14^{hi} cells metabolically labeled with [^3H]leucine. Cells (2×10^8) were labeled with 10 mCi of [^3H]leucine (48) and lysed in 0.5 percent Nonidet P-40; the lysate was applied to a column of *Lens culinaris* lectin conjugated to Sepharose 4B. The glycoprotein-enriched pool was eluted with 0.3M methyl- α -D-mannopyranoside. Precipitation of 5×10^6 cell equivalents and SDS-PAGE analysis was as described in (A). O, Precipitation with MEL-14. ●, Precipitation with IgG2a isotype-matched rat monoclonal antibody control 30G12 (antibody to T200). □, Precipitation with 9B5 monoclonal antibody to a human lymphocyte surface marker (26, 49). The molecular weight markers were phosphorylase b, 97,400; bovine serum albumin, 68,000; and ovalbumin, 43,000.

adherent pool of an EL-4/MEL-14^{hi} lysate labeled metabolically with [^3H]leucine are shown in Fig. 1B. While a number of nonspecific bands are present, a single specific species (94,000 daltons), identical in molecular size to that precipitated from cell surface iodinated lysates, is found.

The gp90^{MEL-14} thus isolated was subjected to two-dimensional gel analysis (Fig. 2). This internally labeled material migrates with the identical isoelectric mobility as cell surface iodinated gp90^{MEL-14} (26), in the pH range 4.0 to 4.5, suggesting further identity between the MEL-14 antigen isolated from preparations that were iodinated at the cell surface and labeled internally. The microheterogeneity in the internally labeled material appears similar to that seen in iodinated material, with a pattern consistent with glycosylation differences. This indicates that the material isolated from internally labeled lysates for amino acid sequence analysis is unlikely to represent a selected subset of the antigen isolated from cell surface iodinated material. Nor does it appear, by these criteria, to be detectably more complex than the iodinated material.

Partial NH₂-terminal amino acid sequence analysis of the gp90^{MEL-14} glycoprotein. Cells were labeled (Fig. 1) with a number of different essential amino acids. Affinity- and gel-purified material (20,000 to 50,000 count/min) was subjected to automated NH₂-terminal Edman degradation (27) (Fig. 3). Positions containing radioactivity significantly above background indicated the presence of the particular tritiated amino acid residue at that position within a polypeptide chain. Amino acid sequence determination of metabolically labeled gp90^{MEL-14} indicated the presence of two types of amino termini, easily distinguishable by their relative initial yields. A demonstration of this is given in Fig. 3 for the purified gp90^{MEL-14} labeled with [^3H]lysine. The predominant sequence is shown (Fig. 3) by radioactive peaks at positions 8, 17, 20, and 32. A reproducible secondary sequence is also present at a lower specific activity at positions 6, 11, 27, and 29. This secondary sequence corresponds precisely with the positions of lysines in the NH₂-terminal sequence of ubiquitin (12). The pattern of a major and minor NH₂-terminal sequence in absolute yields and relative proportions similar to that in Fig. 3 has been obtained with all the amino acids labeled. A summary of the data for the minor sequence is given in Fig. 4 and compared to the known NH₂-terminal sequence of ubiquitin.

The selection of cDNA clones in the bacteriophage λ gt11 expression system suggested that MEL-14 may recognize a ubiquitin determinant (10). It was therefore important to demonstrate that the relatively minor ubiquitin sequence was covalently associated with a specific core polypeptide, and not representative of a background of ubiquitin or of ubiquitinated proteins precipitated by MEL-14 and distributed over the entire SDS gel from which the specific MEL-14 species was isolated. To rule out this possibility, the remainder of the gel fractions in preparative runs from which gp90^{MEL-14} had been isolated were similarly eluted and collected in four separate pools. The pools were subjected to NH₂-terminal amino acid analysis. There were no detectable sequences corresponding to a ubiquitin NH₂-terminus in any other portions of the gels, and the relatively minor sequence of ubiquitin in the MEL-14-specific species could not be explained by contamination with a neighboring molecule containing ubiquitin as a major sequence. In addition, an immunoglobulin kappa light chain biosynthetically labeled with [^3H]leucine was isolated from the B-cell lymphoma line 38C13, which also bears gp90^{MEL-14} (8). Amino terminal sequence analysis revealed the expected mouse kappa chain leucine positions at 11 and 15, with no evidence of a minor sequence at position 8 to suggest the presence of a ubiquitin NH₂-terminus. By the same analysis, we exclude the possibility that after immunoprecipitation ubiquitin-core polypeptide conjugates spontaneously dissociate. This follows from amino acid sequence analysis of pools containing

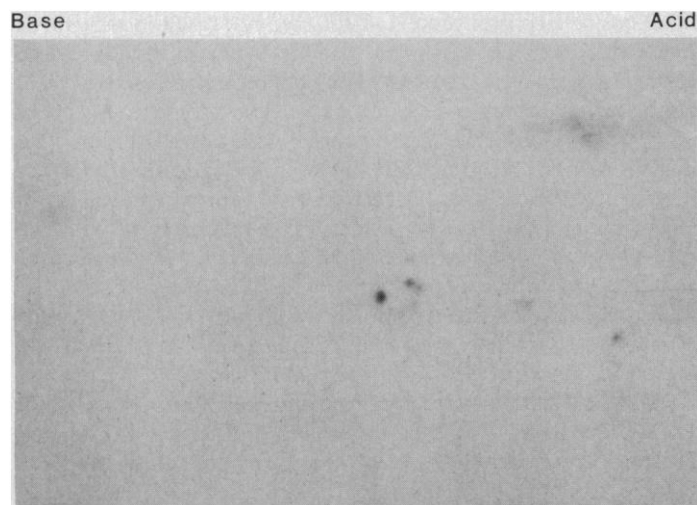


Fig. 2. Two-dimensional polyacrylamide gel analysis of gp90^{MEL-14}. [³H] Phenylalanine labeled gp90^{MEL-14} (1×10^5 count/min) was solubilized in 20 μ l of isoelectric-focusing sample buffer (50). The first-dimensional charge separation was done by nonequilibrium pH gradient electrophoresis (NEPHGE) (51), from pH 3.5 to 10, and the second dimension was done as described by O'Farrell (50). The slab gel was fixed, treated with the fluorographic medium Enhance (New England Nuclear), and exposed to Kodak XAR-5 film for 14 days. The NEPHGE direction is represented horizontally and the SDS direction vertically. The slight size heterogeneity of gp90^{MEL-14} is consistent with variations often seen in one-dimensional SDS-PAGE analysis (Fig. 8), and may result from glycosylation differences shown in Fig. 8.

the smallest species on SDS-polyacrylamide gels, which would be expected to contain free ubiquitin. No trace of such a ubiquitin sequence is found. Thus, two such divergent approaches as amino acid sequence analysis and filter paper immunoselection to select cDNA clones (10) provide evidence that ubiquitin is indeed a component of this MEL-14 reactive cell surface protein associated with lymphocyte homing.

A summary of the other (major) sequence obtained is given in Fig. 5. The repetitive yield of lysine calculated from the two peak heights at positions 8 and 17 (92 percent) compares well with the repetitive yield calculated, minus background, for the lysines in the ubiquitin chain at positions 6 and 11. This observation is consistent with the interpretation that the major sequence, like the ubiquitin sequence, is derived from a single polypeptide chain. This interpretation is reinforced by our failing to encounter any evidence for more than a single amino acid determination at any position for this major sequence in all single amino acid labeled preparations that we examined. In computer searches of data files, the sequence appears to be unlike any other, although the limited information at present prevents adequate searches for homologies. The relatively lysine-rich NH₂-terminus should be regarded as a minimal estimate of the number of lysine positions. Ubiquitin conjugates to proteins in an isopeptide bond between its COOH-terminal glycine and ϵ -amino groups of lysines (16). Should such a conjugated lysine position exist in the NH₂-terminal 35 residues of the core polypeptide, it is likely that, after Edman degradation, a 77-residue ubiquitin polypeptide (or ubiquitin multimer) attached to this lysine would not be extracted from the filter and would remain undetected by this analysis.

The finding that ubiquitin may be synthesized as a polyubiquitin transcriptional unit in head-to-tail array (10, 28-31) raises the issue of whether ubiquitin exists as a concatamer in association with mature proteins as well. We initially addressed this issue by taking advantage of the single methionine at position one in ubiquitin and

performing cyanogen bromide (CNBr) cleavage of filter-bound gp90^{MEL-14} (Fig. 6). Glass fiber filters to which protein samples are applied for amino acid sequence analysis in gas phase sequencers can be subjected to CNBr cleavage reactions even after NH₂-terminal sequence analysis of the protein on the filter has been performed (32). The generation of new NH₂-termini in this fashion indicates the presence of internal methionine residues. After CNBr cleavage, Edman degradation was again performed. This CNBr maneuver was conducted on singly labeled purified MEL-14 antigen after 30 Edman degradation cycles. The profiles for the initial ten residues for [³H]valine before and after CNBr digestion are given in Fig. 7. There is a shift by one in the valine from position 5 in the native form to position 4 in digested material. Amino-terminal analysis of CNBr-digested polyubiquitin would result in such a shift in sequence by one position, while single ubiquitin subunits would generate no additional NH₂-termini in ubiquitin. A similar shift by one after CNBr digestion was seen for the first ubiquitin lysine and threonine positions. Background counts are relatively higher after CNBr digestions, and ubiquitin positions later in the sequence tend to be somewhat obscured by background and apparent high yield determinations, probably as a result of internal cleavages in the major chain. Since the NH₂-terminal ubiquitin sequence through the first 30 positions is presumably absent, a second, shifted sequence after CNBr digestion may suggest that a ubiquitin sequence exists internally within this complex, consistent with a head-to-tail arrangement. Alternatively, it is possible that one or more ubiquitin chains is present exclusively as a monomer and that a proportion of the ubiquitin NH₂-termini, initially blocked to Edman degradation, are available for Edman degradation after CNBr digestion. Isolation of purified CNBr-derived fragments will likely be required to resolve this question.

Endoglycosidase F reveals extensive N-linked glycosylation of gp90^{MEL-14}. Characterization by SDS gels of the gp90^{MEL-14} isolated from cell surface iodinated or intrinsically labeled, lectin-adherent, precipitated preparations reveals a somewhat broad molecular size for this species, about 85 to 95 kD. Isoelectric focusing reveals extensive microheterogeneity in an acidic molecule (*pI* of approxi-

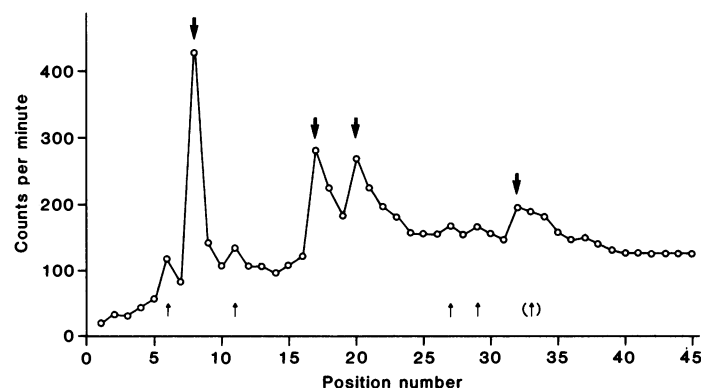


Fig. 3. Amino-terminal automated Edman degradation of gp90^{MEL-14} intrinsically labeled with [³H]lysine. Automated sequence analysis was performed on an Applied Biosystems model 470A gas-liquid protein sequinator. Entire butyl chloride extracts at each step were transferred into vials directly for scintillation counting in a mixture of toluene and PPO (2,5-diphenyloxazole). Each sample was counted (in duplicate) for 10 minutes. Positions containing radioactivity above background indicate the presence of [³H]lysine at that position. A plot of position number against the total number of counts per minute at that position is given. Downward arrows indicate the major high specific activity assignments. Upward arrows indicate the lower specific activity assignments. The upward arrow in parenthesis indicates a tentative determination.

5 10 15 20 25 30 35
M Q I F V K T L T G K P T I L E V E P S D T I E N V K A K I Q D K E G
M - I F V K T L T - K T I T L - V - - - (T) I - - - K - K - - (K) - -

Fig. 4. Compilation of NH₂-terminal amino acid sequence assignments for the low specific activity component in gp90^{MEL-14}. The sequence is compared to the previously reported sequence of the NH₂-terminus of ubiquitin (12) given in the top line. The second line represents positions determined from independent single amino acid labels. A dash indicates that no assignment at that position has been made. Parentheses indicate tentative assignments.

5 10 15 20 25 30
- T - H - - - K - M - - - - - K F - K - - - - - V (V) I - L K - -

Fig. 5. Compilation of NH₂-terminal amino acid sequence assignment for the major high yield sequence in gp90^{MEL-14}. Determinations were made from independent single amino acid labels. Dashes indicate that no assignment at the position has been made. The parentheses at position 28 indicate a tentative assignment.

mately 4 to 4.5), suggesting the possibility of a highly glycosylated molecule.

In order to ascertain the relative contribution of oligosaccharide side chains, we performed endoglycosidase F (Endo F) digestions of isolated receptor. Endo F cleaves high mannose as well as complex type *N*-linked oligosaccharide groups (33). The gp90^{MEL-14} species from cell surface iodinated lysates of EL-4/MEL-14^{hi} was isolated by bulk precipitation with MEL-14 antibody directly conjugated to Sepharose 4B. Endo F digestions of eluted material were performed (Fig. 8) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). After 1 hour, at least three digestion products—bands at 55 kD and 60 kD and a faint band at 76 kD—were seen. All resolve to the major 55-kD species after 22 hours. Since parallel Endo F

digestions of a control protein, T200, did not result in proteolytic fragments, it can be concluded that the species identified indeed resulted from glycosidase activity and not proteolysis. The reduction in microheterogeneity, as judged by the relative compactness of the lowest 55-kD product, is also consistent with this conclusion. This 55-kD size determination after digestion was also observed with material precipitated from lysates of normal lymphocytes and the B-cell lymphoma 38C13 (34). The stepwise reduction in molecular size suggests a minimal estimate of the number of *N*-linked glycosylation sites to be three.

The sequence of the rigidly conserved ubiquitin polypeptide contains no canonical amino acid triplets (Asn-X-Ser/Thr) which would accommodate *N*-linked glycosylation. Therefore, the homing receptor must contain another polypeptide to which both ubiquitin and oligosaccharide side groups are linked. The evidence implies that the polypeptide represented by the major amino acid sequence is this polypeptide core. Since *O*-linked groups are unaffected by Endo F under these conditions, and since analysis was performed on immune complexes where access to some Endo F susceptible groups might be sterically hindered, it should be emphasized that the 55-kD estimate represents an upper limit to the size of the core polypeptide. Subtraction of a minimum of one ubiquitin subunit leaves about 46.5 kD for the size of the ubiquitinated core polypeptide as an upper limit. Since exhaustive digestion results in a single species, and not in two or more products separated in size by 8500 daltons (the unit size of ubiquitin), it is unlikely, although not absolutely excluded, that this core polypeptide is ubiquitinated with a variable number of ubiquitin subunits. However, we cannot, on the basis of this evidence, ascertain the number of subunits modifying the core polypeptide.

Monoclonal antibodies to ubiquitin recognize a set of cell surface iodinated proteins. In order to establish whether this specific instance of cell surface ubiquitination is a property characteristic of this cell surface molecule alone, we treated cell surface ¹²⁵I-labeled lymphoid cell lysates with a group of monoclonal antibodies to independent epitopes on human ubiquitin (35). A group of cell surface species were specifically precipitated (Fig. 9), one of which corresponds to the molecular size of gp90^{MEL-14}. The two larger specific species have mobilities indicating sizes of approximately 150 kD and 125 kD. Although these monoclonals have specificity for isolated peptides of ubiquitin (35), the above experiments do not exclude the possibility that recognition of these species is a result of fortuitous cross-reactions with determinants unrelated to ubiquitin. However, since at least two of the specific bands are in common between antibodies recognizing independent ubiquitin determinants, fortuitous cross-reactions are unlikely. Thus, it is probable that ubiquitination of cell surface molecules is not an exclusive feature of gp90^{MEL-14}, but is a more general phenomenon.

Structural considerations of gp90^{MEL-14}, a receptor associated with lymphocyte homing. Lymphocyte migration occurs in a regulated fashion and with a high degree of specificity. Populations of lymphocytes exhibit preferential homing patterns in vivo, and preferential adherence to sites of entry from blood into secondary lymphoid organs, the high endothelial venules of peripheral lymph node and Peyer's patches in vitro (36–39). This adherence is mediated by way of cell surface molecules in a presumed ligand-receptor interaction, the lymphocyte component of which has been given the operational designation the "lymphocyte homing receptor." The monoclonal antibody, MEL-14, by functional assays, apparently binds to this receptor and identifies a single predominant cell surface species, designated gp90^{MEL-14}. Our studies present initial NH₂-terminal amino acid sequence analysis and structural characterization of this molecule, which appears to undergo a complex series of posttranslational modifications. Our evidence

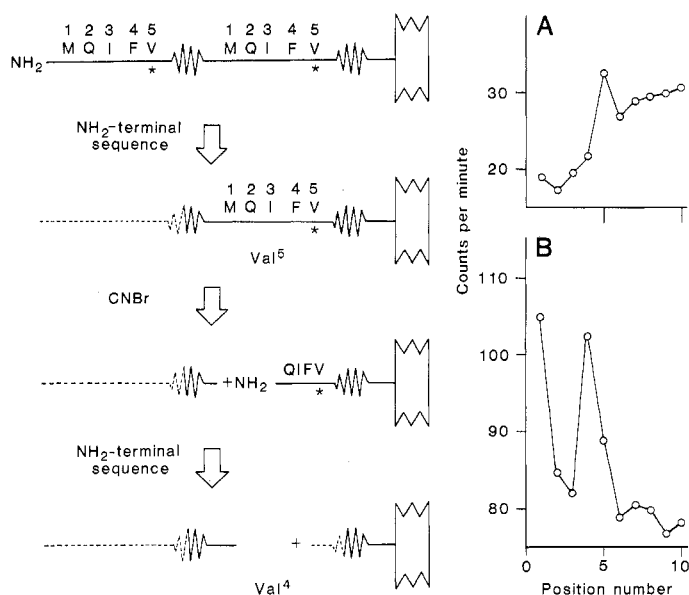


Fig. 6 (left). Representation of an interpretation of CNBr experiments, exemplified by a hypothetical [³H]valine preparation of gp90^{MEL-14}. The diagram shows two ubiquitin monomers connected in linear arrangement. The first five NH₂-terminal amino acids of each subunit are given. The COOH-terminus of the COOH-terminal ubiquitin unit is shown attached to a truncated core polypeptide. Automated NH₂-terminal sequencing is performed as described in Fig. 3. The CNBr digestion was performed basically as described (32). Fig. 7 (right). Automated Edman degradation of [³H]valine-labeled gp90^{MEL-14} (A) before and (B) after CNBr digestion. Amino acid sequence analysis and digestion was performed as described in Fig. 6 and in the text. Position number is plotted against the total number of counts per minute at that position.

suggests that the receptor is a branched chain polypeptide consisting of a core polypeptide most likely in isopeptide linkage to ubiquitin and highly glycosylated in at least *N*-linked form as well. This represents, to our knowledge, the first description of a ubiquitinated cell surface protein. Furthermore, we provide evidence that ubiquitination of cell surface proteins is not reserved to this particular molecule, but may be a more general phenomenon.

A schematic model for the overall structure of the murine lymphocyte homing receptor for peripheral lymph nodes is given in Fig. 10. A core protein with a maximum size of 46.5 kD is modified by a minimum of three *N*-linked glycosylations, carbohydrate therefore constituting approximately 45 percent of the mass of this molecule. This is a lower estimate of the extent of glycosylation, in that we do not take into account the presence of *O*-linked oligosaccharides. A major and rather unexpected structural feature is the covalent association of ubiquitin with the core polypeptide, inferred from the identification of a ubiquitin NH₂-terminal amino acid sequence clearly present in addition to that of a putative core polypeptide. A precedent for ubiquitin-protein conjugates was established for the nuclear protein A24, which was sequenced in its entirety, including a detailed analysis of the ubiquitin-H2A junction, deduced to be a novel linkage between the COOH group of the COOH-terminal glycine residue of ubiquitin to the ϵ -amino group of a lysine at position 119 of histone H2A (16). Since isolation of gp90^{MEL-14} is performed after complete reduction and since ubiquitin contains no cysteine residues, a disulfide protein linkage between the chains is not possible. We therefore expect that a similar isopeptide bond exists linking ubiquitin to the core polypeptide of this murine lymphocyte homing receptor.

Alternatively, it might be considered that the two NH₂-terminal sequences derive from completely separate and unassociated chains, the ubiquitin sequence from a ubiquitin protein polymer of approximately 10 to 12 sequential ubiquitin subunits, yielding species of the appropriate molecular size. The other sequence would derive from a completely unrelated glycoprotein, possibly representing the "true" homing receptor, which fortuitously bears a determinant cross-reactive with, but unrelated to, ubiquitin sequences. Both chains would be recognized and precipitated by the MEL-14 antibody. This alternative can be regarded as highly unlikely for several reasons. (i) There is no evidence for two polypeptides (polyubiquitin and the presumed core glycoprotein) in that it migrates as a single major species on two-dimensional gel analysis. (ii) Ubiquitin contains no canonical sequences that would serve as an acceptor site for *N*-linked glycosylation; therefore polyubiquitin should be selected against in the lectin adherent step used for purification. In addition, if the ubiquitin polymer were glycosylated in *O*-linked form, it should be insensitive to Endo F digestion; but gp90^{MEL-14} is sensitive to Endo F (Fig. 8). (iii) Ubiquitin contains a single methionine at position one, so that complete CNBr digestion of a hypothetical 10- to 12-unit concatamer should result in an increase in the yield of ubiquitin-associated sequence by a factor of 10 or 12. Although ubiquitin was identified (Fig. 7), this extent of possible concatamerization was not seen. (iv) The gp90^{MEL-14} migrates in nonreducing SDS-PAGE more rapidly than under reducing conditions (8), a property expected of proteins rich in internal disulfide bonds; ubiquitin contains no cysteines. (v) Although it is apparent that the potential for large polyubiquitin protein polymers exists (10, 28–31), these in fact have not as yet been found. Therefore, we favor the ubiquitin-core polypeptide relation indicated in Fig. 10. Detailed peptide analysis is required to test this model.

The details of the relation of ubiquitin to the core polypeptide remain to be ascertained. We cannot, from our available data, determine the number of ubiquitin units associated with the receptor, since the stoichiometry in the yield in counts per minute in the

two polypeptide chains is unlikely to reflect their molar ratios (see below). Experiments with purified isopeptidase, an activity in whole cell lysates that removes ubiquitin specifically from proteins (40, 41), when available, should allow us to determine the molecular weight of the core peptide and the number of ubiquitin subunits present. Another unresolved issue is whether ubiquitin exists in linear polymeric arrangements of more than one ubiquitin unit in association with this receptor (Fig. 10). From the evidence presented in Fig. 7, such head-to-tail organization might exist. More direct evidence for this type of structure requires isolation of CNBr fragments of the receptor complex for sequence analysis.

The amino acid sequence analysis revealed a much higher yield in counts per minute for the non-ubiquitin chain. We expect that MEL-14 only recognizes a ubiquitinated form of the receptor [see also 10)]. Therefore, we would predict at least a 1:1 molar ratio of ubiquitin to core protein, assuming at least one ubiquitin molecule per receptor complex. One explanation would require that the two

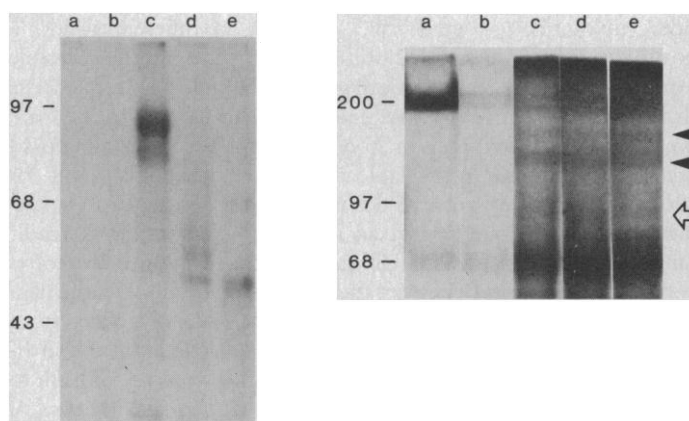


Fig. 8 (left). Endoglycosidase F digestion and SDS-PAGE analysis of cell surface ¹²⁵I-labeled gp90^{MEL-14} immunoprecipitates. EL-4/MEL-14^{hi} cells (2×10^7) were labeled with 2 mCi of ¹²⁵I (by lactoperoxidase-catalyzed surface radioiodination) and solubilized and clarified as described in Fig. 1A. Portions (1 ml) of this lysate were incubated with Sepharose 4B conjugated to MEL-14 antibody or with Sepharose 4B conjugated to R7D4, an isotype matched rat monoclonal antibody negative control (52). Endoglycosidase F (Endo F) digestions were carried out according to Elder *et al.* (33). Samples were eluted and then incubated for 1 or 22 hours at 37°C either with or without addition of 5 μ l of purified protease free Endo F (53). The reaction was terminated by the addition of SDS to a concentration of 1 percent and the samples were analyzed on a 9 percent SDS-polyacrylamide gel by the method of Laemmli (46). The gel was dried and subjected to fluorography (Kodak XAR-5 film for 7d). Gel lanes: (a) control R7D4 immunoprecipitate without Endo F, (b) control R7D4 22 hours after Endo F digestion, (c) MEL-14 precipitate without Endo F digestion, (d) MEL-14 precipitate after 1 hour of Endo F digestion; and (e) MEL-14 precipitate after 22 hours after Endo F digestion. Fig. 9 (right). Immunoprecipitation and SDS-PAGE analysis of cell surface ¹²⁵I-labeled EL-4/MEL-14^{hi} with monoclonal antibodies to ubiquitin. EL-4/MEL-14^{hi} cells (1×10^8) were labeled with 4 mCi of ¹²⁵I via lactoperoxidase catalyzed radioiodination. Cell viability was assessed at 99 percent. Lysates were precleared with *Staphylococcus aureus* (IgG-sorb, The Enzyme Center, Inc.) and Sepharose 4B. CNBr-activated Sepharose 4B (Pharmacia) was conjugated to affinity-purified goat antibody to mouse IgG (Pelfreeze, 2 mg antibody per milliliters of gel bed), and this conjugated material was then incubated with $5 \times$ concentrated preparations of mouse hybridoma supernatant to be used for precipitation. Antibody-coated Sepharose was incubated with labeled cell lysate. Samples were subjected to SDS-PAGE analysis on a 9 percent SDS-polyacrylamide gel under reducing conditions. Gel lanes: (a) 3BD7, a monoclonal mouse IgG₁, negative control (52); (b) 2DG12, a monoclonal mouse IgG₃, negative control (52); (c) 4-2D8-BA9, an IgG₁ mouse monoclonal antibody to human ubiquitin; (d) 2-3D7-CG101, an IgG₃ mouse monoclonal antibody to human ubiquitin; and (e) 1-2H11, an IgM monoclonal antibody to human ubiquitin. The upper two thirds of the gel is shown. Open arrow indicates the mobility of gp90^{MEL-14}. Closed arrows indicate examples of species that are precipitated only by antibody to ubiquitin.

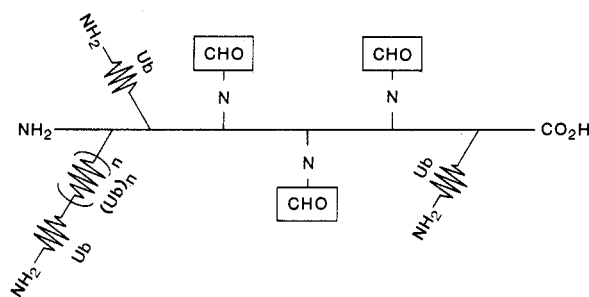


Fig. 10. Hypothetical model of $gp90^{MEL-14}$, a putative lymphocyte homing receptor for peripheral lymph node high endothelial venules. CHO, sites of N-linked glycosylation; Ub, ubiquitin subunits. The latter are represented in both monomeric (Ub), and in head-to-tail arrangement $(Ub)_n$. The disposition and number of ubiquitin moieties is arbitrary as is the placement of carbohydrates. Both carbohydrates and ubiquitin chains are attached to a central core polypeptide.

NH₂-terminal sequences obtained were of two independent peptides (ubiquitin and $gp90^{MEL-14}$) present in different yields, a possibility that we have ruled out experimentally. Another possible explanation for the relatively low yield of the ubiquitin sequence is that the NH₂-termini of most ubiquitin chains in $gp90^{MEL-14}$ are preferentially blocked to Edman degradation. We prefer the possibility that ubiquitin is available in a large intracellular pool for conjugation to many proteins destined for various cellular compartments, or that ubiquitin targeted for membrane sites has a significant lag before it is available. Ubiquitin's refractoriness to proteolytic digestion (12), and compact globular structure by nuclear magnetic resonance and x-ray crystallography (42, 43), suggests a relatively inert polypeptide which may have a relatively long half-life. It follows then, that by intrinsic labeling, it would be difficult to pulse efficiently a given tritiated amino acid through a large pool of long-lived ubiquitin over the 4- to 5-hour period of labeling.

The convergence of observations from two such disparate approaches as amino acid sequence analysis of a cell surface molecule purified with a monoclonal antibody to a lymphocyte homing receptor and the isolation of antibody reactive, expressed cDNA clones (10) provide compelling evidence that this antibody recognizes a ubiquitin-dependent determinant. However, the specificity of this antibody by cell and tissue section staining and by functional assays (36-39) raises a possible paradox. How can a determinant on a polypeptide as prevalent as ubiquitin be so selectively expressed, and what might that determinant be? Although the sequence of ubiquitin itself is highly conserved, novel immunogenic determinants might be generated de novo by conjugation to another species. If this were the case, one might expect antibody responses to ubiquitin to focus preferentially on the COOH-terminal region of ubiquitin where conjugation to other proteins is effected. In the accompanying report (10), we have presented evidence that the MEL-14 antibody reacts with a COOH-terminal peptide comprising positions 64 to 76 of ubiquitin, but not with intact ubiquitin. X-ray crystallography of ubiquitin demonstrates that the COOH-terminal amino acids extend away from the otherwise globular structure, and that this portion of the molecule appears particularly free of constraints on its motion (43). MEL-14 may therefore recognize either a specific determinant at the ubiquitin-receptor conjugate junction or, less likely, a determinant in the ubiquitin moiety proper induced by a conformational alteration due to conjugation to the core polypeptide.

We have only begun to characterize the polypeptide that we suggest is the core polypeptide of this lymphocyte homing receptor (Fig. 10). A striking feature about the initial available NH₂-terminal sequence of this chain is its richness in lysine, with four residues in

the first 32 amino acids. This observation leads to an interesting speculation. If this lysine richness is representative generally of the NH₂-terminus, opportunities for ubiquitination by isopeptide bond formation may exist. If $gp90^{MEL-14}$ is anchored in the membrane by its COOH-terminus, a ubiquitinated portion would likely be available for interactions, perhaps with the cell surface of HEV. Supporting this view, it has been shown that mildly trypsinized murine HEV-binding lymphocytes lose their capacity to bind to HEV, and lose MEL-14 reactivity (26). Perhaps functional lymph node HEV binding requires an NH₂-terminal trypsin-sensitive ubiquitinated peptide. Also, since the murine lymph node HEV ligand is sensitive to sialidase (44), a relatively positively charged NH₂-terminus might help stabilize the lymphocyte-HEV interaction.

Generality of cell surface ubiquitination. As a postranslational modification of cell surface proteins ubiquitination poses a problem in the biosynthesis of membrane proteins. Ubiquitination could occur cotranslationally on the cytoplasmic face of the rough endoplasmic reticulum (ER), necessitating transport of a branched chain polypeptide to the lumen of the ER. Because ubiquitin transcripts lack sequences that could encode a typical NH₂-terminal signal sequence (10, 28-31), ubiquitination within the ER or Golgi would require a new form of transport. Why only a subset of cell surface molecules is ubiquitinated is also unclear. Perhaps isopeptide bond formation occurs only with lysines surrounded by a "canonical" sequence, analogous to glycosylation sites. Resolution of this should reveal new understanding of sites and functions of ubiquitination and protein targeting mechanisms.

Assignments of a particular role for ubiquitin on cell surfaces can still only be a matter of speculation. The degree of conservation of ubiquitin throughout evolution suggests a fundamental cellular function. One proposed role, for which a body of evidence already exists, is that it is required for intracellular protein degradation (17-20). Conceivably ubiquitination of these cell surface proteins is incidental and simply serves as a tag for surface proteins that are destined for degradation. Indeed, it may be that a surface molecule such as a lymphocyte homing receptor has a special requirement for rapid internalization and degradation by either HEV's or the lymphocytes bearing it so that entry into a secondary lymphoid organ is directional and not easily reversible. Ubiquitination may then ensure rapid turnover. This could be a general property of many developmental cell surface molecules which target cells to particular sites.

Consideration should also be given to a possible independent role for ubiquitin at the cell surface. The MEL-14 antibody specifically and completely inhibits binding of lymphocytes to peripheral lymph node HEV (8), and from our evidence, it appears to recognize a ubiquitin-dependent determinant (10). The degree of motion allowed the COOH-terminal portion of ubiquitin (43) and the combinatorial permutations of ubiquitin conjugation to a number of cell surface proteins and to different sites on these proteins may provide a large repertoire of diverse receptors. Alternatively, the homing receptor backbone could provide specificity for a particular cell type, in this instance high endothelium, while ubiquitin might provide additional stability to the adhesive interaction. Such an avid association between two cell surfaces could be important for a lymphocyte to migrate through a vessel wall into a lymphoid organ. This mechanism could be a general requirement in many biological systems in which migration through vessel walls or other tissues is essential.

REFERENCES AND NOTES

1. J. L. Gowans and E. J. Knight, *Proc. R. Soc. London Ser. A*, **159**, 257 (1964).
2. V. T. Marchesi and J. L. Gowans, *ibid.*, p. 283.
3. H. B. Stamper and J. J. Woodruff, *J. Exp. Med.*, **144**, 828 (1976).
4. G. A. Gutman and I. L. Weissman, *Transplantation*, **16**, 621 (1973).

5. J. C. Howard, S. V. Hunt, J. L. Gowans, *J. Exp. Med.* **135**, 200 (1972).
6. S. K. Stevens, I. L. Weissman, E. C. Butcher, *J. Immunol.* **128**, 844 (1982).
7. E. C. Butcher and I. L. Weissman, *Ciba Fdn. Symp.* **71**, 265 (1979).
8. W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Nature (London)* **303**, 30 (1983).
9. R. A. Rasmussen *et al.*, *J. Immunol.* **135**, 19 (1985).
10. T. St. John *et al.*, *Science* **231**, 845 (1986).
11. G. Goldstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 11 (1975).
12. D. H. Schlessinger, G. Goldstein, H. D. Niall, *Biochemistry* **14**, 2214 (1975).
13. ———, *Nature (London)* **255**, 423 (1975).
14. D. C. Watson, W. B. Levy, G. H. Dixon, *ibid.* **276**, 196 (1978).
15. J. G. Gavilanes *et al.*, *J. Biol. Chem.* **257**, 10267 (1982).
16. I. L. Goldknopf and H. Busch, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 864 (1977).
17. A. Hershko and A. Ciechanover, *Annu. Rev. Biochem.* **51**, 335 (1982).
18. A. Ciechanover, C. Finley, A. Varshavsky, *J. Cell Biochem.* **24**, 27 (1984).
19. A. Hershko, *Cell* **34**, 11 (1983).
20. D. Finley, A. Ciechanover, A. Varshavsky, *ibid.* **37**, 43 (1984).
21. I. L. Goldknopf, G. Wilson, N. R. Ballal, H. Busch, *J. Biol. Chem.* **255**, 10555 (1980).
22. R. S. Wu, R. W. Kohn, W. M. Bonner, *ibid.* **256**, 5916 (1981).
23. L. Levinger and A. Varshavsky, *Cell* **28**, 375 (1982).
24. S. I. Matsui, B. K. Seon, A. A. Sandburg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6386 (1979).
25. W. M. Gallatin, in preparation.
26. S. T. Jalkanen *et al.*, in preparation.
27. R. M. Hewick, M. W. Hunkapiller, L. E. Hood, W. J. Dreyer, *J. Biol. Chem.* **256**, 7990 (1981).
28. E. Dworkin-Rastl, A. Shrutkowski, M. B. Dworkin, *Cell* **39**, 321 (1981).
29. E. Ozkaynak, D. Finley, A. Varshavsky, *Nature (London)* **312**, 663 (1984).
30. U. Bond and M. J. Schlessinger, *Mol. Cell Biol.* **5**, 949 (1985).
31. O. Wiborg *et al.*, *EMBO J.* **4**, 755 (1985).
32. D. L. Urdal *et al.*, *J. Chromatog.* **296**, 171 (1984).
33. J. H. Elder and S. Alexander, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4540 (1982).
34. W. M. Gallatin *et al.*, unpublished observations.
35. H. T. Smith and V. A. Fried, in preparation.
36. E. C. Butcher, R. G. Scollay, I. L. Weissman, *J. Immunol.* **123**, 1996 (1979).
37. S. K. Stevens, I. L. Weissman, E. C. Butcher, *ibid.* **128**, 844 (1982).
38. R. A. Reichert, W. M. Gallatin, I. L. Weissman, E. C. Butcher, *J. Exp. Med.* **157**, 813 (1983).
39. M. O. Dailey, C. G. Fathman, E. C. Butcher, E. Pillemer, I. Weissman, *J. Immunol.* **128**, 2134 (1982).
40. S. Matsui, A. A. Sandburg, S. Negoro, B. K. Seon, G. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1535 (1982).
41. A. Hershko, H. Heller, S. Elias, A. Ciechanover, *J. Biol. Chem.* **258**, 8206 (1983).
42. P. D. Cary *et al.*, *Biochim. Biophys. Acta* **624**, 378 (1980).
43. S. Vijay-Kumar, C. E. Bugg, R. D. Wilkinson, W. J. Cook, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3582 (1985).
44. S. D. Rosen, M. S. Singer, T. A. Yednock, L. M. Stoolman, *Science* **228**, 1005 (1985).
45. O. N. Witte, N. Rosenberg, M. Paskind, A. Shields, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2488 (1978).
46. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
47. S. E. Cullen, T. N. Freed, S. G. Nathenson, *Transplant. Rev.* **30**, 236 (1976).
48. E. S. Vitetta, J. D. Capra, D. G. Klapper, J. Klein, J. W. Uhr, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 905 (1976).
49. The monoclonal antibody 9B5 was provided by E. Butcher.
50. P. H. O'Farrell, *J. Biol. Chem.* **250**, 4007 (1975).
51. P. Z. O'Farrell, H. M. Goodman, P. H. O'Farrell, *Cell* **12**, 1133 (1977).
52. Monoclonal antibodies R7D4, 3BD7, and 2DG12 were provided by R. Levy.
53. Protease-free Endo F was provided by J. Elder.
54. We thank Dr. A. Begovich, for assistance with two-dimensional gel electrophoresis, Drs. P. Estess and B. Sher for discussions and critical review of the manuscript, and J. Mason for secretarial help. Supported by NIH grant AI 19512; a grant from the American Cancer Society, California Division (W.M.G. and T.St.J.); Tumor Biology training grant CA 09151 (T.St.J. and M.S.); NIH grant GM 31461 (V.A.F.), and the American Lebanese Syrian Associated Charities (V.A.F.). Amino acid sequencing was carried out at and supported by the DNAX Research Institute.

20 August 1985; accepted 20 December 1985

AAAS–Newcomb Cleveland Prize

To Be Awarded for an Article or a Report Published in *Science*

The AAAS–Newcomb Cleveland Prize is awarded to the author of an outstanding paper published in *Science*. The value of the prize is \$5000; the winner also receives a bronze medal. The current competition period begins with the 3 January 1986 issue and ends with the issue of 29 May 1987.

Reports and Articles that include original research data, theories, or syntheses and are fundamental contributions to basic knowledge or technical achievements of far-reaching consequence are eligible for consideration for the prize. The paper must be a first-time publication of the author's own work. Reference to pertinent earlier work by the author may be included to give perspective.

Throughout the competition period, readers are invited to

nominate papers appearing in the Reports or Articles sections. Nominations must be typed, and the following information provided: the title of the paper, issue in which it was published, author's name, and a brief statement of justification for nomination. Nominations should be submitted to the AAAS–Newcomb Cleveland Prize, AAAS, 1333 H Street, NW, Washington, DC 20005, and **must be received on or before 30 June 1987**. Final selection will rest with a panel of distinguished scientists appointed by the editor of *Science*.

The award will be presented at a ceremony preceding the President's Public Lecture at the 1988 AAAS annual meeting to be held in Boston. In cases of multiple authorship, the prize will be divided equally between or among the authors.