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The major histocompatibility complex class I heavy chain as a structural subunit of the human cell membrane insulin receptor: Implications for the range of biological functions of histocompatibility antigens

(insulin receptors/β_2-microglobulin/capping/immunoprecipitation)

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ABSTRACT **Monoclonal antibodies against some of the monomorphic determinants of major histocompatibility complex (MHC) class I molecules reduce insulin binding and precipitate ^{125}I-labeled insulin receptor preparations. A monoclonal antibody with specificity for the insulin binding site on the cell membrane insulin receptor of human cells was used to precipitate insulin receptors from human cell lines and resulted in distinct bands of M_r ≈130,000, 90,000, and 45,000. The M_r 45,000 molecules thus precipitated were subjected to NaDodSO$_4$/PAGE, eluted from the gels, and found to react with monoclonal antibodies against monomorphic and a polymorphic MHC class I determinant known to be expressed on the cell line used as receptor source. Moreover, a murine thymoma line (RI) with MHC class I expression bound significant amounts of insulin, whereas a MHC class I-negative variant had low insulin binding capacity. Reduction in the density on human cells of the MHC class I heavy chain was obtained by capping with antibodies to β_2-microglobulin or to the MHC class I heavy chain and resulted in decreased insulin binding, whereas down-regulation of insulin receptors induced increased density of MHC class I molecules. It is concluded that the MHC class I heavy chain and the tetrameric insulin receptor are structurally associated in the cell membrane and suggested that this association may occur by displacement of β_2-microglobulin by the insulin receptor.**

The heavy chain of major histocompatibility complex (MHC) class I molecules normally associates in the membrane with β_2-microglobulin (β_2m) (1–5), and an interchange between β_2m in plasma and on cells takes place *in vivo* (6–8). MHC class I molecules may also associate with other cell membrane components such as viral antigens (9–11). Ohno (12) has suggested that MHC class I molecules may serve as an anchorage protein in the membrane for organogenesis directing proteins (12), and it has been suggested that biologically active insulin receptors may include the MHC class I heavy chain as a subunit (13–15). This hypothesis was mainly based on experiments demonstrating interference of monoclonal antibodies against MHC class I molecules with insulin binding to viable cells and is supported by observations indicating a structural association between insulin receptors and MHC class I molecules on the surface of murine hepatocytes (16).

We now report more extensive experimental support of this hypothesis. Reduction of the density of cell surface MHC class I molecules reduced the amount of functional insulin receptors, and down-regulation of insulin receptor expression increased the density of cell surface MHC class I molecules. Further, a monoclonal antibody with specificity for the insulin binding site on the α chain of the insulin receptor immunoprecipitated both the α and the β chain of the well-described tetrameric insulin receptor (17–19) and also molecules of M_r ≈45,000. The latter molecules were shown to bind monoclonal antibodies against monomorphic and polymorphic class MHC I determinants. It is concluded that the MHC class I heavy chain can occur as a subunit of the insulin receptor on the intact cell surface.

MATERIALS AND METHODS

Cell Lines. Five established human cell lines were used: (*i*) U937 was established from a histiocytic lymphoma and has the phenotypic attributes of monocytes/macrophages (20); its HLA type is -A3, -B5,18, -Cw1; (*ii*) HL-60 is a promyelocytic leukemia line (21); (*iii*) IM-9 is a lymphoblastoid cell line (17) with HLA-A2,25, -B21,22, -Cw4; (*iv*) RH-L4 is a B-lymphoma line that expresses, but does not secrete, a γ heavy chain and κ light chain (22); (*v*) Daudi is a Burkitt lymphoma line that is MHC class I negative (23). A MHC class I-positive murine T-lymphoma cell line (RI) and a MHC class I-negative subclone (RIε) were used in some experiments (24). All lines grow as floater cultures, are maintained in RPMI 1640 medium supplemented with 10–15% fetal calf serum and 0.03% L-glutamine, and are seeded at a density of 10^5/ml and split at a cell concentration of 1–2 × 10^6/ml.

Antibodies. Monoclonal antibodies (mAbs) against histocompatibility antigens were obtained from various sources. PA 2.6 (monomorphic MHC class I) and PA 2.1 (A2 of MHC class I) were obtained from P. Parham (Stanford University, CA) (25), and BB 7.5 (monomorphic MHC class I), BB 7.7 (monomorphic MHC class I), BB 7.6 (broad spectrum of the *B* locus of MHC class I), GAP A3 (A3 of MHC class I), and MA 2.1 (A2/B17 of MHC class I) were from American Tissue Culture Collection (Rockville, MD). L243 (monomorphic MHC class II) (26) and anti-Leu 2a, anti-Leu 3, and anti-Leu 4 [all against T-lymphocyte cell surface molecules (27)] were obtained from Becton Dickinson. OKT9 (Ortho Diagnostics) was used as anti-transferrin receptor antibody. A monoclonal antibody directed against H-2K^b antigens was kindly provided by R. S. Goodenow (University of California, Berkeley). Furthermore, a monoclonal antibody directed against a metastasis-associated protein in Lewis lung carcinoma cells was used as another control antibody (28). All mAbs were purified. A polyclonal antibody against the insulin receptor (designated AHA) (29) was a gift from J. Nerup (Niels Steensens Hospital, Copenhagen). The antibody was ob-

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Abbreviations: MHC, major histocompatibility complex; β_2m, β_2-microglobulin; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody.

Table 1. Inhibition of insulin binding to U937 cells by various antibodies and reactivity of those antibodies with the purified insulin receptor

Antibody	Antibody specificity	Inhibition of insulin binding, %		Immunoprecipitation of insulin receptor preparations,* cpm
		At 1.0 nM	At 10.0 nM	
PA 2.6	MHC class I/monomorphic	≈70	≈55	10,928 ± 1,825
BB 7.5	MHC class I/monomorphic	0	0	3,431 ± 1,367
BB 7.7	MHC class I/monomorphic	0	0	4,983 ± 1,454
BB 7.6	MHC class I/B locus	0	0	6,473 ± 1,622
MA 2.1	MHC class I/A2/B17	0	0	842 ± 173
PA 2.1	MHC class I/A2	0	0	437 ± 268
L 243	MHC class II/monomorphic	0	0	628 ± 324
Leu 4	Anti-T-lymphocyte	0	0	781 ± 318
Leu 2a	Anti-T-lymphocyte	0	0	671 ± 257
Leu 3	Anti-T-lymphocyte	0	0	418 ± 269
OKT-9	Anti-transferrin receptor	0	0	293 ± 144
4F2	Anti-insulin receptor α-chain	≈65	≈60	12,196 ± 3,418
AHA (polyclonal)	Anti-insulin receptor	≈70	≈65	16,104 ± 2,516
Mouse Ig	Negative control	0	0	394 ± 298
Human Ig	Negative control	0	0	418 ± 316

*One microgram of purified ^{125}I-labeled insulin receptor suspended in 100 μl of phosphate-buffered saline/Triton X-100 was mixed with 1–5 μg of specific antibody in 100 μl of phosphate-buffered saline and incubated under constant shaking for 2 hr at room temperature. Then, 100 μl of the second-step antibody (rabbit anti-mouse or rabbit anti-human) was added and incubation was continued for 2 hr at room temperature. Finally, protein A-Sepharose (Pharmacia, Sweden) was added and the precipitates were collected by centrifugation. The amount of precipitated receptor was determined in a γ counter.

tained from serum of a patient with severe insulin resistance. Antibody AHA inhibited ≈70% of insulin binding to human monocytes and immunoprecipitated from U937 cells bands of M_r ≈90,000 and ≈130,000. A mAb (designated 4F2) with specificity for the insulin binding site on human cells was also used and will be described elsewhere. Briefly, mAb 4F2 was produced by conventional murine hybridoma technology after immunization of mice with chicken lymphoid cells and was shown to inhibit insulin binding to both avian and human cells, to immunoblot a molecule of M_r ≈130,000 (the insulin-binding α chain of the insulin receptor has M_r ≈130,000), and to immunoprecipitate molecules of M_r ≈130,000, ≈90,000, and ≈45,000 from human cell lines (see Fig. 4). Rabbit antibodies against β_2m, against mouse Ig, and against human Ig were obtained from DAKO (Copenhagen).

Insulins. Semisynthetic human insulin and ^{125}I-labeled insulin (labeled at Tyr-A14, specific activity 200–300 μCi/μg; 1 Ci = 37 GBq) were obtained from NOVO Industry A/S (Bagsværd, Denmark). Fluorescein isothiocyanate (FITC)-conjugated insulin was prepared as described (30).

Insulin Receptors. Purified insulin receptor material was a gift from Y. Yamaguchi, who purified the receptor from human placenta tissue (18).

Insulin-Binding Assay. ^{125}I-labeled insulin binding studies were performed as reported (30). Briefly, cells were suspended in assay buffer (100 mM Hepes, pH 7.8/120 mM NaCl/1.2 mM $MgSO_4$/1.0 mM EDTA/10 mM dextrose/15 mM sodium acetate/1% bovine serum albumin) with ^{125}I-labeled insulin (1.0–25.0 pM) in the absence or presence of excess unlabeled insulin (1 μM) at 15°C for 90 min. Cells were pelleted by centrifugation, the supernatant was removed, and the radioactivity of the pellet was determined. Specific binding was calculated by subtracting the values obtained from cells continuously incubated with excess unlabeled insulin (nonspecific binding) from the values obtained from cells incubated without native insulin. Of the total radioactivity bound, 2–4% was nonspecifically bound. All assays were done in duplicate. Cell viability was >95% as measured by trypan blue dye exclusion. Inhibition of insulin binding was tested by incubation of the target cells with specific antibody at 0.5–5.0 μg/ml for 2 hr at room temperature, then washing twice with cold phosphate-buffered saline assaying for insulin binding. Control antibodies included irrelevant monoclonal antibodies against leukemia-associated antigens that do not bind to the target cells (31) and antibodies directed against lung cancer cells (32). Antibody AHA was the positive control.

Fluorescence-Activated Cell Sorter (FACS) Analysis. The cell surface density of various specific antigens was measured by FACS analysis after staining of the cells with FITC-conjugated specific mAbs or in a two-step procedure with the specific mAb as the first-step reagent and FITC-conjugated rabbit anti-mouse Ig as the second. Briefly, 10^6 cells were incubated for 30 min with ≈1 μg of mAb (in phosphate-buffered saline/0.1% azide/0.1% bovine serum albumin) at 4°C, washed twice with phosphate-buffered saline and either analyzed directly or incubated with FITC-conjugated rabbit anti-mouse Ig for 30 min at 4°C, washed twice with phosphate-buffered saline, and then analyzed.

Down-Regulation of Insulin Receptors. Cells were grown for 16 hr in RPMI 1640 medium supplemented with 1 μM insulin, 10% fetal calf serum, and antibiotics. They were then washed three times with 0.1 M phosphate buffer, pH 6/0.5% bovine serum albumin over a period of 1.5 hr at 30°C to remove bound insulin (17, 33).

Capping of Cell Membrane Molecules. Cells were incubated with rabbit anti-FITC-conjugated β_2m (DAKO) antiserum that had been dialyzed (Spectrapor, cutoff M_r 14,000) against

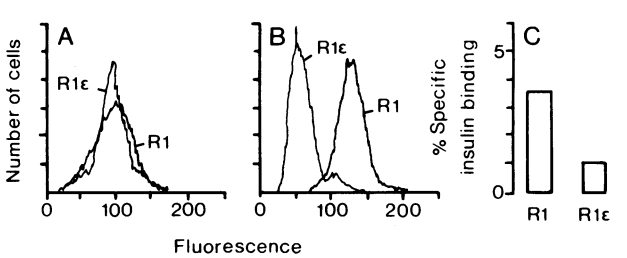

FIG. 1. (*A* and *B*) Binding of an anti-insulin-receptor mAb (4F2) and of an anti-H-2K^b mAb to R1 and R1ε mouse thymoma cells. (*C*) Specific binding of ^{125}I-labeled insulin to R1 and R1ε cells. mAb 4F2 conjugated to FITC and used in a one-step staining procedure, whereas binding of the anti-H-2K^b mAb was detected with a FITC-conjugated polyclonal rabbit anti-mouse Ig. Fluorescence is indicated in arbitrary units. Autofluorescence signals were in all tests <30 channels.

phosphate-buffered saline at various dilutions for 1 hr at 4°C. They were then washed with phosphate-buffered saline/0.5% bovine serum albumin and incubated at 4°C, 20°C, or 37°C for 30 min before microscopic examination, FACS analysis, and ^{125}I-labeled insulin binding assays. Prior to immunofluorescence microscopy, cells were fixed in 1% formaldehyde, which inhibits dissociation and rearrangement of immunoglobulin on the cell surface.

Electroimmunoblotting. Analytical NaDodSO$_4$/PAGE was performed according to Laemmli (34) with a 4% stacking gel and a 10% analytical gel. The samples were boiled prior to analysis for 5 min in 2% NaDodSO$_4$/5% 2-mercaptoethanol/0.01% bromophenol blue/10% (vol/vol) glycerol. Gel-electrophoresed molecules were transferred to nitrocellulose sheets by electrophoresis at 4°C in 25 mM Tris·HCl/192 mM glycine/3 mM NaDodSO$_4$, pH 8.3, for 12 hr at 20 V. The nitrocellulose sheets were washed with phosphate-buffered saline/0.05% Tween 20/30% fetal calf serum and incubated in sealed plastic bags for 2 hr at room temperature with ^{125}I-labeled or with unlabeled antibody. Binding of iodine-labeled mAb was detected by autoradiography and binding of unlabeled mAb was detected by staining with a 1:500 dilution of a peroxidase-conjugated rabbit anti-mouse Ig light chain (DAKO) in phosphate-buffered saline/Tween/30% fetal calf serum for 2 hr at 20°C. The peroxidase stain was developed with 0.06% diaminobenzidine/0.01% H_2O_2 in 50 mM Tris·HCl, pH 7.4. In some experiments, the radioactivity of the bands was determined by excising them from the gel and counting in a γ counter.

Immunoprecipitation. Cells (2×10^6) were labeled with ^{125}I by the Iodo-Gen procedure (Pierce) and solubilized in 1% Triton X-100/50 mM Hepes, pH 7.6/2 mM phenylmethylsulfonyl fluoride for ≈30 min at room temperature. Insoluble material was removed by centrifugation at 150,000 × *g* for 30 min at 4°C and the supernatant was incubated for 4 hr at 4°C with different antibodies. Immunoprecipitation with protein A-Sepharose was done by addition of protein A-coupled polyclonal rabbit anti-mouse Ig (DAKO). After 1 hr at 4°C, the immunoprecipitates were isolated by centrifugation at 300 × *g* for 1 min at 4°C and washed once with high-salt buffer (10 mM Tris·HCl/1% Nonidet P-40/0.50 mM NaCl/2 mM EDTA/0.1% NaDodSO$_4$) and twice with low-salt buffer (10 mM Tris·HCl/1% Nonidet P-40/0.12 mM NaCl/2 mM EDTA/0.1% NaDodSO$_4$). In some experiments, labeled cell extracts were preabsorbed with protein A-Sepharose CL4B-coupled polyclonal rabbit anti-mouse Ig.

RESULTS

Effect of Prior Incubation with mAb on Binding of Insulin. mAbs against MHC class I and class II molecules and against some differentiation antigens were examined for interference with binding of ^{125}I-labeled insulin. Only one mAb (PA 2.6) reduced insulin binding significantly and precipitated the purified insulin receptor (Table 1). The Daudi cells and the R1ε cells did not bind insulin (30), whereas the R1 cells bound significant amounts of insulin (Fig. 1).

Capping of MHC Class I Molecules and of β_2m and Insulin Binding. Capping of cell membrane components and subsequent analysis of co-capping of other molecules has been used to identify structural associations between molecules and complexes in the membrane. A polyclonal rabbit anti-β_2m antibody was used to cap membrane-associated β_2m. At 4°C, this antibody did not interfere with (*i*) binding of mAbs against monomorphic determinants of the MHC class I heavy chain, (*ii*) binding of mAbs against MHC class II chains, or (*iii*) insulin binding (data not shown). At 37°C, the polyclonal anti-β_2m antibody resulted in capping of membrane-bound β_2m (Fig. 2). However, co-capping of the MHC class I heavy chain occurred only for certain antibody dilutions and was dependent of the time of incubation. Capping of β_2m did not result in co-capping of MHC class II molecules. The polyclonal anti-β_2m antibody affected binding of insulin to cells only under conditions that resulted in co-capping of the MHC class I heavy chains (Fig. 2). Reduction of the amount of MHC class I heavy chain was invariably associated with a reduction of insulin binding (Table 2), whereas capping with antibodies to MHC class II molecules or to the transferrin receptor had no effect on insulin binding. No effects were observed on MHC class II antigens or on transferrin receptors after capping with antibodies to MHC class I molecules or β_2m (data not shown).

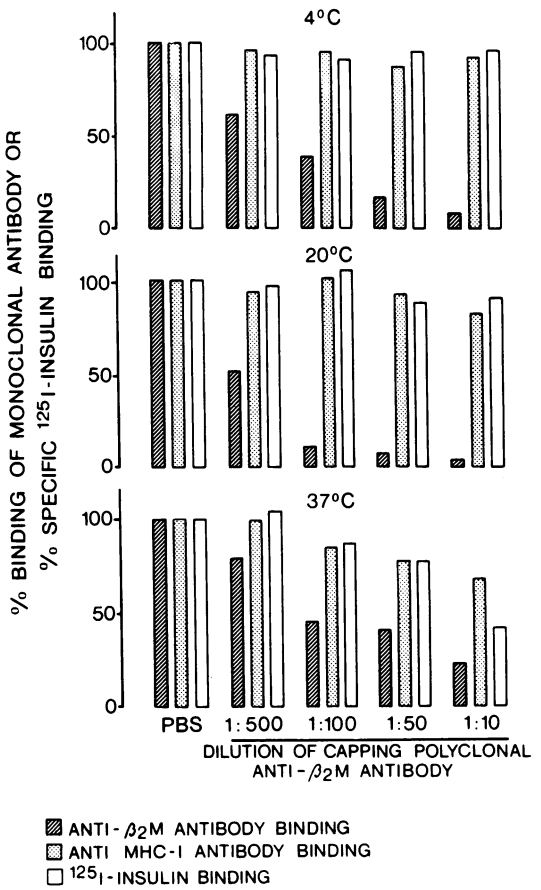

FIG. 2. Effect of capping of β_2m by a polyclonal anti-β_2m mAb on expression of the MHC class I heavy chain (MHC-I), on ^{125}I-labeled insulin binding, and on β_2m expression. The experiments were performed with U937 cells at different dilutions of the capping antibody and at different temperatures. Pilot experiments indicated that 1 hr of incubation resulted in maximal capping of β_2m at 37°C and this incubation period was therefore used throughout. Capping was verified microscopically. The MHC class I heavy chain and β_2m were detected by FITC-conjugated mAbs against a monomorphic determinant MHC class I (PA 2.6 mAb) and against β_2m, respectively.

Insulin Receptor Down-Regulation. The insulin receptor on IM-9 cells can be down-regulated by exposure of the cells to insulin (17). Fig. 3 shows that down-regulation of the insulin receptor is associated with a marked decrease in binding of mAb 4F2, and a significant increase in binding of the anti-HLA-A3 mAb. Comparable results were obtained with mAb PA 2.6. The average number of total binding sites for insulin and for mAb PA 2.6 were estimated to 4×10^4 and 5×10^5, respectively. A complete down-regulation of the insulin receptor can therefore maximally result in an ≈10% increase in binding sites for anti-MHC class I mAbs.

Immunoprecipitation and Electroimmunoblotting. Immunoblots of a Triton X-100 extract of U937 cells and mAb 4F2 resulted in staining of one band with a M_r of ≈130,000 (Fig. 4). mAb PA 2.6 could not be used in immunoblots but

Table 2. Effect of capping of specific cell membrane molecules on expression of other cell membrane components

Capping antibody	Cell membrane component* MHC class I	MHC class II	Transferrin receptor	Insulin molecules per cell,† no.
Phosphate-buffered saline	184	141	155	125
Anti-MHC class I	35	182	191	64
Anti-MHC class II	186	47	158	127
Anti-transferrin receptor	165	154	57	166
Anti-insulin receptor	169	148	160	42

*Determined by FACS channel number on a linear scale. The autofluorescence channel number (0–15 in all experiments) was subtracted from the experimental value.
†FITC-labeled insulin was prepared as described (30) and used to estimate the number of insulin binding sites on viable cells.

immunoprecipitated from ^{125}I-labeled cell membrane material of U937 cells a distinct band with a M_r of ≈45,000, and faint bands in the area of M_r ≈90,000 and 130,000. From the same iodine-labeled U937 cell membrane material, mAb 4F2 precipitated a prominent band at M_r ≈45,000 and distinct, although weaker, bands of M_r ≈90,000 and 130,000. The M_r 45,000 band was eluted from NaDodSO$_4$/PAGE gels and found in a dot-blot assay to bind the mAb GAP A3 (Fig. 4), which is specific for A3 of HLA (U937 cells are HLA-A3 positive). mAb PA 2.6 reacted with proteins that immunoprecipitated with mAb 4F2 from U937 cells before NaDodSO$_4$/PAGE, whereas denaturation of the precipitate resulted in loss of this reactivity (Fig. 4).

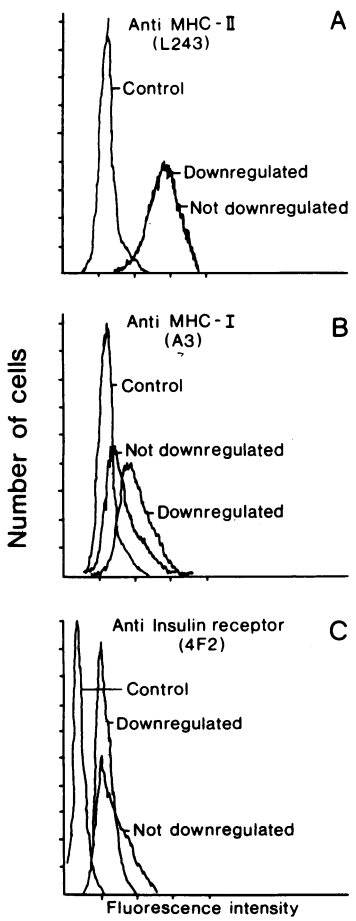

FIG. 3. Effect of insulin-mediated down-regulation of insulin receptor expression on binding of mAbs against MHC class II antigens (*A*), against MHC class I antigens (*B*), and against insulin receptors (*C*). All assays were done on U937 monocytic leukemia cells. mAb 4F2 was used FITC conjugated, whereas staining with the anti-MHC class II and MHC class I mAbs was done in a two-step procedure with FITC-conjugated rabbit anti-mouse Ig as second-step reagent.

DISCUSSION

MHC molecules perform two main kinds of biological role in the cell membrane: (*i*) the universally accepted, though imperfectly understood, immunological functions associated with antigen presentation to T lymphocytes and (*ii*) a group of still more ill-defined, non-immunological functions associated with the binding and/or effect of various hormonal substances. Earlier studies demonstrating correlations between H-2 polymorphism and quantitative physiological traits (for example, body weight and testis weight) have been summarized and reviewed (35). Recently, several reports have indicated more directly that MHC class I molecules interact with receptors for glucagon (36), epidermal growth factor (37), and insulin (13–15) in such a way as to influence the binding of the respective peptide ligands. In the case of γ-endorphin, however, the data suggest a direct binding of this peptide to the polymorphic part of the MHC class I molecule itself (38). Studies on the insulin receptor are so far the most compelling with respect to an interaction in the membrane, and even in detergent-solubilized liver cell membranes, between MHC class I molecules and a hormone receptor (15). The present work extends earlier studies of insulin binding to human cells (13–15) and strengthens the case of the insulin receptor as a paradigm for non-immuno-

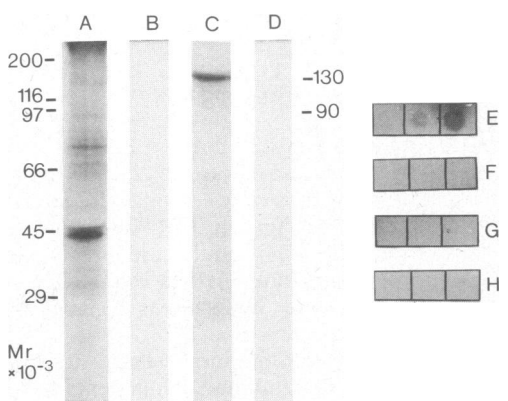

FIG. 4. Immunoblotting and immunoprecipitation of a U937 cell extract. Lane A: immunoprecipitation of an ^{125}I-labeled cell extract with mAb 4F2. Lane B: immunoprecipitation of the ^{125}I-labeled cell extract with mouse serum. Lane C: immunoblotting of U937 cells with mAb 4F2. Lane D: immunoblotting of U937 cells with control mouse serum. The M_r 45,000 band of the mAb 4F2 immunoprecipitate was eluted from the gel, dot-blotted with anti-HLA mAbs (increasing dilution from right to left), and detected with ^{125}I-labeled rabbit anti-mouse Ig: dot-blot E, anti-HLA-A3; dot-blot F, anti-HLA-monomorphic (PA 2.6); dot-blot G, BB77; dot-blot H, normal mouse serum.

logical interaction of MHC molecules with other membrane constituents.

One of the key experiments in the present work consisted in reducing insulin binding to the cell by reducing the concentration of MHC class I heavy chains expressed in the membrane. This was achieved in two ways: capping with antibodies to the heavy chain and capping with antibodies to β_2m. Insulin binding in the cold (4°C) was unaffected by either antibody. The possibility was investigated that the capping effect on insulin binding might be due to immunological crossreaction of the antibodies with the insulin receptor itself. However, the expression of the insulin receptor was reduced by anti-β_2m mAb (Fig. 2). The simplest explanation for the capping effect seems therefore to be that the binding of insulin is furthered by association in the membrane of the insulin receptor with MHC class I molecules. Hence, the sudden and massive removal of MHC class I molecules by capping will necessarily decrease the amount available and thus reduce insulin binding.

The role played by β_2m during the postulated formation of physiologically active MHC class I molecules and insulin receptors is conjectural. Our favored hypothesis (13–15) is that β_2m is replaced by the insulin receptor when the latter associates with the MHC class I heavy chain. Two recent observations support this hypothesis. (*i*) Addition of excess β_2m to the culture medium may reduce insulin binding to the cells in a dose-dependent manner (data not shown), which is the expected result on the assumption of competitive binding in a system in dynamic equilibrium. (*ii*) Immunoprecipitation of detergent-solubilized U937 cells with mAb 4F2, supposedly reacting with or near the insulin binding site on the α chain of the insulin receptor, yielded a precipitate that, on NaDodSO$_4$/PAGE, gave bands corresponding in position to the α and β chains of the insulin receptor, as well as a M_r 45,000 band, but no β_2m band. However, in an immunoblotting experiment, mAb 4F2 stained the putative α chain of the insulin receptor exclusively. The assumed specificity of mAb 4F2 is further supported by the fact that its binding to the cell is markedly reduced by down-regulation of the receptor with insulin and by its blocking effect on insulin binding (15).

We conclude that the insulin receptor associates structurally with MHC class I molecules in the membrane and that the complex thus binds insulin with high affinity. Whether the influence of MHC class I molecules on insulin binding is by conformational change in the insulin receptor molecule or by another mechanism remains to be elucidated.

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