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PRIMARY STRUCTURE OF HUMAN CLASS II HISTOCOMPATIBILITY ANTIGENS

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR INTEGRAL MEMBRANE PROTEINS

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SUMMARY

The amino acid sequence of the human HLA-DR2 class II antigen was determined with the aid of high-performance liquid chromatography. By applying exclusively Hypersil-ODS as the stationary phase in combination with three buffer systems, the amino acid sequences of the extracellular parts of the DRα- and DRβ-chains could be established. Moreover, the sensitivity of the procedure allowed the determination of the primary structures of additional class II polypeptides isolated together with the DR2α- and the DR2β-chains. Significant insight into the heterogeneity of class II molecules was thereby obtained. In addition, a simple, rapid and selective method of general applicability is described for the isolation of membrane fragments of integral membrane proteins. Using Sep-Pak cartridges with a mobile phase containing 60% formic acid and varying 1-propanol concentrations, the highly hydrophobic peptides can selectively be separated from the hydrophilic fragments.

INTRODUCTION

Class II histocompatibility antigens represent a group of molecules which is probably actively involved in both the humoral and the cellular immune responses. In contrast to class I HLA-antigens, class Ii antigens have a confined cellular distribution. They are primarily found on B cells, macrophages and activated T cells. Among other features, they are possibly responsible for the extent to which the immune system attacks bacteria, viruses, proteins or peptides invading the human organism. By mediating the interactions between lymphocyte subsets (B cells and T-

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helper or suppressor cells) these antigens have a regulatory rôle in antibody production and the cell-mediated immune response. Recently, the primary structures of class II antigens have been determined[1-3]. They consist of two non-covalently associated polypeptide chains. Both the α- and β-subunits are integral membrane glycoproteins. These are assumed to be embedded as bimolecular complexes in the plasma membrane.

The α-chains (molecular weight, M_r 34,000) and the β-chains (M_r 29,000) are organized in three regions: first, a hydrophilic extracellular N-terminal portion; secondly, a short, possibly helical stretch of exclusively hydrophobic amino acids spanning the membrane and thirdly, a short hydrophilic cytoplasmic tail, representing the C-terminus.

Class II antigens were isolated from a lymphoblastoid B-cell line homozygous at the HLA loci (HLA-A3, 3; B7, 7; Dw2, 2; DR2, 2; MT1, 1; MB1, 1; DC1, 1; SB4, 4) by a three-step chemical procedure[4,5]. Having solubilized the crude membrane with the non-ionic detergent NP-40, the α–β complex was considerably enriched by ion-exchange chromatography on CM-cellulose. Low- and high-molecular-weight contaminants were removed by gel filtration over Sephacryl S300 in sodium dodecyl sulphate (SDS) and finally the α/β complex was separated into α- and β-chains on a hydroxylapatite column in the presence of SDS.

Isoelectric focusing and two-dimensional electrophoresis on an analytical scale revealed at least two species for the α-chain fraction and an even higher degree of heterogeneity for the β-chain pool. Employing reversed-phase chromatographic systems of high sensitivity and resolution power, we succeeded in elucidating the primary structures of class II HLA-antigens. This was possible despite the heterogeneity of the starting material.

EXPERIMENTAL

High-performance liquid chromatography (HPLC)

A Du Pont Model 850 or 870 microprocessor-controlled single-pump liquid chromatograph in the binary gradient version equipped with a Du Pont UV detector and a DuPont column oven was used.

The stainless-steel columns (250 × 4.6 mm) were packed by us with 5-μm Hypersil ODS (Shandon, Runcorn, U.K.). They were washed daily with at least ten column volumes of methanol.

Enzymatic digestions

A 10-mg amount of either the α- or the β-chain fraction was digested with trypsin, α-chymotrypsin or staphylococcal protease (SV-8) in 0.05 *M* NH_4HCO_3 at pH 8.0 (in the case of SV-8 protease, pH 7.8) at a substrate/enzyme ratio of 50/1 and concentrated in a rotary evaporator to 0.5 ml.

Buffers

All separations were carried out at a flow-rate of 1.9 ml/min at elevated temperatures (60°C), the UV detector was set at 220 nm and sensitivity 0.08, 0.16 or 0.64 a.u.f.s. The following three buffer systems were employed:

(1) Buffer A: 0.025 *M* ammonium acetate, pH 6.0 (acetic acid)

Buffer B: 0.05 *M* ammonium acetate, pH 6.0–acetonitrile (40:60)
Gradient: 0–70% B within 60 min

(2) Buffer A: 0.005 *M* potassium phosphate, pH 6.0
Buffer B: 0.005 *M* potassium phosphate, pH 6.0–acetonitrile (40:60)

(3) Buffer A: water, trifluoroacetic acid, pH 2.15
Buffer B: A–acetonitrile (40:60)

System 1 was used for all primary separations while Systems 2 or 3 were applied for rechromatographies, *i.e.*, 3 for the peptide fractions eluted early, and 2 for the remaining fractions.

Amino acid analyses

Amino acid analyses were performed by means of a Durrum analyzer (Model D 500; Dionex, Palo Alto, CA, U.S.A.), and peptides were hydrolyzed with 6 *M* hydrochloric acid at 110°C for 24 h in sealed glass tubes which had been previously evacuated.

Amino acid sequence determinations

Peptides purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and characterized by amino acid analysis were subjected to the modified form of the Edman degradation by employing the phenylisothiocyanate–dimethylamino-azobenzene-isothiocyanate (PITC/DABITC) double coupling procedure according to Chang *et al.*[6]. Dimethylamino-azobenzene thiohydantoin (DABTH) derivatives of amino acids were identified by two-dimensional thin-layer chromatography (TLC) on 3 × 3 cm polyamide sheets. Isoleucine/leucine determinations were performed according to Yang[7].

Experiments with Sep-Pak cartridges

C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were washed with 10 ml of methanol and 10 ml of water prior to use. Peptide mixtures, dissolved in 0.05 *M* NH_4HCO_3, were repeatedly passed through the cartridges by using a 10-ml plastic syringe and were eluted stepwise with 5-ml portions of 60% formic acid varying in *n*-propanol content between 0 and 80%. The collected fractions were concentrated by a stream of nitrogen and characterized by appropriate methods.

RESULTS AND DISCUSSION

The lyophilized α- or β-chain fraction was dissolved in 0.1 *M* Tris–HCl buffer (pH 8.0), containing 8 *M* urea, then reduced with dithiothreithol (DTT) and alkylated with recrystallized iodoacetic acid. After extensive dialysis, aliquots were digested with trypsin, α-chymotrypsin or staphylococcal protease. The resulting peptide mixtures were concentrated and 60-μl portions were subjected to RP-HPLC in buffer system 1. Figs. 1 and 2 show representative chromatograms of the peptide mixtures resulting from the α- and β-chains.

Fractions collected manually during these primary separations were successfully rechromatographed in either buffer system 2 or 3 (see Experimental). The high resolving power of this combination had been previously demonstrated during an investigation of the tryptic digests of Bence–Jones proteins[8,9], Substituting 0.05 *M* ammonium acetate for 0.005 *M* potassium phosphate, the fractions were rechro-

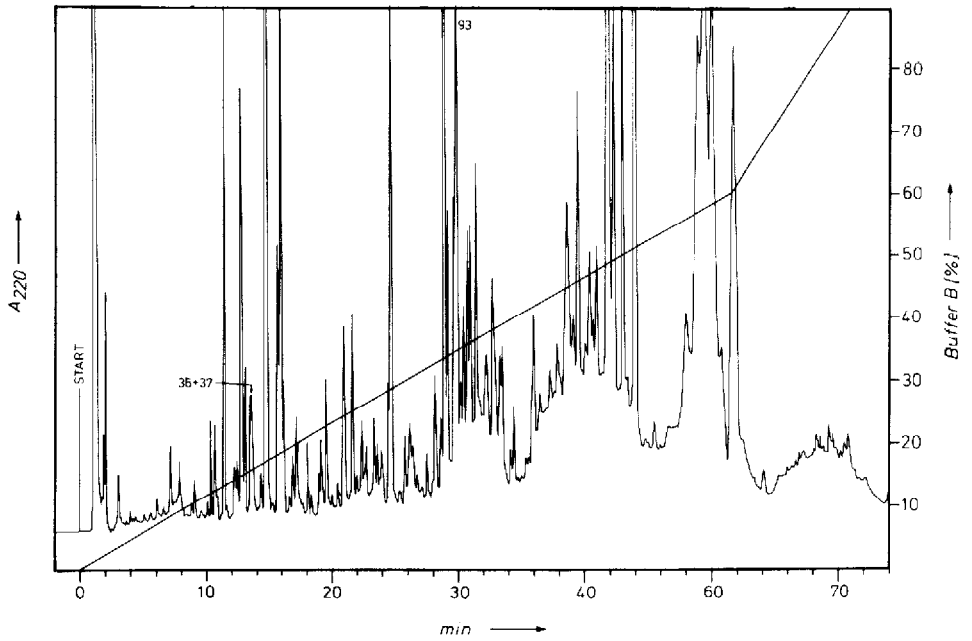

Fig. 1. Micropreparative separation of a tryptic digest of the human class II α-chain fraction, derived from a human lymphoblastoid B-cell line homozygous at the HLA loci, in solvent system 1. Sixty microlitres of concentrated digest were injected at a flow-rate of 1.9 ml/min. The column temperature was 60°C. The gradient conditions are indicated as changes of buffer 2.

matographed with solvent system 2 on the same reversed-phase support (ODS-Hypersil) at the same pH, flow-rate and column temperature. Excellent resolution of the peptides unresolved in the initial chromatogram could be achieved. Amino acid analyses performed with aliquots of these rechromatographed fractions resulted in integral numbers for each amino acid, indicating a high degree of purity. Only a few fractions were resolved by applying the trifluoroacetic acid system at pH 2.15. The theoretically predicted increase of capacity factor (k') for acidic and decrease of k' for basic peptides[20] proved to be advantageous for fragments eluted early in buffer system 1. Examples of such rechromatographies are exhibited in Fig. 3.

The α-chain fraction

All the peptides isolated with the combined HPLC systems were subsequently characterized by amino acid analyses and their sequence was determined by the manual DABITC/PITC degradation[6]. Tryptic, α-chymotryptic and SV-8 protease fragments allowed the correct arrangement of the α-chain peptides to be determined. In Fig. 4 the complete amino acid sequence of the extracellular portion of the HLA-DR2α-chain is shown. In this sequence is included the nearly complete amino acid sequence of a second α-chain which could be established simultaneously. This polypeptide defines the DC subclass, a second class II α type, which was isolated together with the DR2α chain. As estimated from the yields of the peptides, the DRα and DCα chains represent 80 and 20%, respectively, of the total α fraction.

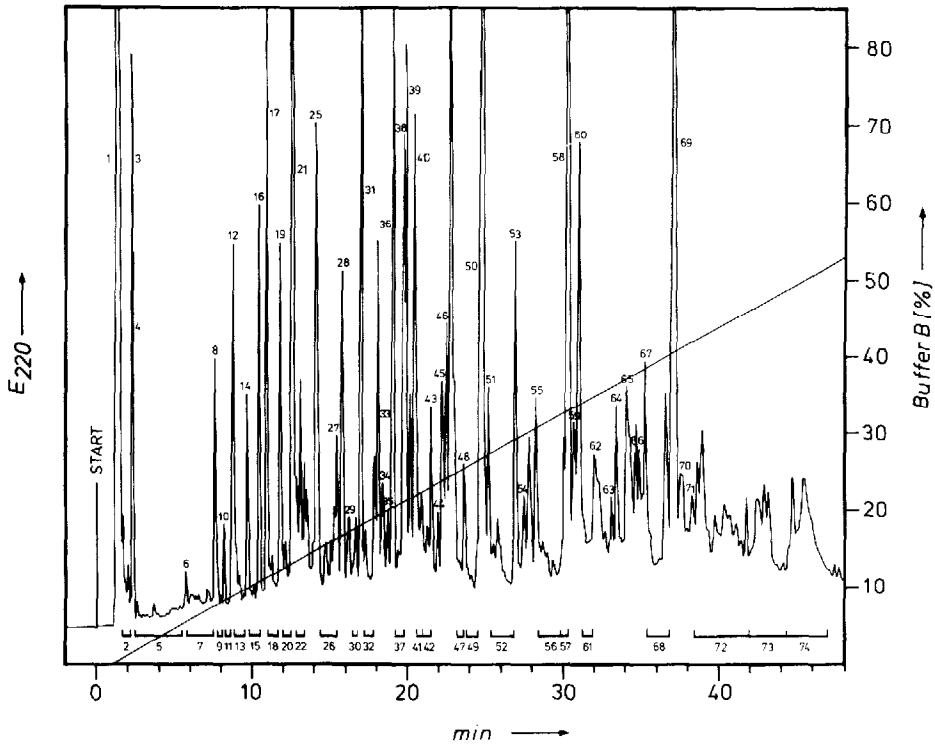

Fig. 2. Micropreparative separation of peptides generated by digesting the human HLA class II β-chain fraction with protease, *Staphylococcus aureus*, V8. Chromatography was performed on Hypersil-ODS in a laboratory-packed column (250 × 4.6 mm) with a flow-rate of 1.9 ml/min. The temperature of the column heater was set at 60°C. Gradient conditions are indicated as changes of buffer B (solvent system 1). Manually collected fractions are consecutively numbered. For repeated chromatography see Fig. 3.

The β-chain fraction

By applying the described chromatography–rechromatography system to the digestion mixtures of the β fraction we were able to establish the amino acid sequence of the extracellular portion of the DR2β chain[1]. This second subunit completes the extracellular portion of the HLA-DR2 antigen.

From the overlapping peptide sequences the complete extracellular portion of the DR2β-chain could be determined. Additional homologous fragments could be isolated and also characterized by amino acid analysis and Edman degradation. From these numerous peptides, isolated in relatively low yields, many could be ordered by overlaps or sequence homology to the nearly complete amino acid sequence of the extracellular portion of a second and major portions of a third β-chain. Additional peptides revealed that at least six β-chains were isolated along with the main DR2β-chain. These amino acid sequences are shown in Fig. 5. This result demonstrates that heterogeneous peptide mixtures including highly homologous fragments are successfully resolved by RP-HPLC systems. From the yield of homologous fragments occupying positions 60–69 of six different β-chains, these β-chains are estimated as 65, 14.5, 5.6, 2.7, 8.8 and 3.2% of the total β pool.

Recently, amino acid sequences deduced from genomic and cDNA encoding

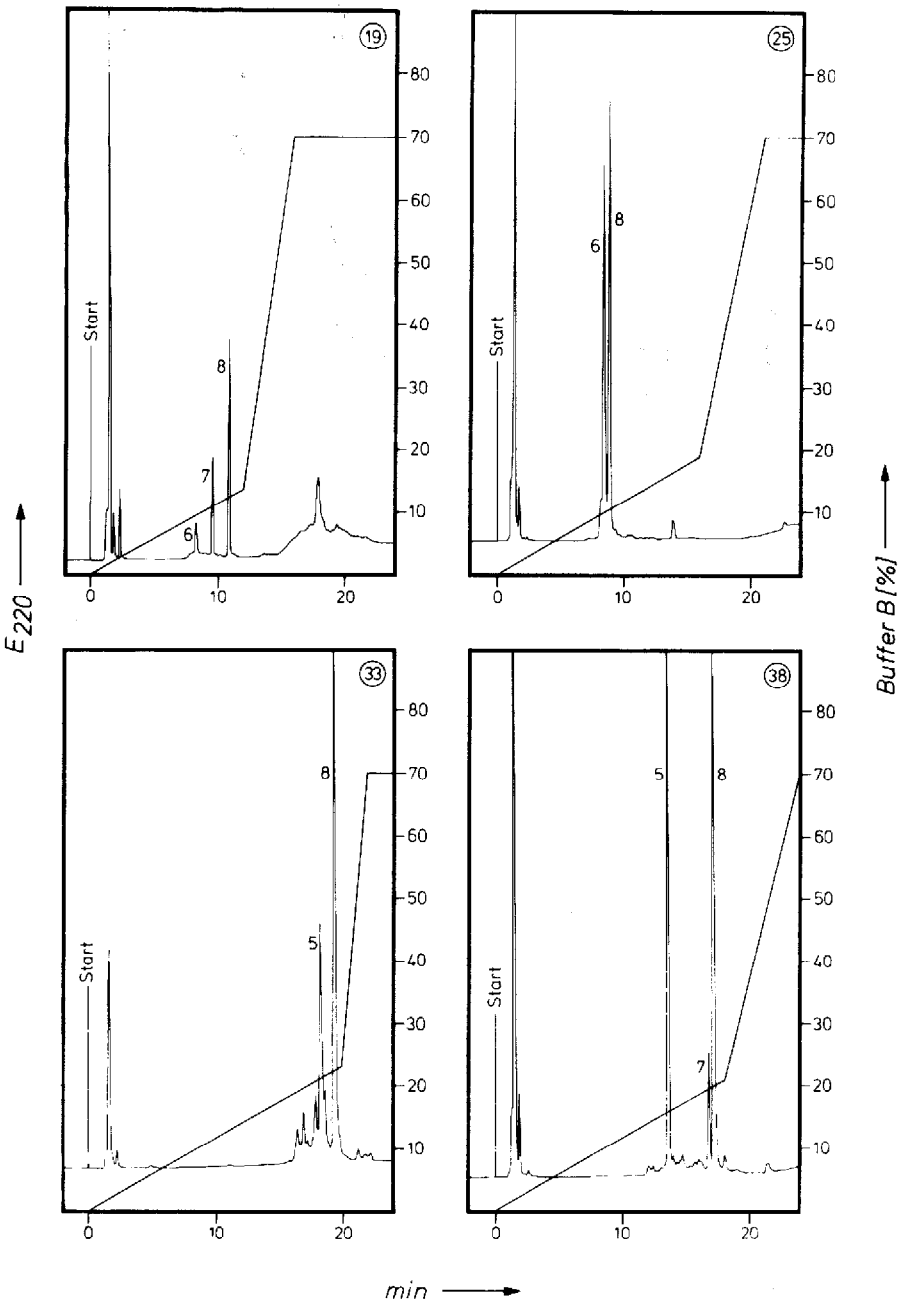

Fig. 3. Repeated chromatography of Fractions 19, 25, 33 and 38, collected from the chromatogram depicted in Fig. 2. These separations were performed with buffer system 2 and gradients as indicated.

histocompatibility antigens were reported. The data for the DRα^{10-13} and DC$\alpha^{14,15}$ chains are in complete agreement with sequences established in our laboratory. This demonstrates that the described RP-HPLC system enabled us to resolve peptide mix-

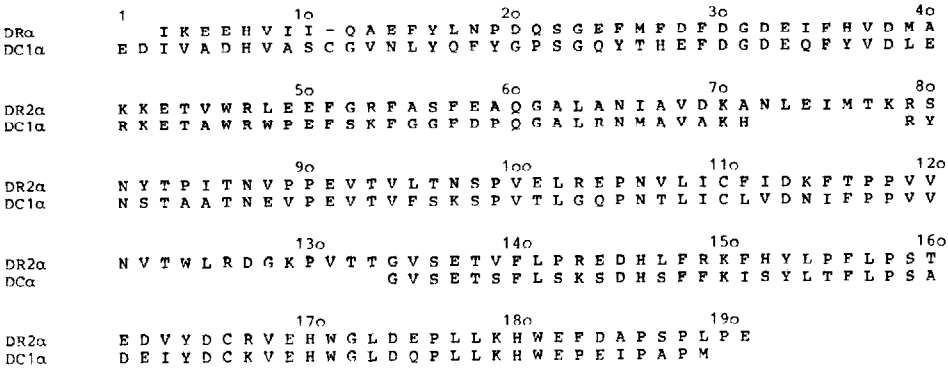

Fig. 4. Amino acid sequences established for the HLA-DR2α- and HLA-DC1α-chain. These are based on overlaps derived from peptides generated by digestion with trypsin, α-chymotrypsin and protease, *S. aureus*, V8.

tures derived from enzymatic digests of as many as seven proteins which display up to 85% homology.

Furthermore, these results show that the elevated temperatures used provide excellent peak shape and resolution and are compatible with peptide separations. All amides were assigned correctly. Previously, it was unclear whether the single discrepancy between nucleotide and protein sequence found in position 127 of the DRα-chain was due to the temperature of 60°C employed during reversed-phase chromatography. Since asparagine in the Arg-Asn-Gly sequence has the shortest half-life as compared with asparagine residues adjacent to other amino acids[16], deamidation may have occurred during cell culture or isolation of the polypeptide chain by conventional methods.

As described above, the primary structure of the extracellular hydrophilic part from both HLA class II subunits could be established. The recently published DNA

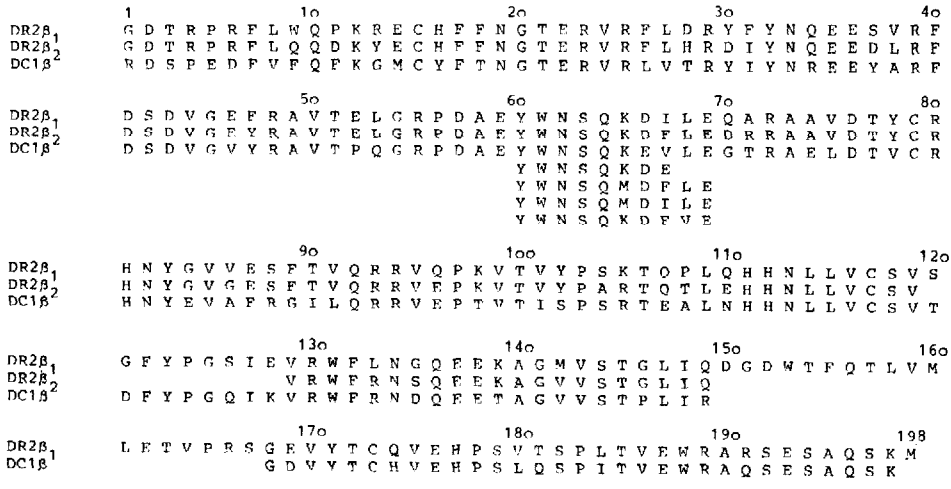

Fig. 5. Amino acid sequences established for HLA-DR/DC β-chains. To elucidate these sequences, tryptic, α-chymotryptic and digests with protease, *S. aureus*, V8, were necessary, With the heterogeneity shown in positions 60 69, seven β-chains were detected.

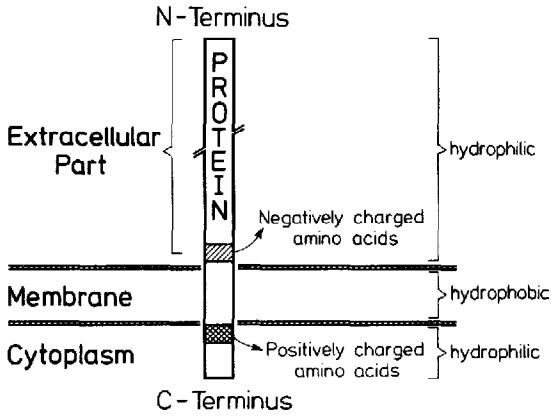

Fig. 6. Principal structure of integral membrane proteins, as exemplified by the histocompatibility antigens.

sequences[10–15] of these antigens revealed that by the present RP-HPLC procedure most of the peptides corresponding to the C-terminal hydrophilic tail could be isolated. However, tryptic, α-chymotryptic or digests generated with staphylococcal protease did not result in fragments including the 10% membranous portion of these molecules. Peptides which contain longer segments of exclusively hydrophobic amino acids are probably irreversibly bound to the reversed-phase support under the applied chromatographic conditions. This hypothesis was supported by the fact that melittin, a highly hydrophobic polypeptide, could not be eluted by any of the three buffer systems described above[17]. Melittin was used as a model compound since its principal structure, shown in Fig. 6, resembles those found in membrane segments of integral membrane proteins. Its hydrophobicity index, calculated according to Segrest and Feldmann[18], is of the same order of magnitude as those calculated for peptides penetrating the membrane. Another feature common to melittin and membrane-derived peptides is that the hydrophobic amino acids spanning the membrane are followed by a cluster of basic residues which may interact via hydrogen bonding with the surface silanol groups of the alkylsilica matrix[19,20] and consequently hinder elution.

Experiments carried out with various buffers demonstrated that, despite its water solubility, melittin required drastic conditions in order to be eluted from ODS-Hypersil. Using acetonitrile as the organic modifier, the most effective elution was observed in acidic buffers of high salt concentration[21,22]. However these buffers were non-volatile. Substituting 1-propanol for acetonitrile in volatile buffers results in mixtures of high viscosity which shorten column life and considerably reduce resolution. Consequently, in spite of being volatile, these systems are also less suitable for primary separations.

To utilize most effectively the described chromatographic procedures for peptide mixtures including extremely hydrophobic fragments, the best approach involves a pre-separation of the hydrophilic from the hydrophobic peptides prior to RP-HPLC. Preliminary experiments with Sep-Pak cartridges were promising. When melittin was added to the tryptic digest of a Bence–Jones protein (M_r = 23,000) in molar amounts and absorbed in a C_{18} cartridge, all the hydrophilic peptides could be eluted with 60% formic acid containing as much as 8% 1-propanol. With 9% 1-propanol in 60% formic acid, impure melittin was eluted. Analytically pure melittin was re-

covered with a 56% yield by washing the cartridge with 5 ml of 25% 1-propanol in 60% formic acid. Since this buffer system is volatile, fractions containing the hydrophilic peptides can be evaporated, redissolved and chromatographed with the well established HPLC procedures.

With the following procedure we succeeded in isolating the membranous fragment of the DRα-chain: 1.7 mg (50 nmol) of reduced and alkylated α-chain were digested with α-chymotrypsin for 6 h in 0.05 *M* ammonium bicarbonate at pH 8.3. The 2-ml solution was then absorbed in a C_{18} cartridge which had been previously washed with 10 ml methanol and equilibrated with 10 ml water. Elution was performed stepwise with 10 ml formic acid (60%) and ten 5-ml portions of 60% formic acid mixtures containing increasing concentrations of 1-propanol. In this case the hydrophilic peptides were eluted between 0 and 5% 1-propanol. Between 6 and 9% propanol a glycopeptide appeared which during chromatography of the α-chymotryptic digest on the ODS-Hypersil support with buffer system 1 had been eluted in only small quantities at the end of the gradient. With 25% propanol, 16 nmol (30%) of the intramembranous α-chymotryptic peptide could be eluted. The tight binding of this peptide to the hydrophobic support is obvious since with 40, 60 and even 80% propanol the portions of this fragment which were eluted corresponded to a total yield of only 58%.

The amino acid analysis indicating a high content of hydrophobic residues (Gly, Val, Ile and Leu) and the N-terminal sequence, determined by liquid-phase sequencing, undoubtedly classify this α-chymotryptic peptide as the membrane fragment always lost during previous chromatographic experiments. To test whether this hydrophobic moiety is bound to the ODS-Hypersil essentially irreversibly, we eluted the column used for primary separations of the α-chymotryptic digest of the DRα fraction with 60% formic acid and increased the 1-propanol concentrations stepwise. Between 0 and 8% propanol nothing was eluted, indicating that indeed all hydrophilic peptides were eluted during chromatography with buffer system 1. With 9 and 25% propanol the α-chymotryptic membrane fragment was eluted in about 19% yield. Interestingly, this elution was observed although the column had been washed daily with 50% methanol in water, methanol alone and even once by a gradient from 0 to 70% ethanol in 4.5% formic acid. The intact peptide was still detected, although the column had been stored for 2 years at room temperature.

CONCLUSIONS

The use of three solvent systems in combination with ODS-Hypersil and elevated temperature has proved to be successful in resolving heterogeneous peptide mixtures derived from enzymatic digests of class II HLA-antigens which were coisolated because of their highly homologous character. Having isolated single pure peptides by RP-HPLC, these peptide sequences could be ordered to the complete amino acid sequence of the HLA-DR2 antigen α-chain, the extracellular part of the HLA-DR2 antigen β-chain and major portions of additional class II antigen α- and β-polypeptides.

During the determination of the primary structure of class II human histocompatibility antigens, a procedure has been developed which is of general usefulness in amino acid sequence investigations of integral membrane proteins. Preceding

RP-HPLC of enzymatic digests, these peptide mixtures are first separated into a hydrophilic and a hydrophobic portion. Examination of the hydrophilic fraction by use of the well established volatile buffer systems no longer results in losses of hydrophobic fragments since they have been isolated in advance. If necessary, the hydrophobic moiety can be subjected to more drastic conditions for further purification, *i.e.*, reversed-phase chromatography including high salt concentrations, stronger acids or organic modifiers. These methods generally require additional handling, such as desalting steps, and therefore they should be avoided for analysis of hydrophilic peptides.

Most interesting is the procedure for integral membrane proteins which penetrate the membrane only once. Compared with all other regions, the membrane segment has the most pronounced hydrophobicity and therefore it is easily isolated in high yield and purity as a single peptide from enzymatic digests. Subsequently, these fragments can be used to determine the positions of post-translational modifications, such as phosphorylation and acylation with fatty acids.

In spite of advanced DNA methodology, the present analyses demonstrate that protein sequence analysis not only compares well with nucleotide sequencing, it is the only tool for elucidating the complete phenotype.

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REFERENCES

1 H. Kratzin, C. Y. Yang, H. Götz, E. Pauly, S. Kölbel, G. Egert, F. P. Thinnes, P. Wernet, P. Altevogt and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1665.
2 C. Y. Yang, H. Kratzin, H. Götz, F. P. Thinnes, T. Kruse, G. Egert, E. Pauly, S. Kölbel, P. Wernet and N. Hilschmann, *Hoppe-Zeyler's Z. Physiol. Chem.*, 363 (1982) 671.
3 H. Götz, H. Kratzin, F. P. Thinnes, C. Y. Yang, T. Kruse, E. Pauly, S. Kölbel, G. Egert, P. Wernet and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 364 (1983) 749.
4 F. P. Thinnes, G. Egert, H. Götz, E. Pauly, P. Altevogt, S. Kölbel, P. Wernet, H. Kratzin, C. Y. Yang and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, in press.
5 G. Egert, F. P. Thinnes, H. Götz, E. Pauly, H. Kratzin, C. Y. Yang, S. Kölbel, P. Altevogt, P. Wernet and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, in press.
6 J. Y. Chang, D. Brauer and B. Wittmann-Liebold, *FEBS Lett.*, 93 (1978) 205.
7 C. Y. Yang, *Hoppe-Seyler's Z. Physiol. Chem.*, 360 (1979) 1673.
8 H. Kratzin, C. Y. Yang, J. U. Krusche and N. Hilschmann, *Hoppe-Zeyler's Z. Physiol. Chem.*, 361 (1980) 1591.
9 C. Y. Yang, E. Pauly, H. Kratzin and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1131.
10 D. Larhammar, K. Gustafsson, L. Claesson, P. Bill, K. Wiman, L. Schenning, J. Sundelin, E. Widmark, P. A. Peterson and L. Rask, *Cell*, 30 (1982) 153.
11 J. S. Lee, T. Trowsdale, P. J. Travers, J. Carey, F. Grosveld, J. Jenkins and W. F. Bodmer, *Nature (London)*, 299 (1982) 750.
12 A. J. Korman, C. Auffray, A. Schamboeck and J. L. Strominger, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 6013.
13 H. K. Das, S. K. Lawrance and S. M. Weissman, *Proc. Nat. Acad. Sci. U.S.*, 80 (1983) 3543.
14 C. Auffray, A. J. Korman, M. Roux-Dosseto, R. Bono and J. L. Strominger, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 6337.

15 H-C. Chang, T. Moriuchi and J. Silver, *Nature (London)*, 305 (1983) 813.
16 A. B. Robinson, J. W. Scotchler and J. H. McKerrow, *J. Amer. Chem. Soc.*, 95 (1973) 8156.
17 H. Tschesche (Editor), *Modern Methods in Protein Chemistry, Review Articles*, Walter de Gruyter, Berlin, New York, 1983, p. 207.
18 J. P. Segrest and J. Feldmann, *J. Mol. Biol.*, 87 (1974) 853.
19 R. P. W. Scott, *Advan. Chromatogr.*, 20 (1982) 167.
20 M. T. W. Hearn, in Cs. Horváth (Editor), *HPLC—Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 86.
21 J. L. Meek, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1632.
22 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.