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Regulation of MHC class II invariant chain expression: induction of synthesis in human and murine plasmocytoma cells by arresting replication

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The expression of the H2 Ia-associated invariant chain (Ii) has been determined by pulse labeling cells with [^{35}S]methionine and resolving the proteins. Expression is maximal in non-cycling peripheral B lymphocytes and is reduced upon maturation of B lymphocytes to plasma cells. B cell-derived cell lines behave correspondingly: IgM$^+$ non-secretor cell lines synthesize Ii while plasmocytoma cells do not. Pre-B cell lines are also negative. In all Ii-negative cell lines, the synthesis of Ii is selectively induced by treating the cells with inhibitors of replication.

Key words: H2 Ia invariant chain (Ii)/pre-B cells/mitomycin C/monoclonal anti-Ii

Introduction

The MHC genes on chromosome 17 of the mouse and on chromosome 6 of man code for three classes of proteins (Hood *et al.*, 1982; Klein *et al.*, 1981): class I comprises the classical transplantation antigens. The class II antigens (coded by Ia,DR) are involved in the interactions of T cells, B cells and macrophages (Zinkernagel and Doherty, 1979). Class III represents members of the complement system. Both the class I and II molecules consist of polymorphic subunits in non-covalent association with another polypeptide chain: class I antigens are associated with β2-microglobulin (Rask *et al.*, 1974), the class II antigens are associated intracellularly with a subunit designated 'invariant chain' (H2) or 'gamma chain' (HLA) (Jones *et al.*, 1978; Moosic *et al.*, 1980; Shackelford and Strominger, 1980). β2-microglobulin is not encoded within the MHC complex (Goding and Walker, 1980; Robinson *et al.*, 1981) and recent experiments indicate that the gene for the invariant chain is also not present on mouse chromosome 17 (Koch *et al.*, 1982; Day and Jones, 1983). However, the association of β2-microglobulin with class I antigens is specific, because lack of β2-microglobulin prevents the expression of transplantation antigens on the cell surface (Goodfellow *et al.*, 1975). It is not yet clear what is the function of the association of Ii with the class II antigens.

During screening for mutagen-induced mammalian proteins, we have detected a B cell-specific 35-kd glycoprotein (XM1) which is expressed only in the non-proliferating state (Rahmsdorf *et al.*, 1982). The synthesis of XM1 in proliferating myeloma cells is induced by treatment with mutagens and/or by inhibition of replication. Resting peripheral B lymphocytes express XM1 constitutively. Upon polyclonal stimulation, the synthesis is reduced. Because of the tissue specificity shown by XM1, we investigated its relationship to B lymphocyte markers. We now present evidence that XM1 is identical with the Ia-associated invariant chain and that Ii expression can be turned-on by inhibiting proliferation. We also show that constitutive expression or inducibility varies in B cell lines according to the stage of B cell maturation.

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Results and Discussion

Identity of mutagen-induced 35-kd protein (XM1) with the invariant chain

The identity of XM1 with Ii was proven by the co-migration of the radiolabeled XM1 and the Ii chain immunoprecipitated by specific antibody in two-dimensional gels and on lentil lectin columns, and by comparing the peptide patterns of the two proteins after limited proteolysis or after cleavage with N-chlorosuccinimide.

The invariant chain which was precipitable from lentil lectin eluates of B lymphocyte extracts with monoclonal antiserum, migrated to the same position in non-equilibrium pH gradient electrophoresis (NEPHGE) (O'Farrell *et al.*, 1977) (Figure 1B and C) as did XM1 (Figure 3C). The position was identified by staining marker proteins (not shown). From peripheral B lymphocytes, the whole spectrum of Ia-associated antigens was precipitated. The same protein pattern was produced with all the following monoclonal antibodies used: antibodies directed against the invariant chain, the I-A region polymorphic proteins (Figure 1) or the I-E region protein Eα. In all cases, Ii, α and β chains were co-precipitated from the extract (example in Figure 1A, C). The gel system as displayed in Figure 1A revealed, in addition, proteins in covalent association (Koch and Haustein, 1980; Koch and Hämmerling, 1982). In this gel system, the first (horizontal) dimension contains no 2-mercaptoethanol, while the second dimension does. Thus, the diagonal represents the peptides not associated by S-S bridges. Spots elsewhere represent peptides that had originally been associated by covalent S-S bonds.

Limited proteolysis of both proteins XM1 and Ii yielded identical peptides. The protein spots were cut out of dried gels, and subjected to limited proteolysis (Cleveland *et al.*, 1977). The result of such an experiment is shown in Figure 2A. The major mutagen-induced 35-kd protein from a 2D resolution of total cell homogenate (lane b) and the invariant chain obtained by immunoprecipitation (lane c) yielded the same two characteristic fragments of 28 kd and 18 kd. Also cleavage of the proteins with N-chlorosuccinimide/urea (NCS/urea) (Lischwe and Ochs, 1982) produced identical fragments (Figure 2B). N-Chlorosuccinimide cleaves proteins selectively at the tryptophanyl peptide bonds. After [^{35}S]-methionine labeling and NCS cleavage, both proteins generated three characteristic fragments of ~32, 27 and 23 kd (Figure 2, lanes b and d).

The identical behavior of XM1 and Ii on lentil lectin (Rahmsdorf *et al.*, 1982), their size and isoelectric point, and their peptide fragments after limited proteolysis and after treatment with NCS prove that the two proteins are indeed identical.

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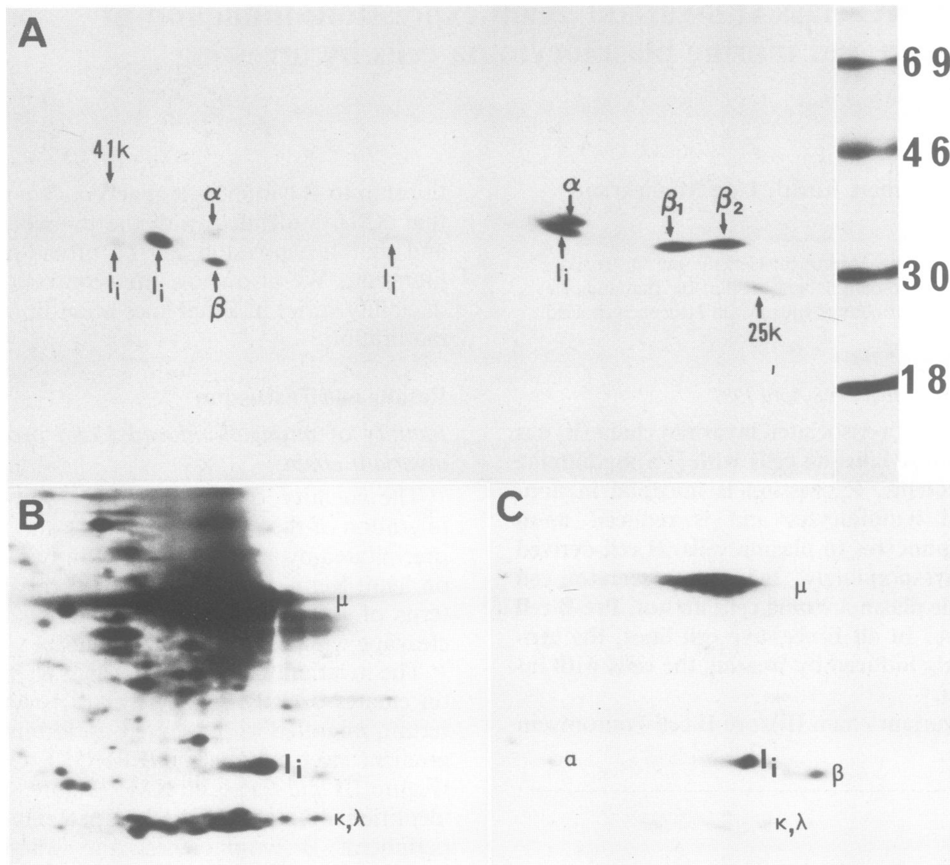

Fig. 1. Ia-associated antigens immunoprecipitated from peripheral B cells. Mouse spleen lymphocytes treated and labeled as described, were subjected to lentil-lectin chromatography (Koch *et al.*, 1982). The fractions eluted by α-methyl-D-mannoside were subjected either directly to NEPHGE (**B**) or were treated with a monoclonal antiserum against I-A (17/227). The immunoprecipitated proteins were either separated in a 2D gel using non-reducing conditions in the first dimension and reducing conditions in the second dimension (**A**) or by NEPHGE (**C**). Fluorograms of the dried gels are shown. Relevant spots are marked: Ii = invariant chain, dissociated from its covalent protein partners: 41 K, Ii (dimer), 27 K (below the dimer position, barely visible, not marked), 10 K (not visible but indicated by a Ii spot), and the monomeric Ii (in the diagonal just below α). In **C**, contaminations of heavy and light immunoglobulin chains (κ, λ, μ), the I-associated polymorphic proteins α and β are visible (see also Koch and Hämmerling, 1982).

Induction of Ii synthesis by mitomycin C

Ig-secreting cells which do not synthesize XM1 but can be induced to express XM1 using mutagenic treatment (Rahmsdorf *et al.*, 1982), were examined for Ii synthesis. Proliferating hybridoma cells were either labeled directly or treated with mitomycin C prior to labeling. After labeling, the hybridoma cells were divided. One part was disrupted and the total protein resolved by NEPHGE (Figure 3A and C). The larger portion of the cells was lysed by sonication and a detergent extract of the membrane-bound proteins was chromatographed on lentil lectin. Material eluted by 0.2 M α-methylmannoside was treated with monoclonal antibodies directed against the invariant chain, and the precipitate analysed by two-dimensional SDS-gel electrophoresis. Reducing conditions, to separate S-S associated subunits, were present only in the second dimension (Figure 3B and D). As described earlier (Rahmsdorf *et al.*, 1982), XM1 was only detectable in the pattern obtained from mitomycin C-treated cells (arrow in Figure 3C). The rate of synthesis was close to that of actin (A in Figure 3A and C). The invariant chain and its covalently associated partners could only be precipitated from extracts of mitomycin C-treated cells (Figure 3D) but not from exponentially growing cells (Figure 3B). Thus, invariant chain synthesis in these hybridoma cells is only detectable after proliferation stops. In the mitomycin C cells, no detectable amounts of labeled α- or β-chains were co-precipitated (Figure 3B and D).

The induction of Ii expression is regulated at the level of translatable mRNA. In *in vitro* translation experiments, RNA from resting lymphocytes and from mitomycin C-arrested hybridoma cells but not from proliferating hybridoma cells, directed the synthesis of a 28-kd protein (this is the unglycosylated precursor of Ii, Rahmsdorf *et al.*, 1982) which was precipitated by antibodies against the invariant chain (Figure 4). Protein synthesis in a reticulocyte extract was directed by equal amounts of poly(A)$^+$ RNA from either spleen cells (lane a), from growing hybridoma cells (lane b) or from the same cells after mitomycin C treatment (lane c). The c.p.m. in the 28-kd peptide precipitated by antibody reflect the amount of specific RNA under these conditions. The 28-kd peptide was only precipitated from the mixtures corresponding to lanes a and c. The c.p.m. in lane b migrated slightly slower than 28 kd, at the position of contaminating light chains. It is thus clear that the RNA level was affected by the treatment with mitomycin C. The position of the mature protein can be seen in lane d, where an immunoprecipitate of labeled spleen cell proteins has been resolved. We have not identified the peptide which migrated slightly more slowly than the mature invariant chain (lane b and c).

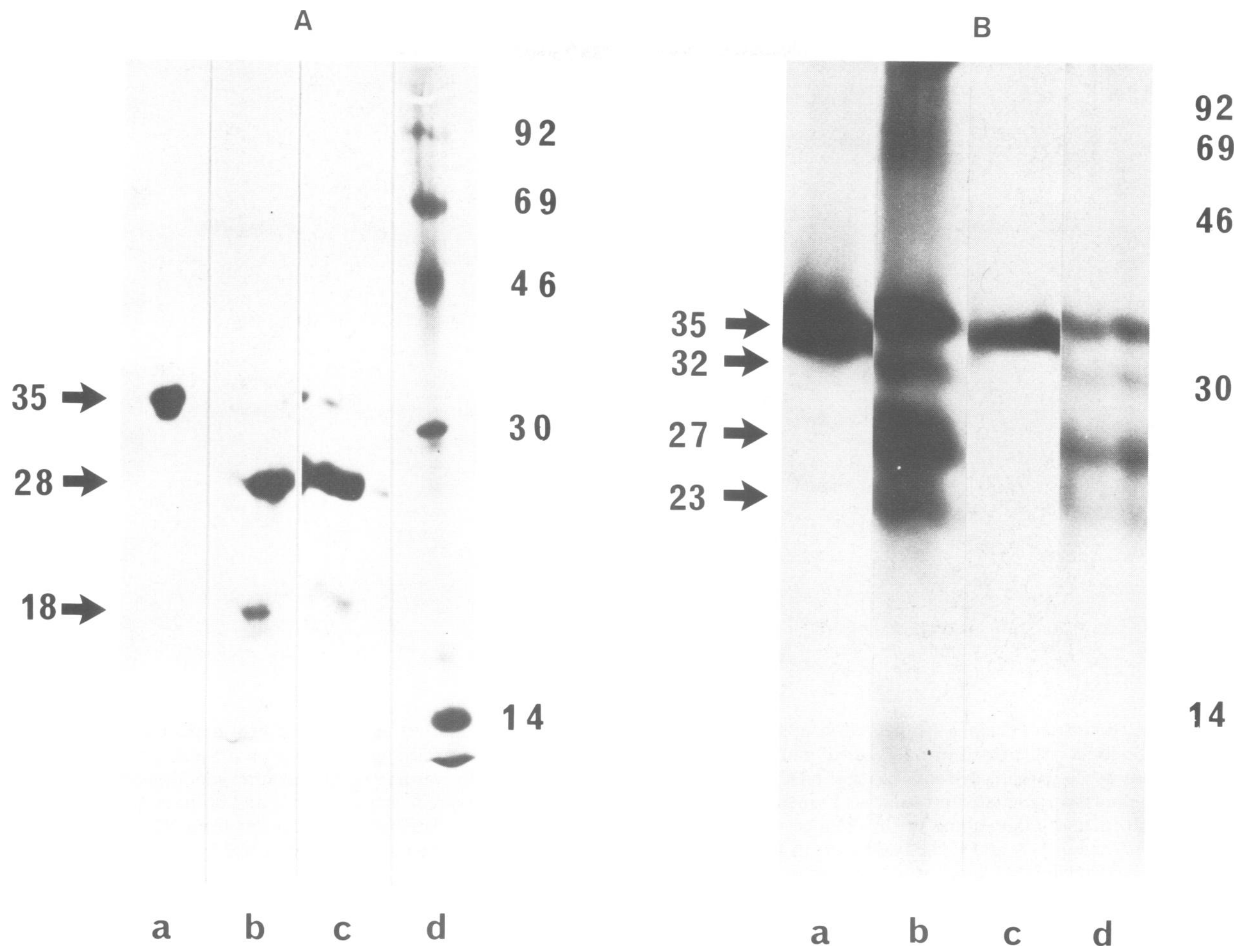

Fig. 2. The protein XM1 detected in cell homogenates of mitomycin C-arrested B cell lines and the immunoprecipitated invariant chain show identical peptide digest patterns. To prove identity of protein XM1 and of the invariant chain, we cut XM1 spots out of a NEPHGE gel as displayed in Figure 3C and the invariant chain from a one-dimensional 10–20% gradient gel (after immunoprecipitation from labeled spleen cells as shown in Figure 1) and compared their peptide pattern after limited proteolysis with V8 staphylococcal protease (Cleveland *et al.*, 1977 **(A)**) or after cleavage with N-chlorosuccinimide/urea (Lischwe and Ochs, 1982 **(B)**). The peptides were resolved on a 15% polyacrylamide slab gel. A fluorogram is shown. **A: Lane a**: XM1 non-digested; **lane b**: XM1 digested; **lane c**: invariant chain, digested; **lane d**: protein markers as indicated. **B: lane a**: XM1 non-treated; **lane b**: XM1, NCS-treated; **lane c**: invariant chain, non-treated; **lane d**: invariant chain, NCS-treated; protein markers are indicated.

Enhanced transcription, as suggested for Ii synthesis here, has been reported for MHC class I genes including the gene for β2-microglobulin (Lindahl *et al.*, 1973; Heron *et al.*, 1978; Fellous *et al.*, 1982; Wallach *et al.*, 1982; Burrone and Milstein, 1982; Basham *et al.*, 1982) and for class II genes (Sonnenfeld *et al.*, 1981; Steeg *et al.*, 1982) after treatment of various cells with interferons. We wonder whether the response to interferon was not mediated by a mechanism similar to ours, e.g., the anti-proliferative action of the interferons.

Physiology of constitutive and inducible Ii or DR gamma expression

To determine whether the inducibility of Ii was only limited to the Ig-secreting myeloma cells, we examined all accessible stages of B cell differentiation, i.e., peripheral B lymphocytes before and after mitogen stimulation, and cell lines representing distinct stages of B cell maturation. Rates of synthesis were measured as described in Materials and methods. Peripheral B lymphocytes did express Ii, while at later times after lipopolysaccharide (LPS) stimulation, the synthesis decreased (Table I and Rahmsdorf *et al.*, 1982). Following the rate of spontaneous Ii or DR gamma expression, the cell lines can be classified into three groups. Terminally differentiated Ig-secreting plasmocytoma and hybridoma lines no longer synthesized Ii (corresponding in phenotype to the LPS-treated B cells). Cell surface immunoglobulin-positive non-secreting cell lines resembling the peripheral B cells, constitutively expressed high levels of Ii. Among these are the Epstein-Barr virus (EBV)-transformed human cells and non-secreting tumors. The non-secreting tumor cell line 38 C-13 seems to be an exception as it was Ii-negative. The third group which may correspond in phenotype to pre-B cells, i.e., not yet synthesizing cell surface immunoglobulins, was Ii negative – at least for the three examples tested. Probably the cell lines reflect the physiological behavior of B cells in the various stages of differentiation.

All cell lines which did not synthesize Ii or DR gamma spontaneously, could be induced by arresting proliferation (Table I). Although the arrest of replication apparently forms a signal to Ii gene expression, non-proliferation is not the only state compatible with expression since proliferating cell lines can express Ii constitutively (Table I, group II).

Concluding remarks

Mutagenic treatment induces a differentiation process, as

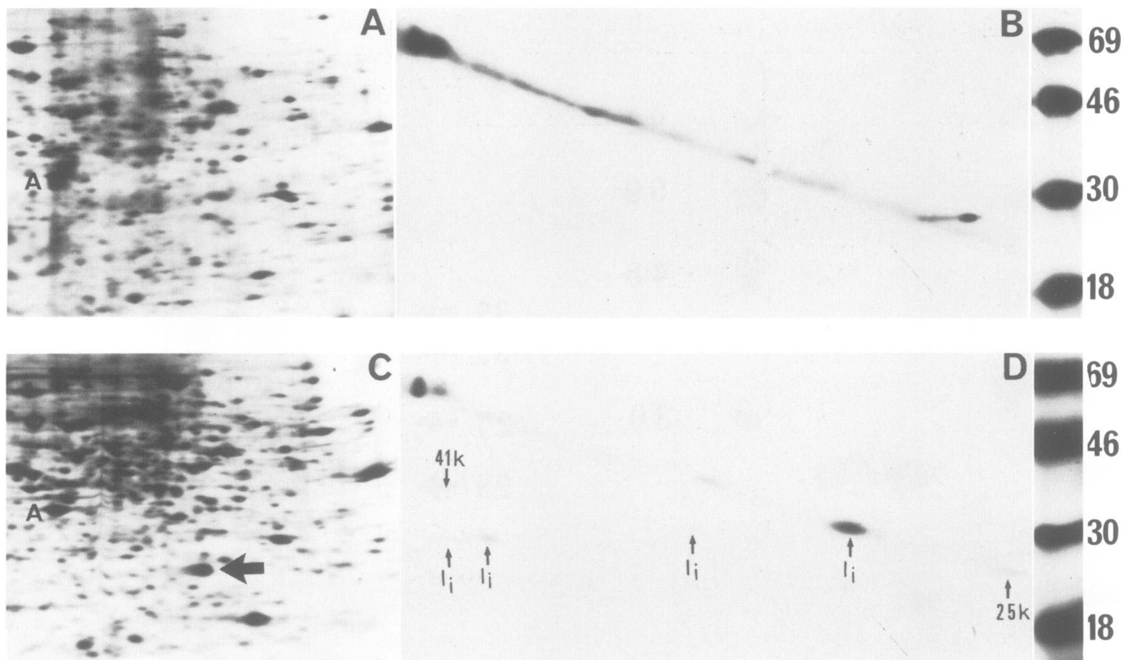

Fig. 3. The Ia-associated invariant chain is synthesized in Ig-secreting cells only after mitomycin C arrest. Proliferating hybridoma cells (PC-I-25-1-3. IgM$^+$, T15$^+$) **(A,B)** and hybridoma cells treated with 0.5 μg/ml mitomycin C for 36 h **(C,D)** were pulse-labeled with 200 μCi/ml [^{35}S]methionine for 2 h. An aliquot of the labeled cells was processed directly for NEPHGE **(A,C)**. The remaining portion of the cells was immunoprecipitated with monoclonal antibodies against Ii. **(B,D)** The immunoprecipitated proteins were separated in the first dimension under non-reducing conditions and in the second dimension in the presence of 2-mercaptoethanol. Fluorograms of the dried gels are shown. The mol. wt. scale (methylated standard proteins from NEN) on the right side applies strictly to **B** and **D** only. In **A** and **C** the basic proteins migrated to the right side. Relevant proteins are marked as in Figure 1. A = actin. The arrow in **C** points to the induced protein XM1 which is identical with the invariant chain.

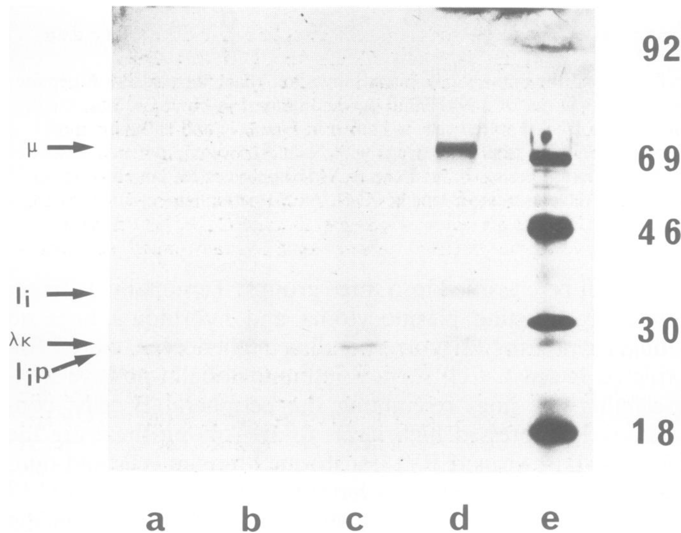

Fig. 4. Poly(A)$^+$ RNA level for the invariant chain is enhanced by mitomycin C. Poly(A)$^+$ RNA prepared as described (Rahmsdorf *et al.*, 1982), from BALB/c spleen cells **(lane a)**, growing hybridoma cells (PC-I-25-1-3, **lane b)** and hybridoma cells treated with 0.5 μg/ml mitomycin C for 40 h **(lane c)** were used to direct *in vitro* synthesis in a reticulocyte system (Amersham). Each mixture received 0.5 μg of poly(A)$^+$ RNA. The mixtures contained [^{35}S]methionine and the incorporation was near linear for 30 min and very similar for all RNAs tested. After this incubation, the proteins were immunoprecipitated by antibodies directed against the invariant chain (In-1) and separated on a one-dimensional polyacrylamide gel. A fluorogram is shown. **Lane d** shows an immunoprecipitate with the same monoclonal antibody from BALB/c spleen cells which have been labeled *in vivo* with [^{35}S]methionine. Relevant proteins are marked on the left: the immunoglobulin chains $\varkappa$, λ and μ, the invariant chain Ii and its precursor Ii p.

exemplified by the induced expression of MHC class II invariant chain. It appears that the signal to the gene is formed when cellular proliferation is arrested (Rahmsdorf *et al.*, 1982). Physiologically, the pre-B cells probably, and the polyclonally stimulated B cells definitely do not synthesize Ii, whereas the non-proliferating peripheral B cells express Ii at a high rate; when 'frozen' into a transformed tumor cell, the expression remains constitutive although the cells proliferate.

Kvist *et al.* (1982) have postulated that the invariant chain has some function in the transport of the polymorphic class II proteins α and β. A functional association of the three proteins is suggested by their intracellular association and by the fact that they are co-expressed in some of the cells which we studied (Table I). It is interesting, however, that not all Ii was precipitated together with the polymorphic subunits. Although the bulk of Ii cannot be iodinated from the outside (Rahmsdorf *et al.*, 1982; Koch *et al.*, 1982), Ii is detectable on the plasma membrane of the cell by binding specific antibodies (Koch *et al.*, 1982). This fraction of Ii is not associated with α or β. From earlier subcellular distribution studies (Rahmsdorf *et al.*, 1982) and from the rate which by far exceeds the synthesis of α and β, a pool of free Ii seems plausible. No function has yet been assigned to free Ii and to the fractions covalently bound to other proteins. Our observations add to this complexity: Ii expression could be linked to the control of proliferation and to terminal differentiation.

Materials and methods

Cells

The cells used are listed and their origin cited in Table I. The mouse myeloma cells MOPC-21 and MPC-11 and the hybridoma PC-I-25-1-3 were

Table I. Constitutive and induced expression of Ii in lymphoid cells

	References	Ig expresion	Ia expression	I_i expression	I_i inducibility
Mouse					
I 'Pre-B' phenotype					
K	Weimann (1976)	–		–	n.d.
4-12-9-7	Burrows *et al.* (1979)	cytopl. μ		–	+
15-29-3	Burrows *et al.* (1979)	cytopl. μ		–	+
II 'Mature B cell' phenotype					
Spleen cells (peripheral B lymphocytes)		surface M^+	+	+	
38 C-13	Bergman and Haimovich (1977)	surface M^+	–	–	+
CH1-1	Lynes *et al.* (1978)	surface M^+	+	+	
Wehi 231	McKean *et al.* (1981)	surface M^+	+	+	
III Ig-secreting cells					
LPS-stimulated spleen cells (peripheral B lymphocytes)		secreted Ig	±	±[a]	+
PC-I-25-1-3	Rahmsdorf *et al.* (1982)	secreted μ,λ	–	–	+
MOPC-21	Potter (1972)	secreted γ_1,k	–	–	+
MPC-11	Potter (1972)	secreted γ_{2B},k	–	–	+
Man					
II 'Mature B cell' phenotype					
Peripheral blood lymphocytes		surface M^+	+	+	
Peripheral blood lymphocytes transformed with EBV		surface M^+	+	+	
BJAB	Klein *et al.* (1974)	surface M^+	+	+	
Daudi 16891	Klein *et al.* (1968)	surface M^+	+	+	
EB 2 19961	Epstein *et al.* (1965)	surface M^+		+	
F89 2587	Jensen *et al.* (1967)	surface M^+		+	
III Ig-secreting cells					
RPMI 1788	Huang and Moore (1969)	secreted μ,λ		±[a]	+

Cell lines were cultured and tested for constitutive or inducible Ii synthesis as described in Materials and methods, with the exception of Wehi 231, the data for which were taken from Koch *et al.* (1982). The mouse pre-B cell lines were from M.Wabl, Tübingen; 38C-13 and CH1.1 from G.Hämmerling, Heidelberg; PC-I-25-1-3 from Ch.Heusser, Basel; MOPC-21 and MPC-11 from the cell distribution Center, Salk Institute for Biological Studies, San Diego; the human cell lines BJAB, Daudi 16891, EB2 19961 and F89 2587 from G.Klein, Stockholm and B.Cohen, Edinburgh; the human Ig-secreting cell line RPMI 1788 from the American Type Culture Collection, Rockville, MD. The peripheral blood lymphocytes were transformed by EBV (B95-8, Miller and Lipman, 1973). EBV was from G.Bornkamm, Freiburg. LPS-stimulated B cells were analysed 3 days after LPS addition. Ia and Ig expression data were taken from the literature indicated in the Table. Ia antigen expression on surface IgM^+ mouse cells has been described by Hämmerling (1976), expression on human peripheral blood lymphocytes and the variable expression during the terminal stages of B cell development has been shown by Halper *et al.* (1978) and Kearney *et al.* (1977). Pre-B cells do not express Ia antigens (Kearney *et al.*, 1977). Absence of Ia antigens on the mouse hybridoma PC-I-25-1-3 has been shown (Figure 3). Presence of Ia-like antigens on BJAB and Daudi cells was described by Trowbridge *et al.* (1976), presence on EBV-transformed peripheral blood lymphocytes by Giphart *et al.* (1977).
[a]Reduced by a factor of 3 – 5; n.d., not determined.

grown in minimal essential medium (MEM) supplemented with Earle's salts, 5% foetal calf serum (FCS), 10% new born calf serum and bykomycin (100 μg/ml). All other cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 50 μM 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml). Preparation, culture and stimulation of primary lymphocytes was as described (Rahmsdorf *et al.*, 1981).

Quantitation of Ii synthesis

Cells grown to mid-exponential phase (2 x 10^5 cells/ml) were labeled and the proteins resolved by NEPHGE as described earlier (Rahmsdorf *et al.*, 1982). Ii and DR gamma were identified by: (i) their specific migration in 2D gel electrophoresis; (ii) their high turnover in a pulse/chase experiment; and (iii) the fact that Ii could be precipitated with specific antibodies. The cross-reaction of anti-Ii with DR gamma was detectable but poor (unpublished). After this identification of Ii and DR gamma spots in the 2D gel, these were cut out and the c.p.m. determined as described (Rahmsdorf *et al.*, 1982). In addition, autoradiograms were evaluated by microdensitometry. To test inducibility of Ii, cells were treated in normal growth medium with 0.5 μg/ml mitomycin C, and then pulse labeled.

Monoclonal antibodies and immunoprecipitation

The monoclonal antibodies used were directed against I-A (17/227, corresponding to Ia 15, Koch and Hämmerling 1982) and against Ii (In-1, Koch *et al.*, 1982). Before immunoprecipitation, extracts from labeled cell were chromatographed on lentil-lectin (Rahmsdorf *et al.*, 1982). The proteins retained were eluted with 0.2 M α-methylmannoside and immunoprecipitated according to Koch *et al.* (1982). The immunoprecipitates were resolved by NEPHGE or in two-dimensional SDS-polyacrylamide gel electrophoresis (non-reducing/reducing) as described by Koch and Haustein (1980). The first dimension is run in the absence of 2-mercaptoethanol to preserve S-S bridges between covalently associated proteins. Before separation in the second dimension, proteins are reduced within the rod gels by treatment with sample buffer containing 3% SDS and 5% 2-mercaptoethanol for 1 h at 60°C.

Poly(A)$^+$ RNA isolation and *in vitro* translation was performed as described (Rahmsdorf *et al.*, 1982). For limited proteolysis of proteins by V8 staphylococcal protease, the method of Cleveland *et al.* (1977) was used. Cleavage of proteins with N-chlorosuccinimide/urea was performed according to Lischwe and Ochs (1983).

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