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Human Class I histocompatibility antigens (HLA-A,B,C)

A small proportion only is phosphorylated

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Human Class I HLA antigens (HLA-A,B,C) were isolated by immune precipitation from cells labelled with ^{32}P, [^{35}S]methionine or ^{125}I (by lactoperoxidase-catalysed cell-surface iodination) and were analysed using both one- and two-dimensional electrophoretic systems. In several B-lymphoblastoid cell lines and in human peripheral blood lymphocytes the electrophoretic mobility of the ^{32}P-labelled HLA-A,B,C heavy chains consistently differed from that of molecules labelled by other means. Thus the ^{32}P-labelled heavy chains appeared to be larger and to possess a more acidic pI than did heavy chains labelled with [^{35}S]methionine or ^{125}I, or identified by Coomassie Blue staining. Phosphatase treatment of immunoprecipitates, under conditions where ^{32}P-labelled antigens were shown to be dephosphorylated, did not affect the mobilities of the [^{35}S]methionine-labelled heavy chains. On glycosidase treatment, the positions of the ^{32}P-labelled heavy chains were affected by neuraminidase but not by *endo-β-N*-acetylglucosaminidase H. These results imply that phosphorylated HLA-A,B,C antigens comprise only a small proportion (relative to the total cellular HLA-A,B,C antigens) of the biosynthetically mature molecules. The possible significance of such heterogeneity is discussed.

It has been known for some time that the 44000 Da glycoprotein heavy chain (p44) of human Class I HLA antigens (HLA-A,B,C) is phosphorylated in Epstein–Barr-virus-transformed lymphoblastoid cell lines and in normal peripheral blood lymphocytes (Pober *et al.*, 1978; Chaplin *et al.*, 1980; Johnstone *et al.*, 1981). Furthermore, purified plasma membrane prepared from these sources contains endogenous protein kinase activity capable of phosphorylating these antigens *in vitro* (Pober *et al.*, 1978; S. Loube, unpublished work). The site of phosphorylation has been localized to (a) serine residue(s) in the hydrophilic intracellular *C*-terminal domain (Pober *et al.*, 1978). Phosphorylation of Class I HLA antigens may be a general phenomenon, as the homologous proteins from mouse (Rothbard *et al.*, 1980; Johnstone *et al.*, 1981) and pig (Johnstone *et al.*, 1981) lymphocytes have also been reported to be phosphorylated.

The functional relevance of this modification remains obscure. It is attractive to speculate that the intracellular site of phosphorylation confers a means of regulating association (that is, transferring information) between this cell-surface-disposed membrane glycoprotein and molecules inside the cell such as cytoskeletal components (Pober *et al.*, 1978). However, such a function has not yet been proven. No specific change in HLA-A,B,C antigen phosphorylation has been shown to occur on mitogen stimulation of whole cells, although specific changes in the phosphorylation patterns of other proteins can be demonstrated under these circumstances (Chaplin *et al.*, 1980). Nonetheless, Class I HLA antigens are known to be involved in cellular recognition functions and in the regulation of cells involved in cell-mediated immunity (Ploegh *et al.*, 1981), and it seems reasonable that antigen phosphorylation may play a role in these phenomena. It is, though, very difficult to conceive of experiments that would provide an unequivocal assessment of such a possibility.

If, however, the modification has structural relevance then the role of phosphorylation may be

Abbreviations used: endo H, *endo-β-N*-acetylglucosaminidase H; i.e.f., isoelectric focusing; G3PD, glyceraldehyde 3-phosphate dehydrogenase; Mes, 4-morpholine-ethanesulphonic acid; n.e.p.h.g.e., non-equilibrium pH gradient electrophoresis; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

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more easily adduced. For example, there may be a subpopulation of HLA-A,B,C antigens, which is defined by a particular localization within the cell, that is phosphorylated to a different extent than the bulk antigen. The molecules in this hypothetical 'compartment' may or may not have a specialized function which is dependent on their state of phosphorylation. Generally speaking, there are a number of situations in which spatially distinct subsets of molecules might be defined in the cell, for instance, during vectorial transport accompanying biosynthesis, during intracellular transport accompanying membrane turnover and antigen degradation, or in association with cytoskeletal proteins within the cell or with viral proteins at the cell surface. Such subpopulations might be expected to be small, dynamic and relatively unstable, and thus difficult to isolate or characterize biochemically. The probability of detection would, however, be increased if the molecules in the subset were specifically modified. Thus the presence of certain specific sugar, fatty acyl or phosphate residues may serve as a useful 'tag' for their identification, quite apart from their functional significance. The present work provides evidence that a small proportion of HLA-A,B,C antigens is phosphorylated *in vivo* in lymphoblastoid cell lines as well as peripheral blood lymphocytes.

Materials and methods

Cells

The human B lymphoblastoid cell lines Maja (HLA-A2, -B35), MST (HLA-A3, -B7), JY (HLA-A2, B7) and the Burkitt-lymphoma line Daudi were cultured in RPMI 1640 medium containing the antibiotics penicillin (100 units/ml) and streptomycin (50 μg/ml) and supplemented with 10% (v/v) foetal-calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.). Maja, MST and JY cell lines are homozygous for HLA-A,B antigen expression (Brodsky *et al.*, 1979). Daudi does not, however, express any HLA-A,B antigens on the cell surface, although incompletely processed forms of the heavy chain are present intracellularly (Ploegh *et al.*, 1979). Human peripheral blood lymphocytes were prepared from whole blood or platelet-depleted blood from multiple donors by layering 2 ml on to an equal volume of Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuging at 2000 ***g*** for 20 min (Boyum, 1968).

Labelling procedures

For labelling with ^{32}P, cells were washed twice in sterile saline (0.9% NaCl) and then resuspended at 5×10^6/ml in phosphate-free RPMI 1640 medium (Gibco) supplemented with 5% (v/v) dialysed foetal-calf serum. The cells were incubated for 1 h at 37°C and then carrier-free [^{32}P]P_i (PBS.11, 10 mCi/ml; Amersham International) was added to a final concentration of 1 mCi (about 40 nmol)/10^8 cells. The cells were labelled for 3 h at 37°C and then harvested by centrifugation. They were usually lysed without washing.

For labelling with [^{35}S]methionine, cells were washed as before then resuspended in methionine-free RPMI 1640 medium supplemented with 5% (v/v) dialysed foetal-calf serum. After preincubating the cells for 1 h at 37°C, [^{35}S]methionine (SJ.204, 600 Ci/mmol; Amersham) was added to a final concentration of 50 μCi/ml. The cells were labelled for 3–4 h at 37°C before harvesting.

Lactoperoxidase-catalysed radioiodination was performed by using glucose oxidase and glucose under conditions previously described (Walsh & Crumpton, 1977). Na^{125}I (IMS.30, carrier-free) was obtained from Amersham.

Preparation of cell lysates

To limit loss of protein-bound phosphate, the buffer used to lyse the cells [50 mM-Tris/HCl (pH 7.3)/50 mM-NaF/5 mM-sodium pyrophosphate/2 mM-EDTA/1 mM-PMSF/1% (w/v) Nonidet P40] contained several inhibitors of phosphatase activity (Chaplin *et al.*, 1980). When samples were to be compared, cells were prepared, labelled and lysed in parallel. Cell pellets were resuspended in ice-cold lysis buffer immediately after harvesting and left for 30 min [$(1–2) \times 10^7$ cells/ml] at 0°C. Nuclei were removed by centrifugation at 2000 ***g*** for 10 min and the lysates were frozen at −20°C for storage if necessary. Before use, supernatants were cleared by centrifugation at 150000 ***g*** for 1 h.

Antisera

The principal antiserum used was W6/32, a mouse monoclonal (hybridoma) antibody that recognizes an antigenic determinant common to all HLA-A,B,C antigens providing the heavy chains are associated with β_2-microglobulin (Barnstable *et al.*, 1978). The hybridoma was grown as an ascites tumour in pristane-primed BALB/c mice.

Immunoprecipitation

Immunoprecipitation was carried out by using fixed *Staphylococcus aureus* (Cowan 1) bacteria to bind antigen–antibody complexes; the method employed has been described previously (Owen *et al.*, 1980). Lysates were precleared by incubation with 50 μl of a washed 10% (v/v) suspension of fixed *Staph. aureus* bacteria/ml. For each precipitate, a volume of cleared lysate corresponding to 10^7 cell equivalents (generally 1 ml) was incubated with 2 μl of W6/32 ascitic fluid. The complexes so formed were collected by binding to a further 50 μl of washed *Staph. aureus*. Sedimented bacteria were washed

sequentially with: (*a*) lysis buffer [1% Nonidet P40/10mM-Tris/HCl buffer (pH 7.4)/0.15M-NaCl/1mM-EDTA/0.1mM-PMSF] supplemented to 0.5M with NaCl; (*b*) lysis buffer plus 0.1% (w/v) SDS; (*c*) 0.1% (w/v) Nonidet P40 in 10mM-Tris/HCl buffer, pH 7.4. After washing, the bacterial pellets were resuspended in 50μl of SDS/sample buffer [0.0625M-Tris/HCl (pH 6.8)/2.5% (w/v) SDS/10% (v/v) glycerol] and heated at 100°C for 4min. The supernatant obtained on centrifugation was either analysed directly by SDS/polyacrylamide-gel electrophoresis in one dimension or stored frozen for subsequent analysis by two-dimensional gel electrophoresis (see below).

Enzymic digestions

When phosphatase digestions were to be performed, bacterial pellets were resuspended (without elution) in the appropriate buffer. Digestions with acid phosphatase were carried out in 50mM-Mes, pH 6.2, containing 0.1% Nonidet P40 (Rangel-Aldao *et al.*, 1979); 5μg of potato acid phosphatase (Calbiochem) were used per immune precipitate (10^7 cell equivalents). Digestions with alkaline phosphatase were carried out in 50mM-Tris/HCl, pH 7.5, containing 1mM-dithioerythritol, 5% (v/v) glycerol, 5mM-$MgCl_2$ and 0.1% Nonidet P40 (Ahmad & Huang, 1981); 10 units of calf intestine alkaline phosphatase (Boehringer) were used per immune precipitate. The final volume of the digests of the control and phosphatase-treated samples was 200μl. After addition of enzyme, digestion was allowed to proceed at 37°C for 30min. The precipitates were then collected by centrifugation, resuspended in SDS/sample buffer and eluted by boiling as described above.

Glycosidase digestions were performed on SDS-eluted immunoprecipitates. Endo H digestions were performed as described previously (Owen *et al.*, 1980). For neuraminidase digestion, the SDS-solubilized proteins (in 50μl) were diluted to 250μl in acetate buffer (0.1M sodium acetate, pH 5.5, containing 0.3M-NaCl and 0.2% $CaCl_2$) and 5μl of a solution (2mg/ml) of bovine serum albumin (Sigma) was added to each sample. The proteins were then recovered by precipitation with an equal volume of 30% (w/v) trichloroacetic acid. The pellet was washed twice with ice-cold acetone, air-dried, and then resuspended in 50μl of water plus 150μl of acetate buffer. Samples were incubated at 37°C and three aliquots (10μl each) of *Vibrio cholerae* neuraminidase (1i.u./ml, Calbiochem) were added over 24h; control samples received acetate buffer only. The reaction was stopped by precipitation with 250μl of 30% trichloroacetic acid. The pellet recovered after washing was dissolved in SDS/sample buffer or, for two-dimensional gel analysis, in Nonidet P40/urea lysis buffer (see below).

Electrophoretic analyses

SDS/polyacrylamide-gel electrophoretic analysis was carried out in 12.5% (w/v) acrylamide slab gels as previously described, as were staining, drying and autoradiographic procedures (Owen *et al.*, 1980).

Two-dimensional gel electrophoresis was carried out by using two different methods for separating proteins in the first dimension, namely i.e.f. (O'Farrell, 1975) and n.e.p.h.g.e. (O'Farrell *et al.*, 1977). Samples (either pelleted *Staph. aureus*-bound immune complexes, or SDS-eluted trichloroacetic acid-precipitated immunoprecipitates) were taken up in Nonidet P40/urea lysis buffer [9.5M-urea, 2% (w/v) Nonidet P40/2% Ampholines (LKB)/5% (v/v) 2-mercaptoethanol]. After 1h at room temperature, and brief centrifugation if necessary to clear, samples (25μl) were loaded on to cylindrical (115mm × 2mm diam.) first-dimension gels. N.e.p.h.g.e. gels (containing 2% Ampholines of pH range 3–10) were run for 1600 V-h. I.e.f. gels (containing 1.6% Ampholines of pH range 5–7 and 0.4% of pH range 3–10) were run for 4500 V-h. pH gradients in the first-dimension gels were estimated by cutting blank gels, run in parallel, into 0.5cm sections. The gel fragments were soaked for 2h at room temperature in 1.5ml of water in tightly capped tubes before reading the pH of the eluate using a pH meter (Beckman Instruments).

Results and discussion

The present study grew out of observations obtained when HLA-A,B,C antigens were isolated by immunoprecipitation from lysates of Maja B-lymphoblastoid cells which had been labelled metabolically with ^{32}P or [^{35}S]methionine. When molecules labelled by the two methods were compared directly by SDS/polyacrylamide-gel electrophoresis, the heavy chains (p44) clearly displayed different mobilities. Thus, as shown in Fig. 1, the heavy chains labelled with ^{32}P appeared to be larger (by about 1000Da) compared with those labelled with [^{35}S]methionine or those identified by staining for protein with Coomassie Brilliant Blue. This result was consistently observed, regardless of the cell line used. Similar results (not shown) were also obtained with human peripheral blood lymphocytes. Furthermore, when precipitates were examined by n.e.p.h.g.e., the heavy chains that had been labelled with ^{32}P showed charge differences from those labelled metabolically with [^{35}S]methionine or by lactoperoxidase-catalysed cell-surface iodination with ^{125}I (Fig. 2). Fig. 2 also shows that, as expected, both the HLA-A and HLA-B antigens are phosphorylated to some extent. Thus ^{32}P-labelled spots were detected in positions corresponding both to the heavy chains of the HLA-A2 antigens (the more basic cluster of peptides) and those of the HLA-B7

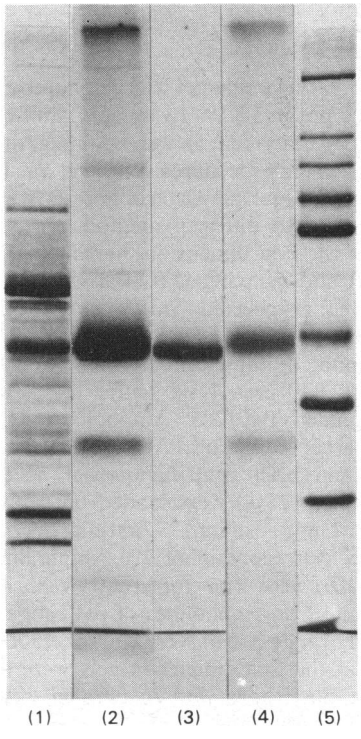

Fig. 1. *SDS/polyacrylamide-gel electrophoretic analysis of HLA-A,B,C antigens precipitated with the monoclonal antibody W6/32 from Maja cell lysates*

One ^{32}P- plus one [^{35}S]methionine-labelled immunoprecipitate were combined and electrophoresed together in the same sample well. The Figure is a composite photograph, designed to show, respectively, total protein (lane 1), all radioactively labelled proteins (lane 2), [^{35}S]methionine-labelled proteins only (lane 3) and ^{32}P-labelled proteins only (lane 4). Thus lane (1) is a photograph of the Coomassie Blue-stained gel. Lane (2) is an autoradiograph of the same track. Lane (3) is an autoradiograph of the same track obtained 70 days after that shown in lane (2); under these conditions only about 3% of the ^{32}P radioactivity remains. Lane (4) is an autoradiograph of the same track obtained 4 days after that shown in lane (2); here a 180 μm-thick acetate sheet (sufficient to block the emission due to ^{35}S) was interposed between the gel and the film. Lane (5) is a photograph of another track from the same gel, showing the Coomassie Blue-stained proteins used as molecular-weight standards. These are as follows: myosin (200000), β-galactosidase (130000), phosphorylase *b* (95000), transferrin (78000), bovine serum albumin (68000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (34000), human immunoglobulin L-chain (25000) and cytochrome *c* (12500). In this gel, cytochrome *c* has migrated with the dye front. A second ^{32}P-labelled band, corresponding to approx. 30000 mol.wt., in addition to that of the HLA-A,B,C heavy chains (p44) is clearly present; this polypeptide does not appear to label well with [^{35}S]methionine and has not been identified. It does not, however, appear to be related to, or associated with, HLA-A,B,C antigens, as it was also found in control precipitates (i.e. no W6/32 antibody added).

antigens (the more acidic cluster of peptides). However, in both instances, the phosphorylated heavy chains corresponded to the most acidic forms of (or were more acidic than) the groups of polypeptides revealed by ^{35}S and ^{125}I labelling. Although in Fig. 2 the ^{32}P-labelled heavy chains displayed a notable decrease in charge microheterogeneity compared with that observed for the ^{35}S and ^{125}I-labelled chains, similar spans of charge microheterogeneity were detected in other experiments (e.g. see Fig. 3*b*). This heterogeneity most probably reflects primarily differences in sialylation (Parham *et al.*, 1974).

The most logical explanation for the lack of coincidence of the ^{32}P- and ^{35}S-labelled polypeptides is that the polypeptides identified by using ^{32}P represent a minor fraction only of the total antigen of the cell. We decided to determine whether this explanation is correct and, if so, to delineate the properties of this unique subfraction.

It was first necessary to demonstrate that the labelling procedures employed reflect accurately the state of phosphorylation of the HLA-A,B,C antigens. Immunoprecipitated ^{32}P- and [^{35}S]methionine-labelled antigens were treated with phosphatase to remove bound phosphate. As can be seen from Fig. 3, such treatment removed a major part of the ^{32}P label but resulted in no detectable shift in the molecular weight of the [^{35}S]methionine-labelled material and in a negligible change in the pattern of spots obtained on two-dimensional electrophoresis. Since the presence of one (or more) charged phosphate groups should have a detectable effect on polypeptide mobility in both pH and molecular-weight dimensions (Racevskis & Sarkar, 1979), it is reasonable to conclude that a large proportion of the [^{35}S]methionine-labelled antigen does not contain bound phosphate.

The fact that Coomassie Blue staining and cell-surface iodination gave results consistent with the above conclusion make it unlikely that metabolic differences during labelling with [^{35}S]methionine or ^{32}P are responsible for the observed results. Furthermore, within an experiment, care was taken to treat all cells in the same way, regardless of the labelling method used. Thus the cells were always lysed in the same buffer and immunoprecipitations were carried out at the same time under the same conditions to limit the possibility that differential activity of endogenous phosphatases might influence the results.

HLA-A,B antigens contain one asparagine-linked oligosaccharide per heavy chain (Ploegh *et al.*,

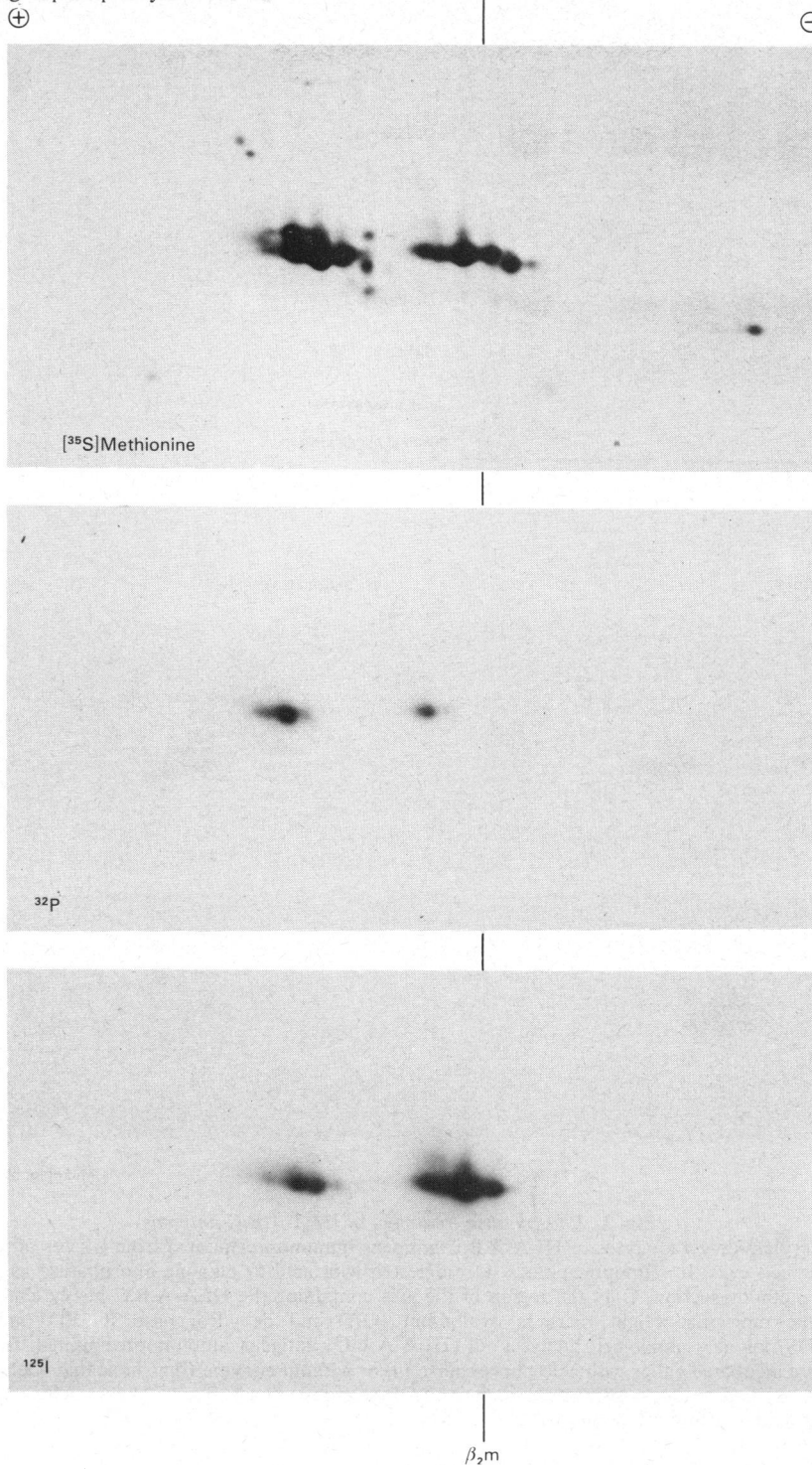

Fig. 2. *N.e.p.h.g.e.–SDS/polyacrylamide-gel electrophoretic analysis of HLA-A,B,C antigens immunoprecipitated from lysates of JY cells*

The gels were aligned according to the observed position of the β_2-microglobulin (β_2m) spot (indicated by the vertical line). The latter polypeptide was labelled biosynthetically with [^{35}S]methionine as well as with ^{125}I by lactoperoxidase-catalysed iodination and was readily detected as a Coomassie Blue-stained spot. Only the section of the gel containing the HLA-A,B,C heavy chains is shown in each case. The more basic cluster of polypeptides represents the heavy chain of the HLA-A2 antigen, whereas the more acidic cluster of polypeptides corresponds to the HLA-B7 antigen.

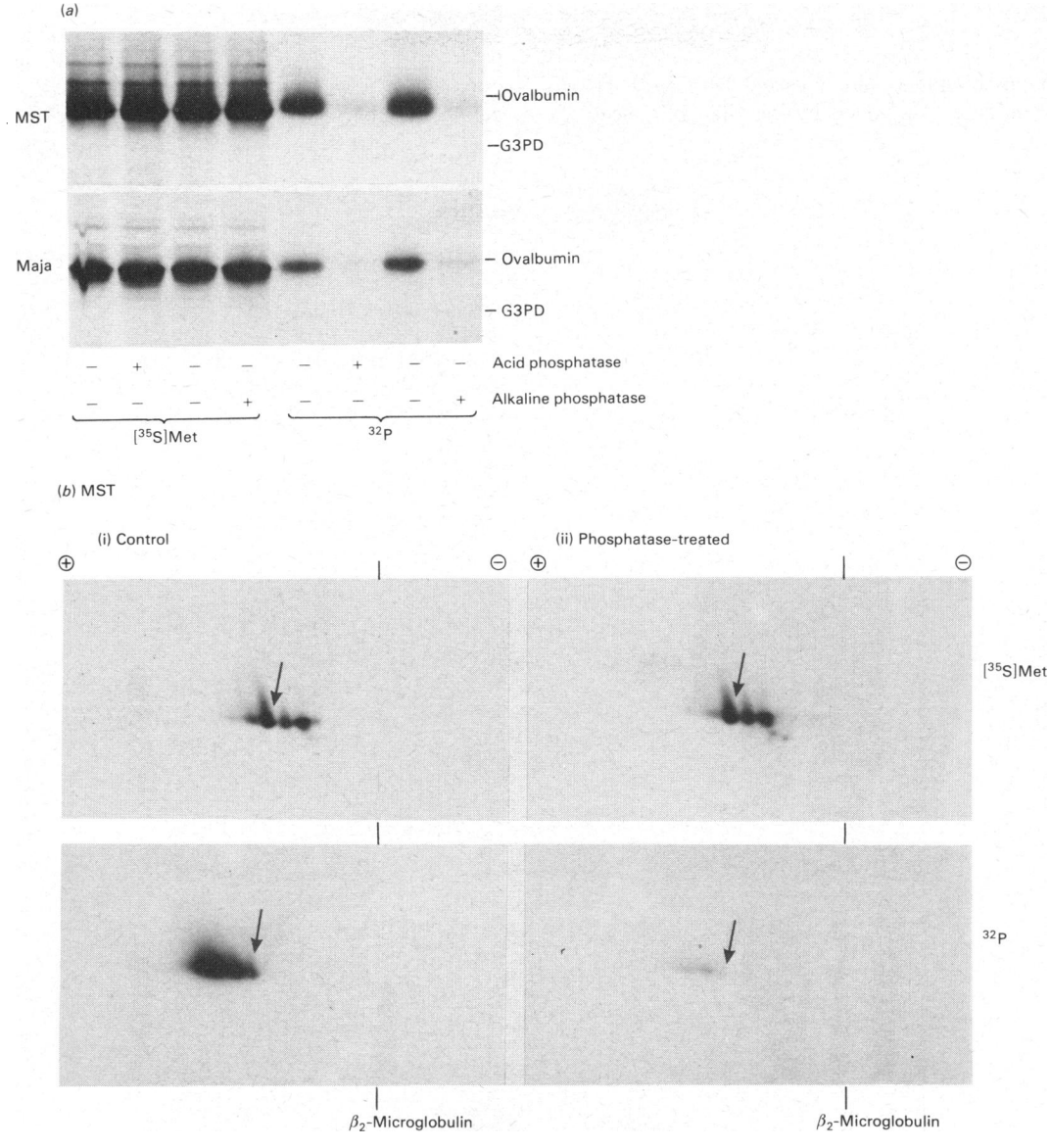

Fig. 3. *Phosphatase treatment of HLA-A,B,C antigens*

(*a*) SDS/polyacrylamide-gel analyses of HLA-A,B,C antigens immunoprecipitated from lysates of MST (upper gel) or Maja (lower gel) cells. Immunoprecipitates were digested with acid or alkaline phosphatase as described in the Materials and methods section. Only the region of the gels containing the HLA-A,B,C heavy chain is shown. The positions of the molecular-weight markers ovalbumin (OA) and dehydrogenase (G3PD) are indicated. (*b*) N.e.p.h.g.e.–SDS/polyacrylamide-gel analyses of HLA-A,B,C antigens immunoprecipitated from MST cells. Precipitates were incubated either with acid phosphatase (ii) or without enzyme (i) as indicated. Gels were aligned on the basis of the position of the β_2-microglobulin spot and were cropped as in Fig. 2. The arrowed spot was judged to represent the same polypeptide chain in each gel.

1981). The synthesis and processing of such *N*-linked oligosaccharides, and the temporal sequence of the processing reactions, are understood to the extent that the state of maturity of an isolated polypeptide chain can be determined by its sensitivity to certain glycosidases. Thus sensitivity to endo H indicates the presence of (early) high-mannose forms of the oligosaccharide, whereas, conversely, sensitivity to neuraminidase indicates the presence of (late) sialylated forms (Hubbard & Ivatt,

1981). To determine the extent of processing of the phosphorylated HLA-A,B heavy chains, duplicate immunoprecipitates were digested with endo H or neuraminidase. As judged by the change in electrophoretic mobility in respect of both molecular weight (Fig. 4) and charge (Fig. 5), the ^{32}P-labelled polypeptides were neuraminidase-sensitive, indicating that the phosphorylated molecules bear sialic acid. In contrast, as shown in Fig. 4, no apparent change in the molecular weight of the ^{32}P-labelled chains was detected on endo H digestion [Cleavage by endo H corresponds to a decrease of about 2500 in molecular weight (Owen *et al.*, 1980)]. The results of these experiments, and the fact that a small proportion of the ^{125}I-labelled spots co-migrate with those labelled with ^{32}P (Figs. 2 and 5) suggest that the phosphorylated subpopulations of HLA-A,B antigens are the biosynthetically mature forms which are present on the cell surface. Support for this interpretation comes from the observation that the immature HLA-A,B heavy chains present in the Burkitt-lymphoma line Daudi were not metabolically labelled with ^{32}P (results not shown).

An important question is whether all the HLA-A,B,C antigens that are located on the cell surface are, or are not, phosphorylated? The proportion of cell-surface [^{35}S]methionine-labelled HLA-A,B,C antigens was determined by measuring the relative amount of antigen precipitated by adding the

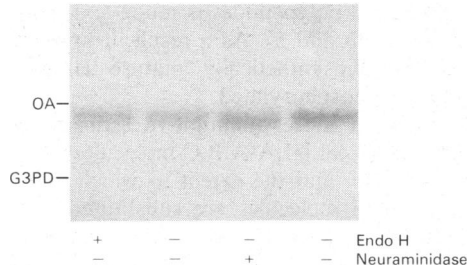

Fig. 4. *SDS/polyacrylamide-gel electrophoretic analysis of ^{32}P-labelled HLA-A,B antigens immunoprecipitated from Maja cells*

Precipitates were digested with endo H or neuraminidase (+) or without enzyme (−) as indicated. Only the region of the gel containing the HLA-A,B heavy chains is shown. The positions of the molecular-weight markers ovalbumin (OA) and G3PD are indicated.

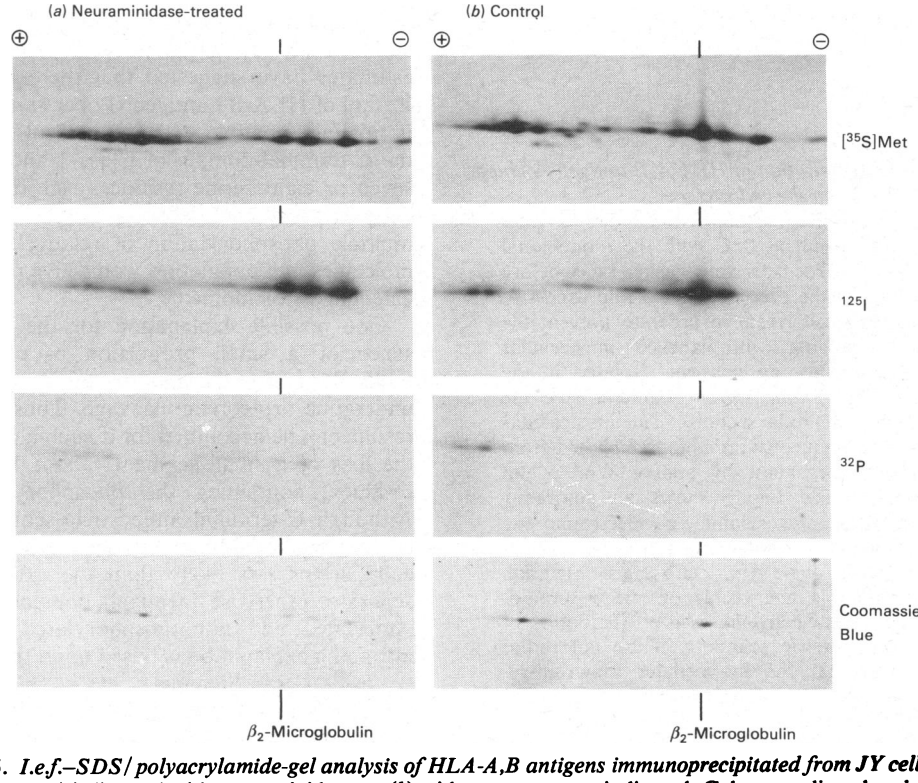

Fig. 5. *I.e.f.–SDS/polyacrylamide-gel analysis of HLA-A,B antigens immunoprecipitated from JY cells*

Precipitates were (*a*) digested with neuraminidase or (*b*) without enzyme as indicated. Gels were aligned and cropped as in Figure 2. The pH gradient extends in each case from approximately pH 5.05 (left-hand side) to pH 6.15 (right-hand side). β_2-Microglobulin focused in these experiments with an apparent pI of 5.9.

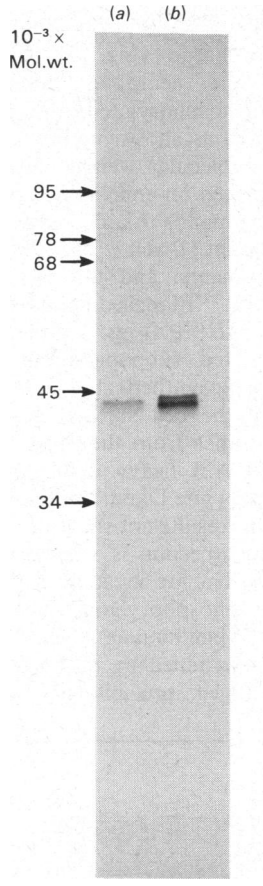

Fig. 6. *Immunoprecipitation of HLA-A,B antigens located on the cell surface*

Maja cells, labelled with [^{35}S]methionine, were incubated for 30min at 0°C with the monoclonal antibody W6/32. The cells were washed twice before detergent lysis in the presence of a 5-fold excess of unlabelled Maja-cell lysate in order to prevent the antibody exchanging with labelled intracellular antigen. The surface antigen was isolated in the usual way with *Staph. aureus* bacteria (see the Materials and methods section). The intracellular antigen was subsequently immunoprecipitated from the resultant supernatant by adding W6/32 and *Staph. aureus*. The Figure shows an autoradiogram of an SDS/polyacrylamide-gel-electrophoretic analysis of the immunoprecipitates. Lane (*a*), intracellular material; lane (*b*), cell-surface material. β_2-Microglobulin is not visible on the autoradiogram, since it was electrophoresed off the botton of the gel. Densitometric scanning of the autoradiogram showed that the intracellular material represented about 18% of the total W6/32-precipitable material, whereas the surface material represented 82% of the total. The arrows indicate the positions of molecular-weight standards run in a parallel track: phosphorylase *b* (mol.wt. 95000), transferrin (78000), bovine serum albumin (68000), ovalbumin (45000) and G3PD (34000).

monoclonal antibody W6/32 to whole cells before cell lysis. The results, shown in Fig. 6, argue strongly in support of the view that the cell-surface antigens represent about 80% of the total ^{35}S-labelled antigen. However, the proportion of the ^{35}S-labelled heavy chains (relative to the total) that is coincident with the ^{32}P-labelled polypeptides is much less than 80% (see Figs. 2, 3*b* and 5). As a result, it appears that not all the biosynthetically mature HLA-A,B,C antigens are phosphorylated.

To obtain a more quantitative estimate of the proportion of total HLA-A,B,C molecules which are phosphorylated, and the extent to which individual phosphorylated molecules are substituted, is not a simple matter. The results obtained by i.e.f. can only lead to underestimates, as HLA-A,B,C antigens have isoelectric points close to the pK_a for the phosphate group in serine phosphate [pK_a = 5.91 (Dawson *et al.*, 1969)]. Thus the protein-bound phosphate will not be fully ionized when the antigens are at their pI. At pH 5.9 half of the phosphate will be electrophoretically 'silent'; at pH 5.3, 80% will be un-ionized. This explains why in Figs. 2, 3 and 5 the more acidic antigens appear to be more uniformly phosphorylated than the more basic molecules. In contrast, after neuraminidase treatment (Fig. 5), all of the molecules possess a more basic isoelectric point, and the difference in mobility between the ^{32}P- and ^{125}I-labelled forms is more striking. Previous estimates have suggested that there are 0.7mol of P_i/mol of HLA-B7 antigen (Pober *et al.*, 1978). This is not incompatible with the present findings, since the *C*-terminal-domain of Class 1 antigens contains seven or eight serine residues, each of which might be phosphorylated (Robb *et al.*, 1978). That is, multiple phosphorylation of relatively few antigen molecules in a population would give rise to the same calculated stoichiometry.

One possible explanation for the apparent presence of a small proportion of phosphorylated molecules is that the majority of the molecules have undergone proteolytic cleavage. Thus the observed results can be accounted for if such a cleavage led to the loss of a small segment (about 10 amino acid residues) containing the phosphorylation site(s). Although *C*-terminal amino acid-sequence analysis is essential to exclude this explanation, it intrinsically seems less likely than the existence of two separate, otherwise identical, populations of phosphorylated and non-phosphorylated antigens. An intriguing explanation is based upon the fairly recent suggestion of Steinmetz *et al.* (1981) that a differential RNA splicing mechanism operates at the 3′-end of Class 1 genes, allowing the synthesis of polypeptides with different cytoplasmic domains. In this case, it is possible that the minor phosphorylated population of Class 1 antigens possesses a unique *C*-terminus. No evidence exists that such a

differential splicing mechanism operates to produce Class 1 antigens heterogeneous at their *C*-termini. However, a similar mechanism is most probably responsible for the generation of the *C*-terminal segments characteristic of membrane-associated and secreted forms of immunoglobulins (Alt *et al.*, 1980; Early *et al.*, 1980). Furthermore, the *C*-terminal cytoplasmic tails of membrane-associated immunoglobulins M and G differ markedly (Rogers *et al.*, 1981; Owen & Kissonerghis, 1982). Lastly, whether or not the minor phosphorylated population of Class 1 antigens possess a unique *C*-terminal tail, it is possible that phosphorylation is correlated with a specific function; that is, the phosphorylated molecules may represent a functionally distinct subpopulation.

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