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Purification and structural characterisation of human *HLA*-linked B-cell antigens

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The human B cell-specific alloantigen which is closely linked genetically to HLA *contains two non-covalently associated, sialogycoprotein subunits of molecular weight (MW) 29,000 (p29), and 34,000 (p34). Although p29 and p34 have different amino-terminal sequences, their tyrosine peptide maps indicate considerable similarity in other portions of their polypeptide chains. Thus the genes for their proteins may have evolved by duplication of a common ancestral gene. Another lymphocyte cell surface protein of MW 16,000 (p16) has also been characterised. Both p16 and p44 (the heavy chain of HLA-A,B antigens) have been compared with p29 and p34.*

A HUMAN system of cell surface alloantigens has recently been identified which is expressed predominantly on B lymphocytes, and is determined by a gene in *HLA* (refs 1–4), the major histocompatibility complex (MHC) of humans. At least two genetic loci for B-cell alloantigens have been identified in the *HLA* region separable by crossing over[5]. One of these loci is either identical to or in very strong linkage disequilibrium with the *HLA*-D locus, the genetic locus causing the strongest stimulation of the mixed lymphocyte culture (MLC) (ref. 1). Human *HLA*-linked B-cell antigens are serologically distinct from the cell surface antigens which are products of the classical *HLA*-A and *HLA*-B loci. It has previously been shown that papain solubilised and purified *HLA*-linked B-cell alloantigens consist of a complex of two polypeptides of 23,000 (p23) and 30,000 (p30) molecular weight (MW) (refs 6–8). Antisera raised against this complex are potent inhibitors of the MLC reaction and precipitate polypeptides of 29,000 and 34,000 MW (refs 6–9) from detergent-solubilised extracts.

This report describes the isolation of detergent soluble *HLA*-linked B-cell alloantigens, analysis of their subunit structure, and characterisation of the subunits by amino acid analysis, NH_2-terminal sequencing, and peptide mapping.

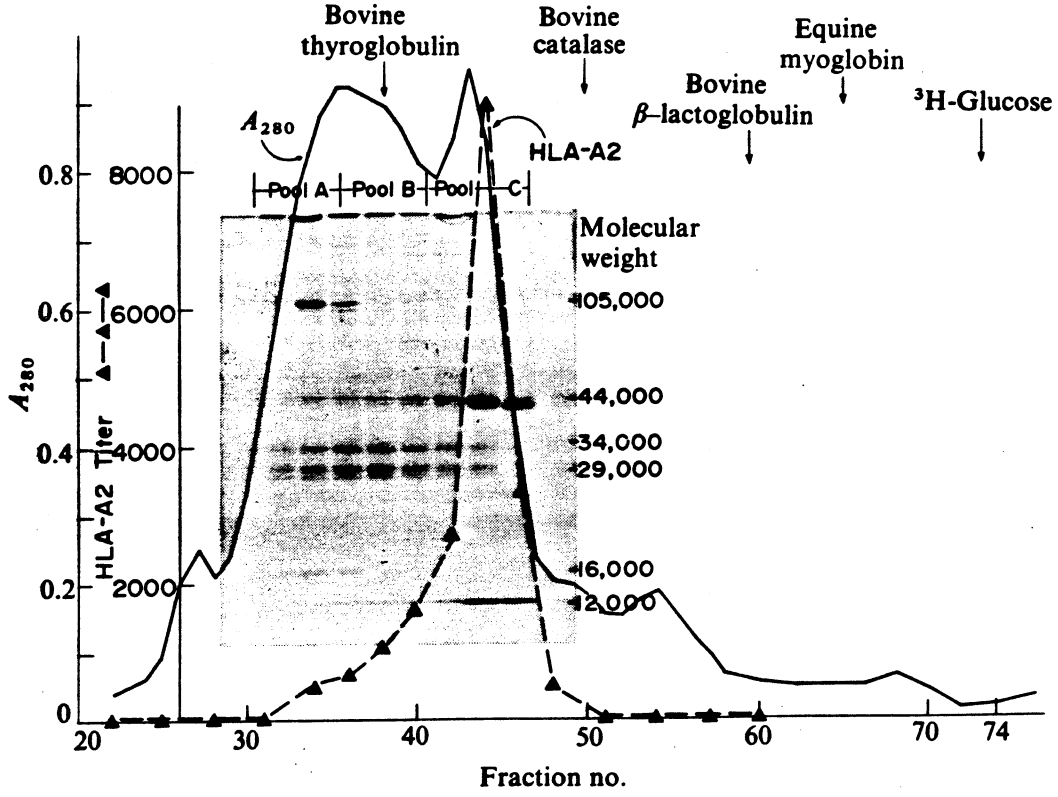

Fig. 1 BioGel A-5m filtration and SDS gel electrophoresis profile of p29, 34. BioGel A-5m filtration of the lectin column eluate was as described[10,11]. Equal volumes of the even numbered fractions 30–48 were precipitated with five volumes of acetone and electrophoresed on Laemmli 11% polyacrylamide–SDS slab gels[12], after boiling in 5% ME sample buffer, and in the figure are aligned with their respective BioGel fractions.

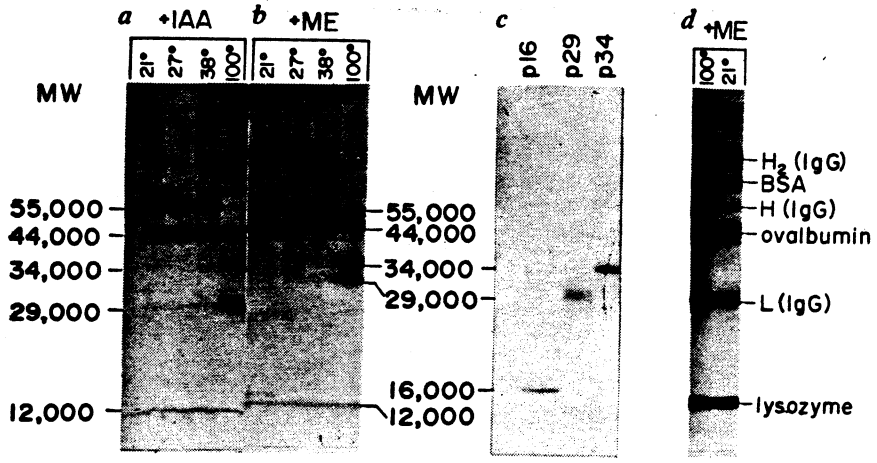

Fig. 2 SDS gels of p29,34 preparations after various treatments. *a*, SDS gel electrophoresis in 10% acrylamide Laemmli gels[12] of material purified in the absence of DTT by lectin affinity chromatography and gel filtration[13,14] after incubation with 0.6% SDS and 10 mM IAA for ½ h at 20, 27 and 38 °C or for 5 min at 100 °C. *b*, As above, except incubation was in the presence of 5% ME instead of IAA. *c*, 2 μg of each polypeptide eluted from gels[19] was electrophoresed in an SDS7–15% acrylamide gradient gel. *d*, A mixture of standards (IgG, bovine serum albumin (BSA), ovalbumin, and lysozyme) were electrophoresed after treatment as in (*b*).

Purification

Membranes were prepared from RPMI 4265 lymphoblastoid cells, solubilised with the non-ionic detergent Brij 99 : Brij 97 (2 : 1), and after high speed centrifugation, the detergent soluble supernatant was adsorbed on to and eluted from a *Lens culinaris* lectin affinity column[10,11]. The eluate was subjected to gel filtration on a BioGel A-5m column (Fig. 1). Fractions from the area of the column containing protein were analysed by sodium dodecyl suphate (SDS) gel electrophoresis (Fig. 1, inset) and placed in pools A, B and C. The material applied to the BioGel A-5m column consisted predominantly of six polypeptides of MW 105,000 (p105), 44,000 (p44), 34,000 (p34), 29,000 (p29), 16,000 (p16), and 12,000 (p12). p105 and p16 were in a sharp peak at 900,000 MW (pool A), and p44 and p12, the subunits of HLA-A,B antigens, were in a sharp peak at 400,000 MW (pool C) with some aggregated p44,12 at higher MW. p29 and p34, which are the subunits of the *HLA*-linked B cell alloantigen (see below), chromatographed at an intermediate MW in a broad peak indicative of partial aggregation (pools A and B) and were largely separated from p44,12. The fact that the ratio of p29 to p34 was constant in all fractions of the column was consistent with non-covalent association between p29 and p34. Membrane suspensions were prepared in the presence or absence of 1 mM dithiothreitol (DDT) (refs 13, 14). The preparations with no DDT were somewhat less pure, but p29 and p34 showed a similar elution pattern during BioGel A-5m filtration.

Subunit structure

After preparation in the absence of DDT, the fractions from BioGel A-5m filtration enriched in p29 and p34 were incubated with 0.6% SDS at various temperatures, in the presence of iodoacetamide (IAA) or 2-mercaptoethanol (ME) and electrophoresed on SDS gels (Fig. 2*a*, *b*). Comparison of the patterns at different temperatures showed that the HLA-A,B antigen subunits, p44 and p12, were dissociated and electrophoresed identically after incubation at all temperatures. But, after incubation at 21, 27 or 38 °C in SDS, p29 and p34 were associated in a complex (p29,34) at an apparent MW of 55,000, but were dissociated at 100 °C. Interestingly, when pooled human IgG was similarly treated with ME and SDS at 21 °C, L chains were dissociated, but H chains remained associated non-covalently (at the ME concentration used[15]) in an H_2 dimer which calibrated at 90,000 MW (Fig. 2*d*). Since dissociation into p29 and p34 occurred at 100 °C in the presence of 10 mM iodoacetamide to prevent disulphide interchange (Fig. 2*a*), it may be concluded that p29 and p34 are not linked by disulphide bonds. The mobilities of the subunits in reduced gels (Fig. 2*b*) were different from those in unreduced gels (Fig. 2*a*), particularly in the case of p29. The identity of reduced and unreduced bands was confirmed by cutting out bands from unreduced gels, reducing and electrophoresing them. Two dimensional SDS gels of the same preparation using electrophoresis without reduction in the first dimension, followed by reduction and electrophoresis in the second dimension, also showed that while p44 disulphide-linked dimers were present in these preparations, p29 and p34 were not disulphide-linked (see Fig. 7 in ref. 13 and Fig. 2 in ref. 14). But, cross-linking experiments with two cleavable cross-linking reagents containing S–S bonds[16,17] and isoelectric focusing showed that p29 and p34 are non-covalently associated with each other and that dimers of p29 alone or of p34 alone are not present (see Figs 3 and 4 in ref. 18).

Purified p29,34 was prepared by incubation of pool B in SDS at room temperature, followed by preparative SDS gel electrophoresis. Immunisation of rabbits with this material elicited a B cell specific antiserum with properties much like the B cell specific anti-p23,30 heteroantisera reported previously (refs 6–8, 18; T.A.S., J.F.K. and A. Fuks, in preparation). Moreover, purified p29,34 antigen specifically inhibited an *HLA*-linked B cell alloantiserum Po[41]. Heat-dissociated p29 and p34 did not elicit a detectable antibody response in rabbits and did not inhibit either the human alloantiserum or the rabbit heteroantiserum prepared against the p29,34 complex.

Table 1 Amino terminal sequences

	Residue 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
p16	Thr	Glu	Thr	[Thr]		Phe				Lys	Phe		Pro	Asp	
p29	Gly	Asp	Thr	Pro			Phe	Leu	Glu	Gln	Val				
p34	Ile	Lys	Glu	Glu	[Arg]	Val	Ile		Gln	Ala	Glu	Phe	Tyr	Leu	Asn

Peptides (10–30 nmol) were sequenced using ^{35}S-phenylisothiocyanate (New England Nuclear) in an updated Beckman 890 B Sequencer[21]. Phenylthiohydantoins were identified after thin-layer chromatography on polyamide sheets by visual inspection and autoradiography. Arginine, lysine, isoleucine and leucine were identified on the amino acid analyser after back hydrolysis. Peptides were also sequenced using the SDS–Edman–dansyl technique[19].

Table 2 Amino acid and sialic acid composition

Amino acid	mol per 100 mol p16	mol per 100 mol p29	mol per 100 mol p34	mol per 100 mol p44**	residues per molecule p16	residues per molecule p29	residues per molecule p34
CM-Cys	0.55	1.50	1.21	1.31	0.8(1)	3.1(3)	3.0(3)
Asp	12.14	7.87	8.93	8.73	17.0(17)	16.2(16)	22.3(22)
Thr*	10.78	6.70	6.65	6.87	15.1(15)	13.8(14)	16.6(17)
Ser*	7.32	6.87	5.32	6.77	10.2(10	14.2(14)	13.2(13)
Glu	7.80	12.62	11.61	12.65	10.9(11)	26.0(26)	29.0(29)
Pro	4.37	4.82	6.26	5.06	6.1(6)	9.9(10)	15.6(16)
Gly	7.04	7.93	6.89	7.6 7	9.9(10)	16.4(16)	17.2(17)
Ala	7.16	4.85	5.27	8.32	10.0(10)	10.0(10)	13.1(13)
Val†	7.90	8.14	8.48	6.19	11.1(11)	16.8(17)	21.1(21)
Met	0.63	1.52	1.46	1.51	0.9(1)	3.1(3)	3.6(4)
Ile†	5.31	3.33	4.89	3.45	7.4(7)	6.9(7)	12.2(12)
Leu	5.94	8.56	9.17	6.94	8.3(8)	17.7(18)	22.9(23)
Tyr	3.58	3.76	2.73	4.32	5.0(5)	7.8(8)	6.8(7)
Phe	6.21	5.31	5.98	3.06	8.7(9)	10.9(11)	14.9(15)
His	1.34	2.83	2.70	2.91	1.9(2)	5.8(6)	6.7(7)
Lys	5.79	3.88	4.45	3.87	8.1(8)	8.0(8)	11.1(11)
Arg	3.24	7.01	5.56	7.19	4.6(5)	14.5(14)	13.8(14)
Trp‡	2.90	2.51	2.44	(3.2)¶	4.1(4)	5.2(5)	6.1(6)
Polarity §	48.4	47.8	45.3	49.0			
Sialic acid ‖					0.5	2.2	2.6

Values in parentheses rounded to the nearest integer.
* Extrapolated to zero time values.
† 72-h values.
‡ Determined by using a modification of the method of Edelhoch (23) in 0.01% SDS.
§ Calculated according to ref. 24.
‖ Determined by the method of Warren (25).
¶ Assumed to be the same as for the papain product (21).
**From refs 10 and 11.

Chemical characterisation

The subunits of the *HLA*-linked B-cell alloantigen, p29 and p34, as well as p16, an unrelated protein which also bound to *Lens culinaris* lectin affinity columns and was obtained in good yield, were chemically characterised.

Materials in pools A and B were combined, alkylated with 3 mM iodoacetic acid and heated at 100 °C in the presence of SDS. Proteins were then purified by preparative electrophoresis in 0.8×22 cm, 10% polyacrylamide tube gels using precautions described by Weiner *et al.*[19]. Advantage was taken of the greater separation of p29 and p34 in the unreduced state (see Fig. 2*a*). After elution, the proteins were chromatographed twice on a 0.9×40-cm Sephadex G-50 column equilibrated with 20 mM NH_4HCO_3 to remove SDS and substances which interfered with the Edman degradation, and were lyophilised for amino acid and NH_2-terminal sequence analysis. Equimolar amounts of p29 and p34 were recovered, as measured by either the Lowry assay[20] or amino acid analysis. Analytical electrophoresis of the eluted polypeptides (Fig. 2*c*) showed them to be homogeneous.

The peptides were also shown to be homogeneous by amino-terminal analysis using the SDS–dansyl technique[19] and by automatic amino-terminal sequencing (Table 1). The amino-terminal sequences of p29 and p34 are quite different from one another, and from p16. Strikingly, the NH_2-terminal amino acids of p29 and p34 are identical to those of p44 and p12 (refs 11, 21, 22) although no further homologies are obvious. Much more extensive sequence data for all of these polypeptides will be required to examine the possibility of homology among them.

The amino acid compositions of p16, p29 and p34 were determined by performing hydrolysis for 24, 48 and 72 h and applying samples at two concentrations to a Beckman 121 M analyser equipped with a microbore single column system (Table 2). The composition of p44 from detergent soluble HLA-A,B antigens (a mixture of three specificities)[10,11] is included in Table 2 for comparison. The amino acid compositions of p29 and p34 are strikingly similar, suggesting that these proteins may be related. The amino acid compositions showed that p29 and p34 do not contain an unusually low percentage of polar residues, suggesting that only a short segment of their polypeptides is involved in membrane binding. This is consistent with the finding that papain-solubilised *HLA*-linked B cell alloantigens (p23,30) contain polypeptides of only slightly shortened length[6–8].

The sialic acid content (Table 2) was 2.2 and 2.6 mol per mol for p29 and p34 respectively, and thus they are both sialoglycoproteins with amounts of sialic acid similar to that of HLA-A,B antigen heavy chains[21]. Staining of SDS gels with periodic acid-Schiff reagent (PAS) also showed that p29 and p34 are glycoproteins. Since glycan moieties which have affinity for the *Lens culinaris* lectin are generally linked by a glycosylamine bond to asparagine and are branched oligosaccharides with galactosyl-*N*-acetylglucosamine sequences in the outer chains and mannose residues in the core[26], the carbohydrate compositions of p29 and p34 may be similar to that of p44 (ref. 21). p16 contained 0.5 mole sialic acid per mol, but PAS staining was not detected.

Since p29 and p34 are glycoproteins, their true MWs may be lower than the MW estimated by SDS gel electrophoresis, as is the case for the heavy chain of HLA-A,B antigens[21]. Using these molecular weights as a starting point, the peptide MWs of p29, p34 and p16 were calculated from the amino acid compositions[27] and found to be 23,600; 28,400; and 15,600 respectively, and the latter MWs were used for calculation of amino acid composition in residues per molecule (Table 2). Three cysteines were found in p29 and p34 (although cysteine may have been underestimated since p12 contained 0.8 residue per mol). p29 and p34 which had been treated with 1 mM DTT and 3 mM iodoacetic acid in the absence of SDS in order to alkylate easily reduced S–S bridges and free SH groups, were also analysed. They contained 0.8 and 1.0 residue of carboxymethyl-cysteine per mol respectively. The additional cysteines of p29 and p34 were either in intrachain disulphide bridges or unreactive. The fact that the mobilities of p29 and p34 in SDS gels (Fig. 2*a*, *b*) are slower after reduction suggests that they each contain at least one intrachain disulphide bridge. Even during purification in the absence

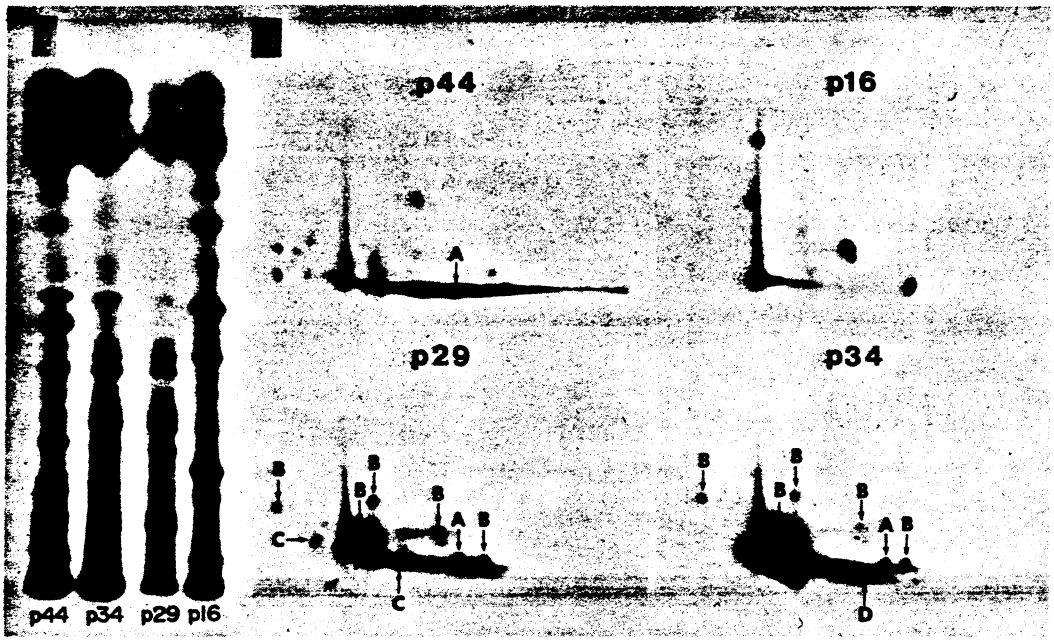

Fig. 3 ^{125}I-tyrosine-labelled tryptic peptide maps of p16, p29, p34 and p44. Peptides were labelled with ^{125}I in the presence of SDS, digested with trypsin, and separated on silica gel G (ref. 28). HLA antigens were prepared as described in refs. 10 and 11 and p44 was separated from p12 by preparative SDS gel electrophoresis[19]. *a*, Chromatography dimension only. *b*, Chromatography (upward) followed by electrophoresis (anode to right). The origin is the intensely radioactive spot near the lower left corner. Labelled spots: A, peptides common to p29, p34 and p44; B, peptides common to p29 and p34; C, peptides unique to p29; D, peptide unique to p34.

of DDT, as shown above the easily alkylated cysteines are not oxidised to form interchain disulphide bridges between p29 and p34.

Subunit homologies in tryptic peptide maps

Although limited NH_2-terminal sequencing did not reveal homology between p29, p34 and p44, homologies between polypeptides of dissimilar size do not necessarily begin at the NH_2-terminus. Since the amino acid compositions suggested such an homology, these proteins were compared by tyrosyl peptide mapping.

Carboxymethylated p16, p29, p34 and p44 were heated at 100 °C in the presence of SDS and labelled with ^{125}I. After removal of SDS, they were digested with trypsin[28]. Comparison of the ^{125}I-tyrosine tryptic peptides by one-dimensional silica-gel chromatography (Fig. 3*a*) showed that p29 and p34 had an almost identical peptide pattern. Spots due to free iodine, present in all samples, migrated close to the solvent front in the one dimensional system (and moved out of the area shown on the two dimensional maps illustrated in Fig. 3*b*). Two-dimensional maps (Fig. 3*b*) showed that p29 and p34 shared six major ^{125}I-tyrosine tryptic peptides (labelled A and B). p29 and p34 also each seem to contain unique peptides (labelled C and D) which agrees well with amino acid analysis showing p29 and p34 to contain eight and seven tyrosines, respectively. p44 showed considerable differences but seemed to contain one peptide (A) in the same position as a peptide of both p29 and p34. No significance should be attached to this single similarity without much further study. p16 showed no homology with any of the other peptides and provided evidence that none of the homologies observed in two-dimensional maps were due to artefacts. Thus p29 and p34 are highly homologous to one another. p29 and p34 could not be proteolytic products of p44, since four peptides not present in p44 were found in p29 and p34. Several pieces of evidence show that p29 is not a proteolytic breakdown product of p34. p29 contained one more serine and tyrosine than p34 (Table 2) and two unique tyrosine tryptic peptides (Fig. 3). Additionally, p34 contained a tyrosine residue at position 13 (Table 1), and therefore, proteolytic cleavage from the NH_2-terminus would have produced a product containing two fewer tyrosines than actually found in p29. Proteolysis of p29 and p34 during preparation or storage was never noted, and the ratio of the two polypeptides always remained constant, as determined by SDS gel electrophoresis. Equimolar amounts of p29 and p34 were recovered after preparative SDS gel electrophoresis. Proteolysis would have been expected to result in a mixture of molecules of $(p34)_2$, $(p29)_2$ and $(p29)_1(p34)_1$ structures, but only molecules of the latter structure were found in cross-linking experiments. p29 and p34 each also seemed to be uniquely cleaved by papain digestion, since one polypeptide was converted to p23 and the other to p30. Recent studies of somatic hybrids have also suggested that p29 and p34 are products of different genes[29].

Two likely explanations for the high degree of homology between p29 and p34 remain. A common portion could be encoded by one gene, and unique portions, probably at the NH_2-terminus could be encoded by different genes (a two gene, one polypeptide model, as in antibodies). Alternatively, the genes for p29 and p34 could have arisen by duplication of a common ancestral gene. Since this is the case for two other genes in *HLA*, *HLA-A* and *HLA-B* (ref. 21), this seems a likely explanation. Inhibition of the alloantiserum suggests that at least one of the p29 and p34 subunits is a product of one of the *HLA* gene loci which code for B cell alloantigens. But, since genes which code for homologous subunits are often unlinked (as is the case for the immunoglobulin L and H chain genes[30]), p29 and p34 would not necessarily both be products of the *HLA* region.

If the tyrosine tryptic peptides are representative of the remaining three-quarters of the tryptic peptides of p29 and p34, these molecules would be identical throughout most of their sequences. It will be interesting to determine by further structural analysis whether non-covalent association occurs between homologous regions, and whether differences between the subunits are confined to the NH_2-terminal region. Since RPMI 4265 cells may be heterozygous, and in analogy to the mouse *I* subregions[31], closely linked loci might specify chemically similar yet distinct molecules, p29 and p34 preparations may well contain chemically different populations of molecules, which might explain the electrophoresis of p29 as a doublet in high resolution Laemmli SDS gels (Fig. 1). Such differences must be limited, however, as no obvious heterogeneity was detected by NH_2-terminal sequencing and close to the expected number of major tyrosine tryptic peptides was obtained.

Relationship to murine and guinea pig Ia antigens

Genetic and serological studies have demonstrated the following similarities between murine Ia antigens and human *HLA*-linked B-cell alloantigens. (1) There genes are in the MHC and in intimate association with MLC genes[1,31]; (2) Antisera to these products are potent inhibitors of the MLC reaction[6,7,31] and also block generation of plaque-forming cells *in vitro*[32]. And (3), they have similar tissue distributions[1-9,31]. Our report shows that the two-chain, glycoprotein structure of *HLA*-linked B-cell alloantigens seems similar to that of murine and guinea pig Ia antigens[28,31,33]. Moreover, the NH_2-terminal sequence of the guinea pig Ia antigen smaller chain[33] is identical to that of human p29 in three out of seven positions, strongly suggesting inter-species homology. Similarly, sequence studies have shown that *HLA-A* and *HLA-B* antigens are homologous to murine H-2 D and K antigens[34] and to guinea pig GP-LA B antigens[33]. However, murine and guinea-pig Ia antigens analysed after immunoprecipitation have been found on some but not other occasions to contain a disulphide bridge between their subunits[33,35]. Thus, these rodent Ia antigens might also contain free cysteines in each subunit, but would differ from the human analogues studied here in that their sulphydryls can readily form an interchain disulphide link.

It is not yet known whether *HLA*-linked B cell alloantigens resemble murine and guinea pig antigens in a further respect. The latter alloantigens are termed immune associated (Ia) because their genes co-map with (but are not necessarily identical to) immune response (*Ir*) genes[31]. This type of close association has not been established in humans. However, susceptibility to certain diseases, which may reflect the absence of particular *Ir* genes, is linked to the *HLA* region[36].

Ir gene function may be expressed in T cells, B cells, or both[37-39]. Stimulated T cells express Ia antigens, although in lower amounts than B cells[39,40]. It is thus possible, although not yet demonstrated, that Ia antigens may in fact be the products of *Ir* genes. If so, the homologies between the subunits of *HLA*-linked B-cell alloantigens would have implications for its role in the immune system as a human *Ir* gene product. Proteins with homologous subunits (immunoglobulins, haemoglobins, and isozymes are examples) often can be composed of any of a set of interchangeable subunits which are specified by duplicated genes. Since many unique combinations can be made using a much smaller number of unique subunits, great diversity in functional specificity can be generated in an economical fashion.

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