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Alterations in the Expression of MHC Class I Glycoproteins by B16BL6 Melanoma Cells Modulate Insulin Receptor-Regulated Signal Transduction and Augments Resistance to Apoptosis[1]

Efrat Assa-Kunik,[2]* Daniel Fishman,[2†] Sigal Kellman-Pressman,[†] Sylvia Tsory,[†] Shira Elhyany,[†] Ofer Baharir,[†] and Shraga Segal[3†]

In a variety of malignancies, the immune-escape phenotype is associated, in part, with the inability of tumor cells to properly present their Ags to CTLs due to a deranged expression of MHC class I glycoproteins. However, these molecules were found to possess broader nonimmune functions, including participation in signal transduction and regulation of proliferation, differentiation, and sensitivity to apoptosis-inducing factors; processes, which are characteristically impaired during malignant transformation. We investigated whether the deranged expression of MHC class I expression by tumor cells could affect proper receptor-mediated signal transduction and accentuate their malignant phenotype. The malignant and H-2K murine MHC class I-deficient B16BL6 melanoma cells were characterized by an attenuated capacity to bind insulin due to the retention of corresponding receptor in intracellular stores. The restoration of H-2K expression in these cells, which abrogated their capacity to form tumors in mice, enhanced membrane translocation of the receptor, presumably, by modulating its glycosylation. The addition of insulin to H-2K-expressing melanoma cells cultured in serum-free conditions precluded apoptotic death by up-regulating the activity of protein kinase B (PKB)/Akt. In contrast, the deficiency for H-2K characteristic to the malignant clones was associated with a constitutive high activity of PKB/Akt, which rendered them resistant to apoptosis, induced by deprivation of serum-derived growth factors. The possibility to correct the regulation of PKB/Akt activity by restoration of H-2K expression in B16BL6 melanoma cells may be considered as an attractive approach for cancer therapy, since an aberrant activation of this enzyme is characteristic to resistant malignancies. ***The Journal of Immunology,* 2003, 171: 2945–2952.**

Major histocompatibility complex class I glycoproteins present peptides originating mainly from intracellular proteins to CTL and are mandatory for the induction and development of an effective immune response against cells infected by viruses or altered by malignant transformation (1). Our previous studies (1–6) followed by studies conducted by other investigators (reviewed in Ref. 1) demonstrated that an immune escape phenotype of many tumors is associated with their inability to properly present Ags due to a diminished or imbalanced expression of MHC class I molecules. Supporting evidence as to the pivotal role played by the deranged expression of these glycoproteins in tumor immune evasion has been provided by our studies (7–9); in these studies, a significant proportion of chemically induced murine sarcomas were found to be deficient for murine MHC class I H-2K region-encoded glycoproteins (7). As a rule, de novo expression of H-2K region-encoded glycoproteins in these cells rendered them highly immunogenic, led to their effective recognition by the hosts immune system, and reduced their capacity to generate tumors and metastases when grafted into syngeneic immunocompetent animals (7). Yet, it is noteworthy, that MHC class I glycoproteins do not only provide a mechanistic framework for the presentation of antigenic peptides, but, rather these molecules were found to possess a broader nonimmune biological significance. The concept of possible nonimmune functions performed by MHC class I molecules was raised due to the demonstration of an MHC class I-related component (β_2-microglobulin) in biological species lacking a conventional adoptive immune system, i.e., *Drosophila melanogaster* (9). This concept was further extended by other studies implicating these glycoproteins in the regulation of proliferation and apoptosis in cells of both lymphoid and nonlymphoid origin (reviewed in Refs. 10–12). It has been demonstrated, as well, that MHC class I glycoproteins tend to form complexes with other membrane-bound molecules including MHC class II (13, 14), intracellular adhesion molecule I (13, 14), and a number of transmembrane receptors for growth factors and cytokines (Ref. 15, reviewed in Refs. 16 and 17). Among these receptors are included those for insulin (IR),[4] insulin-like growth factor (IGF), epidermal growth factor, IL-2, and various additional factors (16). Despite the obvious importance of these data, the precise mechanism by which MHC class I molecules participate in receptor-mediated signal transduction is not completely understood. It has been reported in a number of studies that direct interactions between MHC glycoproteins and the IR

*Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel; and †Department of Immunology and Microbiology, Faculty of Health Sciences, Ben-Gurion University Cancer Research Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel

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[2] E.A.-K. and D.F. equally contributed to this research.

[3] Address correspondence and reprint requests to Dr. Segal Shraga, Department of Immunology and Microbiology, Faculty of Health Sciences, Ben-Gurion University Cancer Research Center, Ben-Gurion University of the Negev POB 653, Beer-Sheva 84105, Israel. E-mail address: ssegal@bgumail.bgu.ac.il

[4] Abbreviations used in this paper: IR, insulin receptor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; PKB, protein kinase B, PTEN, phosphatase and tensin homologue deleted on chromosome 10; SBA, soybean agglutinin; PNGase, glycopeptidase.

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were found to enhance ligand-induced internalization of the receptor (18). Other investigators have shown that the ratio between membrane-expressed MHC class I glycoproteins and IR molecules determines the extent of ligand-induced phosphorylation of the IR and the IR substrate-1 and, as well, the recruitment of phosphatidylinositol 3-kinase to the plasma membrane (11). The aforementioned findings suggest that MHC class I glycoproteins may play an important role in growth factor-mediated cell-cell communication and in receptor-mediated signal transduction; both of these processes are significantly impaired during malignant transformation and tumor progression. The relevance of nonimmune functions assigned to MHC class I for the biology of tumor cells is further substantiated by reports demonstrating an attenuated in vivo growth of de novo MHC class I-expressing tumor cells even when grafted into immune-compromised animals (19- 21). In our present investigation, we addressed the question whether alterations in MHC class I expression by tumor cells could affect proper receptor-mediated signal transduction processes and accentuate the malignant phenotype of these cells. For that purpose, we used B16BL6 murine malignant melanoma cells, which provide an appropriate model illustrating the existence of a strong correlation between a deficiency in H-2K MHC class I expression and the capacity of tumor cells to form primary and secondary tumors when grafted into animals (20). In contrast, all H-2K murine MHC class I-expressing clones were unable to form tumors when grafted into syngeneic animals, regardless of the mode by which the expression of the *H-2K* gene was achieved (20). Based on the existence of the aforementioned functional interactions between MHC class I and IR, we investigated the influence of MHC class I glycoproteins on the cellular compartmentalization of the IR and IR-associated signal transduction pathways in MHC class I-deficient and highly malignant vs MHC class I-expressing and nonmalignant B16BL6 melanoma cells.

Materials and Methods

Cell cultures

B16BL6 melanoma clones of C57BL/6J origin used in this study have been developed and kindly provided by Dr. E. Gorelik (Department of Pathology, University of Pittsburgh, Pittsburgh, PA) and are described in Refs. 20 and 22. The first subset of B16BL6 cells includes 1) the H-2K murine MHC class I-deficient and malignant BL6-8 clone, 2) the H-2K-expressing and nonmalignant BL6–29 clone (a spontaneous H-2K-expressing revertant of BL6-8), and 3) the H-2K-expressing and nonmalignant Kb30 (BL6-8 cells transfected with an H-2K-encoding pRSV vector) clones. The selection of these H-2K-expressing clones was performed according to their enhanced ability to bind the soybean agglutinin (SBA). The second subset of melanoma cells includes 1) the H-2K-deficient and malignant BL6-9 clone (BL6-8 cells transfected with the pRSV control vector carrying a gene for neo resistance), 2) the H-2K-deficient and malignant BL6-22 clone (BL6-8 transfected with an H-2IA murine MHC class II-encoding pRSV vector), and 3) the H-2K-expressing and nonmalignant CL8-1 and CL8-2 clone (BL6-8 cells transfected with an H-2K-encoding pRSV vector). We confirmed the aforementioned malignant characteristics of these melanoma clones by inoculating these tumor cells into syngeneic C57BL/6J mice (usually $4–5 \times 10^5$ cells) intrafoot pads. All tissue culture reagents were purchased from Biological Industries (Bet-Ha-emek, Israel). Tissue culture plasticware was purchased from Corning (Corning, NY). Cells were usually grown in a complete medium (RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin)). For experiments using deprivation from growth factors, cells were plated on tissue culture dishes into complete medium and allowed to attach for 24 h. The complete medium was removed and exchanged for RPMI 1640 medium containing antibiotics and not supplemented with FCS (serum-free medium). Cells were cultivated for an additional 12–24 h. The medium was then replaced by either antibiotics containing serum-free RPMI 1640 supplemented or not supplemented with 10^{-6} M bovine insulin (Sigma-Aldrich, Rehovot, Israel).

Insulin-binding assay

Binding of biotinylated insulin to cells was performed as described previously (23). Briefly, cells were maintained in serum-free conditions for 12 h, harvested by trypsinization, and counted. Five hundred thousand cells were exposed to $6 - 10^{-8}$ M bovine insulin conjugated to biotin (B-Insulin; Sigma-Aldrich) diluted in 100 μl of assay buffer (100 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM sodium acetate, 10 mM D-glucose, and 1% BSA, pH 7.8) for 90 min at 15°C. Cells were washed using the assay buffer and incubated with PE-conjugated streptavidin (SA-PE; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in the assay buffer for 30 min at 15°C. Following incubation, cells were washed three times with the assay buffer and B-Insulin binding was assessed using a FACSCalibur cytometer (BD Immunocytometry Systems, San Jose, CA). The binding specificity was assessed by the inclusion of nonbiotinylated insulin into the reaction mixture. Cells stained only with streptavidin-PE were used as a negative control.

Assessment of apoptosis by examination of nuclear morphology

Cells were starved for serum and exposed to insulin as described above. Five hundred thousand cells were suspended in 25 μl of PBS containing 4 μg/ml ethidium bromide (Sigma-Aldrich) and acridine orange (Sigma-Aldrich). Stained cells were examined for nuclear morphology using a fluorescent confocal microscope (Laser Scanning System LSM 510; Zeiss, Jena, Germany). One hundred fifty cells per field were analyzed. The structure of chromatin stained with acridine orange in cells stained with acridine orange was analyzed. Cells stained with ethidium bromide were considered as damaged (necrotic). The percentage of apoptotic cells was calculated according to the formula: $[(EA + LA) \times 100]/\text{Total}$, where EA = the number of early apoptotic cells, LA = the number of late apoptotic cells, and Total = EA + LA + normal + the number of necrotic cells. Typical normal and apoptotic cells are depicted in Fig. 3, *upper panel*.

Immufluorescent detection of the IR in melanoma cells

Cells were plated on coverslips precoated with poly-L-lysine in complete medium. Following 24 h of culture, cells were washed three times with PBS, fixed in 4% paraformaldehyde in PBS, and permeabilized by incubating in PBS containing 0.1% Triton X-100, 1% BSA (fraction V), and 2% normal goat serum and exposed to Abs directed against IR β subunit (Santa Cruz Biotechnology, Santa Cruz, CA). A secondary step reagent (Cy-3-conjugated anti-rabbit Ab) was purchased from Jackson ImmunoResearch Laboratories. The compartmentalization of the receptor was analyzed using a fluorescent confocal microscope.

Preparation of protein lysates

Total protein lysates were prepared by suspending cells in RIPA buffer (25 mM HEPES (pH 7.5), 0.3M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 0.5 mM sodium deoxycholate, and a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany), 0.6 mM PMSF, 0.1 mM sodium orthovanadate, 20 mM sodium fluoride, and 20 mM glycerophosphate) and incubated for 20 min at 4°C. The cellular suspension was homogenized by passing it several times through a syringe equipped with a 27-gauge needle. Cellular debris was sedimented by centrifugation at 12,000 × *g* for 15 min at 4°C and supernatants were saved. For the preparation of a protein lysate enriched with membrane proteins, cells were suspended in a hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protease inhibitor mixture tablets (Roche Diagnostics), 0.6 mM PMSF, 0.1 mM sodium orthovanadate, 20 mM sodium fluoride, and 20 mM β-glycerophosphate) and incubated for 20 min at 40°C. The cellular suspension was homogenized by passing it several times through a syringe equipped with a 21-gauge needle. Nuclei and debris were sedimented by centrifugation at 12,000 × *g* for 15 min at 40°C, suspended in a hypertonic buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors mixture (Roche Diagnostics), 0.6 mM PMSF, 0.1 mM sodium orthovanadate, 20 mM sodium fluoride, and 20 mM β-glycerophosphate), and passed several times through a syringe equipped with a 21-gauge needle. Lysates were subjected to centrifugation at 12,000 × *g* for 15 min at 40°C and pellets enriched in membrane proteins were saved and used in deglycosylation studies.

Immunprecipitation

Total protein lysates were first precleared by incubation with protein A-conjugated Sepharose for 4 h at 40°C. One milligram of the protein lysate was then combined with anti-IRβ Abs (Santa Cruz Biotechnology) and protein A-conjugated Sepharose and then adjusted to a final volume of 600 μl with RIPA buffer before mixing overnight at 40°C. Immune complexes

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were recovered by brief centrifugation, washed three times with RIPA buffer, and eluted from protein A-conjugated Sepharose by boiling in Laemmli sample buffer.

Deglycosylation of membrane-associated glycoproteins

Membrane protein-enriched fractions containing 100 μg of protein were treated with 4 U/ml glycopeptidase F (Calbiochem, San Diego, CA) in a buffer containing 50 mM Tris (pH 8.5), 1% Triton X-100, 0.1% SDS, and 50 mM 2-ME for 4 h at 40°C.

Immunoblotting

Total protein lysates, immunoprecipitates or deglycosylated membrane proteins were resolved on a 7.5% SDS-polyacrylamide gel, electroblotted to a nitrocellulose membrane, and probed to anti-phospho-Akt, anti-Akt (Santa Cruz Biotechnology), 4G10 anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), and anti-IRβ chain (Santa Cruz Biotechnology) Abs.

Results

The reconstituted expression of H-2K glycoproteins in B16BL6 melanoma cells enhances their capacity to bind insulin

The capacity of H-2K-expressing and H-2K-deficient melanoma cells to bind insulin was assessed by using a biotin-conjugated ligand and flow cytometry. This is a safe and nonradioactive approach and, most important, it provides data corresponding to those obtained by a conventional assay using a radioactive ligand, as described previously (23). The results depicted in Fig. 1*A* (*left* and *right panels*) clearly demonstrate that H-2K-deficient BL6-9, BL6-22, and BL6-8 clones are characterized by a diminished capacity to bind biotinylated insulin, as compared with their nonmalignant and H-2K-expressing CL8-1, CL8-2, Kb-30, and BL6-29 counterparts. It is noteworthy that neither the approach by which the expression of H-2K glycoproteins was achieved (gene transfer, as in the case of CL8-1, CL8-2, and Kb-30 clones, vs selection of a spontaneous H-2K-expressing revertant, as in the case of the BL6-29 clone), nor the approach by which H-2K-expressing clones were isolated (resistance to neo, as in the case of CL8-1 and CL8-2 clones, vs differential capacity to bind SBA, as in the case of BL6-29 and Kb-30 clones) affected the insulin-binding pattern (Fig. 1*A*, *left* and *right panels*). The binding specificity was confirmed by inclusion of indicated concentrations of a competing nonbiotinylated ligand (Fig. 1*B*).

The reconstituted expression of H-2K glycoproteins in B16BL6 melanoma cells affects compartmentalization and glycosylation of the IR

To explain the lower insulin-binding capacity of H-2K-deficient melanoma cells as compared with their H-2K-expressing counterparts, cells were stained with anti-IR Abs and optical sections were analyzed by fluorescent confocal microscopy. As depicted in Fig. 2*A*. (*left panels*), a higher number of IR molecules were expressed at the cell surface of H-2K-expressing cells than in intracellular compartments. In contrast, in H-2K-deficient cells IR molecules were located mainly in intracellular compartments (Fig. 2*A*, *right panels*). It is noteworthy that some IR molecules do appear at the plasma membrane in H-2K-deficient cells, since the IR became phosphorylated on tyrosine residues following the exposure of cells to insulin, albeit to a lesser extent and with a different kinetic than in H-2K-expressing cells (Fig. 2*B*, *lanes 3* and *4* vs *7* and *8*). Most important, bands corresponding to a phosphorylated receptor in H-2K-deficient cells were characterized by a faster electrophoretic mobility than those detected in H-2K-expressing cells (Fig. 2*B*, *lanes 2–4* vs *6–8*). Previous studies of Gorelik et al. (24, 25) demonstrated that the de novo expression of an H-2K region-encoded glycoproteins in B16BL6 melanoma cells affects glycosylation processes. Therefore, the differential electrophoretic mobility of bands corresponding to the phosphorylated receptor (Fig. 2*B*) could be the result of its differential glycosylation status in H-2K-expressing vs H-2K-deficient cells. When analyzing the expression of the IR by immunoblotting, a doublet of two close bands (a minor faster running and a major slower running) corresponding to the IRβ chain was detected in H-2K-deficient cells (Fig. 2*C*, *lanes 1* and *5,* double arrows and asterisks). In contrast, only one band corresponding to the IRβ chain was detected in H-2K-expressing cells; the mobility of this band was slower as compared with that of the doublet detected in H-2K-deficient cells (Fig. 2*C*, *lanes 3* and *7*; single arrow). We examined whether deglycosylation of the IR could affect its electrophoretic mobility by treating protein lysates prepared from H-2K-deficient and H-2K-expressing cells by glycopeptidase (PNGase) F, which removes *N*-linked glycans from glycoproteins. As expected, deglycosylation of the

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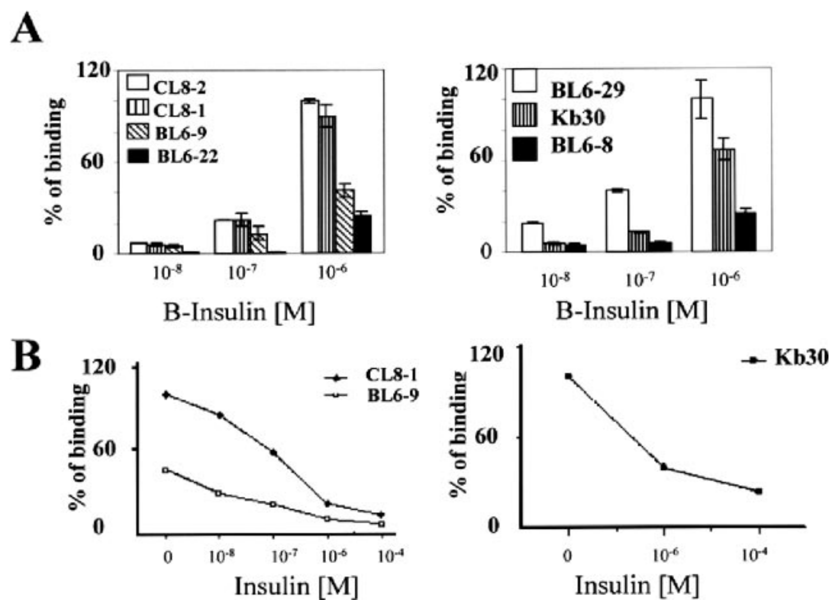

FIGURE 1. Expression of H-2K-encoded glycoproteins by melanoma cells enhances their ability to bind insulin (*A*). Enhanced insulin-biotin binding to H-2K-expressing (CL8-1, CL8-2, BL6-29, and Kb30) melanoma cells as compared with H-2K-deficient (BL6-9, BL6-22, and BL6-8) melanoma cells. *Left panel,* Results obtained with clones that underwent selection for the resistance to G418. Results obtained with cells selected for their capacity to bind SBA lectin are presented in the *right panel*. Cells maintained in serum-free RPMI 1640 medium were exposed to the indicated concentrations of biotinylated insulin (B-Insulin) and then to PE-conjugated streptavidin as described in *Materials and Methods*. The extent of binding was assessed as an intensity of fluorescence by flow cytometry. Maximal fluorescence of CL8-2 cells (*left panel*) and BL6-29 cells (*right panel*) exposed to 10^{-6} M B-insulin is expressed as 100%. Each bar is a mean ± SEM of a triplicate obtained in 1 of 10 independent experiments. *B,* Specificity of binding was confirmed by competition with the indicated concentrations of nonbiotinylated insulin as described in *Materials and Methods.*

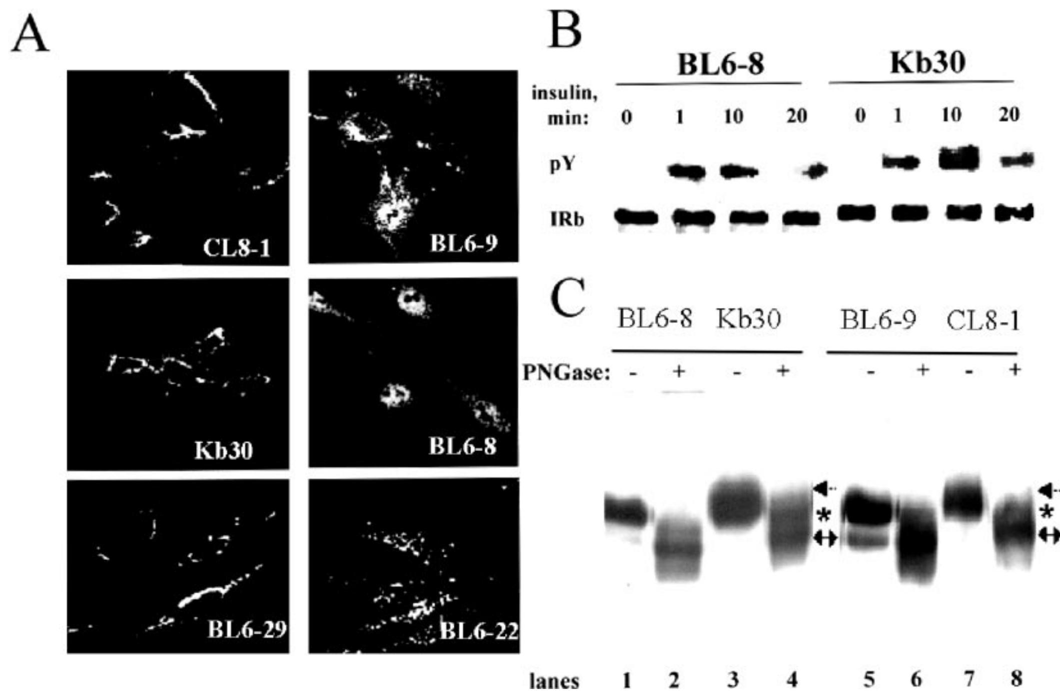

FIGURE 2. H-2K- encoded glycoproteins promote membrane translocation of the IR and modulate its glycosylation. *A*,. Most of IR molecules are located in intracellular compartments of H-2K-deficient cells. Cells were stained with anti-IRβ chain Abs and examined by fluorescence confocal microscopy. Magnification, ×40. The results represent one of five independent experiments. *B*,. Insulin-induced phosphorylation of the IRβ chain in melanoma cells. Serum-starved cells were exposed to 10^{-6} M insulin for the indicated time intervals and protein lysates were prepared. The IR was immunoprecipitated with anti-IRβ Abs and protein A-conjugated Sepharose. Immune complexes were eluted from protein A-conjugated Sepharose by boiling in Laemmli sample buffer. Eluate of each sample was divided into two equal aliquots, and each set of aliquots was subjected to immunoblotting with either anti-phosphotyrosine Abs (*upper panel*) or anti-IRβ Abs (*lower panel*). *C*,. De-glycosylation of the IRβ chain by PNGase. Crude membrane fractions were obtained from melanoma cells, treated with PNGase, and subjected to immunoblotting with anti-IRβ Abs.

IRβ chain by an indicated amount of the enzyme and for an indicated time interval (as depicted in *Materials and Methods*) enhanced its electrophoretic mobility and a number of digestions products were detected (Fig. 2*C*, *lanes 2*, *4*, *6*, and *8*). It is noteworthy, however, that in H-2K-deficient cells a major species of the deglycosylated β-chain was characterized by a similar mobility to that observed for the minor faster running β-chain species in control nondeglycosylated samples (Fig. 2*C*, *lanes 2* and *6* vs *1* and *3*; double arrows). A similar electrophoretic mobility was also characteristic of the major deglycosylated IRβ species derived from H-2K-expressing cells (Fig. 2*C*, *lanes 4* and *8* vs *2* and *6*, double arrows). Although the digestion by PNGase performed at these conditions was incomplete, we concluded that H-2K-dependent modulation of glycosylation in B16BL6 cells described in Refs 24 and 25 affects glycosylation of the IR. Using higher PNGase concentrations and/or more prolonged incubation resulted in protein degradation (data not shown).

H-2K-deficient B16BL6 cells are resistant to apoptosis induced by deprivation from growth factors due to a high constitutive protein kinase B (PKB)/Akt activity

In the next step of our experiments, we investigated the biological significance of the augmented capacity of H-2K-expressing cells to bind insulin. Many malignant cells are characterized by an enhanced glucose uptake which contributes to their growth (26). To assess whether the deficiency for H-2K glycoproteins is associated with a deregulated glucose transport, we examined the basal and insulin-stimulated glucose uptake in either type of melanoma cells by exposing them to 2-deoxy-D-[2,6-^{3}H]glucose. No difference in glucose transport between H-2K-deficient and H-2K-expressing melanoma clones was detected (data not shown). However, the enhanced capacity to bind insulin by H-2K-expressing cells was consistent with the ability of this growth factor to diminish the extent of apoptotic death induced by cultivating these cells in serum-free conditions, as was assessed by the examination of the cellular chromatin structure (Fig. 3, *upper panel*). Indeed, proportions of 50, 52, and 48% of cells with condensed and fragmented chromatin were detected in serum-starved cultures of H-2K-expressing CL8-2, Kb-30, and BL6-29 melanoma cells, respectively, while the addition of insulin to the serum-free culture medium decreased the number of apoptotic cells to 17, 12, and 19%, respectively (Fig. 3, *lower panel*). In contrast, a low percentage of apoptotic cells was detected among H-2K-deficient melanoma cells cultured in serum-free conditions (8, 5, and 5% for BL6-8, BL6-9, and BL6-22, respectively); the addition of insulin to serum-free culture medium failed to affect these cells (Fig. 2*C*, *lower panel*). The function of insulin as a survival factor is associated with the activation of the PKB/Akt signaling module following ligation of the IR by its ligand (27, 28). As expected, the exposure of H-2K-expressing cells to insulin was followed by a rapid and significant elevation in PKB/Akt activity, as was assessed by using anti-PKB/Akt Abs directed against phosphorylated residues of this molecule (pAkt) (Fig. 4, *A* and *B*). In contrast, in H-2K-deficient cells the kinetics of the insulin-induced up-regulation of PKB/Akt activity was slower and to a lesser extent in comparison to the one observed in H-2K-expressing cells (Fig. 4, *A* and *B*). Most important, the basal and noninduced PKB/Akt activity was significantly higher in H-2K-deficient cells than in their H-2K-expressing counterparts.

A high constitutive activation of PKB/Akt is characteristic of a variety of malignancies and renders tumor cells resistant to many cell death-inducing stimuli (28). To assess whether the constitutive

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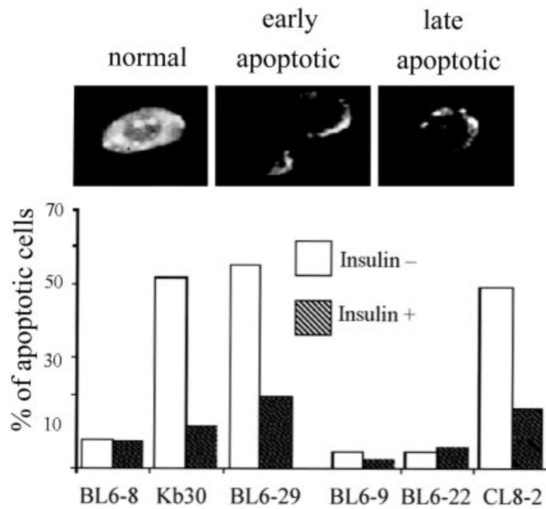

FIGURE 3. Cells deficient for H-2K-encoded glycoproteins are more resistant to apoptosis induced by deprivation from growth factors than H-2K-expressing cells. H-2K-expressing cells are rescued from apoptosis by addition of insulin to serum-free medium. Cells were maintained in serum-free conditions in the presence or absence of 10^{-6} M insulin for 72 h as described in *Materials and Methods*. The evaluation of the extent of apoptosis was performed by the analysis of nuclear chromatin by using the appropriate fluorescent probes and fluorescent confocal microscopy. The *upper panel* represents typical cells considered as normal, early, and late apoptotic, respectively. The *lower panel* represents results obtained in one of four independent experiments.

activation of PKB/Akt in H-2K-deficient cells contributes to their resistance to serum starvation-induced apoptosis (Fig. 3), we exposed these cells to LY294002, which is a potent inhibitor of an upstream regulator of PKB/Akt, i.e., phosphatidylinositol 3-kinase. LY294002, at a concentration of 20 μM, was able to decrease the constitutive PKB/Akt activity in H-2K-deficient cells up to 55% of its basal level, as was assessed by immunoblotting and anti-pAkt Abs (data not shown). When LY294002-treated BL6-8 and BL6-9 cells were maintained in serum-free conditions for 72 h, proportions of 25 and 59% of apoptotic cells were detected, respectively, as compared with 2 and 8% of nontreated cells. The same concentration of LY294002 was unable to induce apoptosis in these cells cultured in a medium supplemented by serum (data not shown).

Discussion

The ability of malignant cells to evade immune surveillance is frequently associated with an impaired or diminished expression of MHC class I glycoproteins. However, these molecules, as reported by several studies, seem to affect the tumor cell phenotype apart from their classical role in the presentation of antigenic determinants to specific T cells. Thus, the reconstituted expression of H-2K murine MHC class I glycoproteins in B16BL6 melanoma cells ultimately diminishes melanin synthesis, abrogates the expression of endogenous retroviruses, alters the pattern of cell surface carbohydrates, and increases the sensitivity to TNF-mediated cytotoxicity (20, 22, 24). Our decision to explore the consequences of MHC class I deficiency for tumor cell responses to growth factors was based on previous studies that have demonstrated the existence of functional interactions between these membrane-associated glycoproteins and the IR (reviewed in Ref. 16). In our present study, we found that a stable expression of an H-2K murine MHC class I gene in B16BL6 melanoma cells augmented their capacity to bind insulin (Fig. 1). The increased insulin-binding

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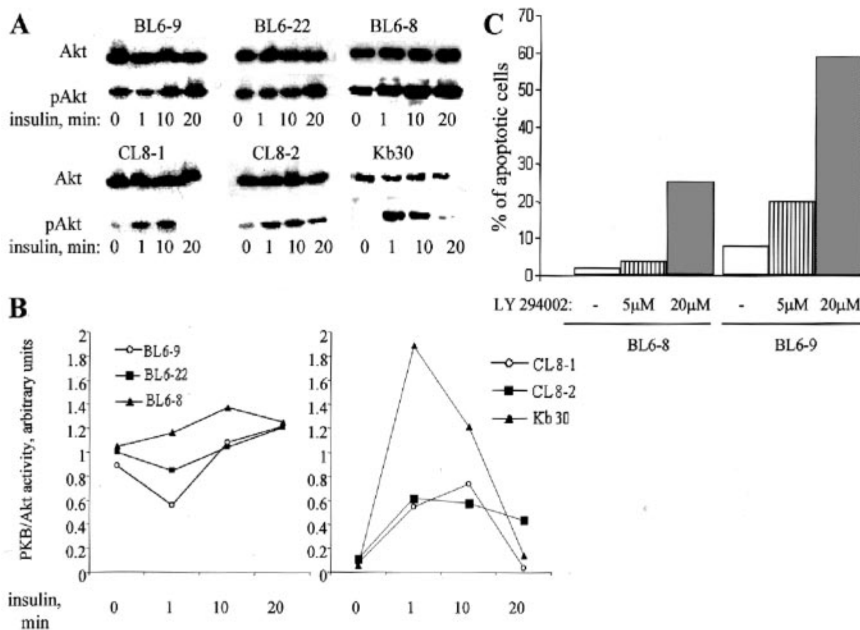

FIGURE 4. The resistance of cells deficient for H-2K-encoded glycoproteins to cell death induced by deprivation from growth factors is mediated by a high basal activity of PKB/Akt. *A,* Basal and insulin-induced PKB/Akt activity in melanoma cells. Cells were maintained in serum-free condition for 12 h and exposed to 10^{-6} M insulin for the indicated time intervals. Protein lysates of each sample were divided into two aliquots, each aliquot (100 μg) was subjected to immunoblotting with Abs recognizing either total (anti-Akt) or phosphorylated PKB/Akt (anti-pAkt). The results are representative of three independent experiments. *B,*. The activity of PKB/Akt in each sample was measured by evaluating the intensity of the bands corresponding to the levels of phosphorylated PKB/Akt, standardized to band intensity corresponding to the total PKB/Akt levels, and expressed in arbitrary units. *C,*. Inhibition of signal transduction through PKB/Akt signaling module induces apoptosis in H-2K-deficient cells when cultured in serum-free conditions. Cells were maintained in serum-free conditions in the presence or absence of indicated concentrations of LY294002 (Cell Signaling Technology, Beverly, MA) and the proportions of apoptotic cells were evaluated after 72 h.

capacity displayed by H-2K-expressing clones of B16BL6 melanoma, as compared with H-2K-deficient clones, was strongly consistent, and was independent of either the approach, by which the expression of these glycoproteins has been achieved, or the mode, by which H-2K-expressing clones have been selected. This observation was consistent with data provided by other research groups, which also relate the MHC phenotype to insulin binding (11, 29-31). The augmented insulin-binding capacity of H-2K-expressing cells correlated well with the cell surface rather than intracellular compartmentalization of the IR in H-2K-expressing cells. In contrast, in H-2K-deficient cells most of the receptor molecules were retained in intracellular stores (Fig. 2*A*) and, therefore, were less available for binding of the externally added ligand (Fig. 1*A*). The ability of MHC class I glycoproteins to promote the translocation of growth factor receptors to plasma membrane from cytoplasmic compartments was also described by other authors. For example, data of Singh et al. (32) and Bian et al. (33) implicate MHC class I glycoproteins in the regulation of compartmentalization of the receptor for fibroblast growth factor (FGFR). These authors demonstrate that the association of MHC class I glycoproteins with a yet unidentified membrane cofactor activates a number of nonreceptor kinases, including Src and the focal adhesion kinase, which, in turn, promote the redistribution of FGFR from cytoplasmic stores to the plasma membrane and nucleus in an actin-dependent manner (32). We suggest that in B16BL6 cells H-2K molecules could enhance membrane translocation of the IR by another mechanism which involves glycosylation processes. Marked alterations in the glycosylation pattern of cell membrane constituents were induced by the re-expression of H-2K glycoproteins in H-2K-deficient B16BL6 melanoma clones, as reflected by the reduction in sialylation and the creation of SBA and GS1B4 lectin- binding epitopes (20, 22, 24, 25). Gorelik and colleagues (22, 24, 25) have shown that these changes are associated at least, in part, with an up-regulated expression of α1,3-galactosyltransferase, which is attributive to the *trans*-Golgi compartment. IR is known to be heavily glycosylated and alterations in this process during the maturation process of newly synthesized receptors led to accumulation of the improperly glycosylated species in intracellular compartments (34, 35). Indeed, distinct patterns of glycosylation of the IR were detected in H-2K-deficient vs H-2K-expressing cells (Fig. 2, *B* and *C*). Moreover, our data support those of Gorelik and colleagues (22, 24) and indicate that the difference in glycosylation patterns between two types of melanoma cells is at the Golgi level, since the fully processed IRβ chain rather than unprocessed species were detected in H-2K-deficient cells. A deranged glycosylation of the receptor in the endoplasmic reticulum achieved by specific inhibitors results in an accumulation of unprocessed receptor species (our unpublished observation). An intracellular retention of growth factor receptors in cancer cells due to an altered glycosylation is not unique for highly malignant H-2K-deficient B16BL6 melanoma cells. Dricu et al. (36) demonstrated the retention of an inadequately glycosylated IGF-1 receptor in MDA231 human estrogen-independent breast cancer cells. The correction of the glycosylation status was followed by an enhanced membrane translocation of the receptor, which significantly increased the IGF-1-binding capacity of these cells and their demand for IGF-1 (36). These authors also suggested that the retained receptor is functional and mediates an intracellular autocrine IGF-1 signaling loop in these cells. The relevance of intracellular migrations of other growth factor receptors (i.e., FGFR and epidermal growth factor receptor) or their fragments for signal transduction in different cellular compartments, including the nucleus, has been also demonstrated (33, 37). We assume that the signaling loop through an intracellular IR may also exist in H-2K-deficient melanoma cells and could be determinative for basic characteristics of the cellular transformed phenotype, including dependence on growth factors. As depicted in Fig. 3, H-2K-deficient BL6-8, BL6-9, and BL6-22 cells were resistant to cell death induced by deprivation from serum-derived growth factors, as compared with clones, where the expression of an *H-2K* gene was reconstituted and most of the IR molecules were translocated to the plasma membrane. The latter cells were characterized by a high demand for growth factors and died when deprived from serum unless insulin was added to the culture medium. Regardless whether the intracellular retention of the IR in H-2K-deficient cells and their diminished dependence for growth factors are related phenomena or not, our results indicate that the deficiency for H-2K glycoproteins accentuates the transformed phenotype of any given B16BL6 melanoma cell by reducing the demand for growth factors. Such malignant cells could have a significant growth advantage over another tumor cell in tumor cell populations which still express H-2K molecules. Consistent with this conclusion, H-2K-deficient B16BL6 melanoma clones were previously characterized by a more aggressive malignant behavior in comparison to their H-2K-expressing counterparts, even when grafted into immune compromised mice (20).

The central role of insulin in the regulation of cell growth and survival is well documented (38–43). For example, a variety of skin lesions associated with atrophic changes and abnormal tissue organization were described in patients carrying a mutant IR or suffering from insulin resistance (42, 43). Insulin is also an important survival factor for cerebellar and retinal neurons (27, 43). The antiapoptotic and growth promoting effects of insulin are mediated by the PKB/Akt serine threonine kinase (28, 44). Since insulin acts as a survival factor for H-2K-expressing melanoma cells (Fig. 3), we proposed that the mode, by which the deficiency for H-2K-encoded glycoproteins renders cells less dependent on this and other growth factors, deregulates signaling through the PKB/Akt. Indeed, a high constitutive activity of this enzyme was detected in H-2K-deficient cells. The exposure of these cells to insulin led to some further up-regulation in PKB/Akt activity (Fig. 4, *A* and *B*). This could be explained by assuming that some IR molecules are still present at the plasma membrane of these cells, albeit hardly detectable by fluorescent microscopy, and underwent phosphorylation following the ligation of insulin (Fig. 2*B*). Nevertheless, the extent of insulin-induced phosphorylation of the IR in H-2K-deficient cells was less prominent than in cells where the re-expression of H-2K glycoproteins led to the translocation of a significant fraction of the IR molecules to the plasma membrane (Fig. 2*B*). In the latter cells, an exposure to insulin resulted in a prominent increase in PKB/Akt activity. Most important, H-2K-expressing cells were characterized by a low basal activity of PKB/Akt as compared with H-2K-deficient cells. The high basal activity of PKB/Akt detected in H-2K-deficient cells was responsible for their low dependence on growth factors, since when the activity of PKB/Akt was inhibited these cells underwent apoptosis following deprivation from growth factors (Fig. 4*C*). The mechanism underlying the abnormal high basal PKB/Akt activity in B16BL6 H-2K-deficient melanoma cells is yet unknown and requires further investigation. One possibility is that some intracellular IR could be activated by yet unidentified intracellular ligands and up-regulate PKB/Akt. We also cannot exclude the possibility that the presence of MHC class I molecules at the plasma membrane could recruit the negative PKB/Akt regulator, i.e., phosphatase and tensin homologue deleted on chromosome 10 (PTEN), to this cellular location and preclude the basal high activity of PKB/Akt. Both MHC class I glycoproteins and PTEN molecules are associated with detergent-insoluble microdomains (lipid rafts) of the plasma membrane (45, 46) and the precise composition of rafts has been shown

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to be determinative for PKB/Akt activity (47, 48). Moreover, it has been shown that even in PTEN-deficient tumor cells the activity of PKB/Akt is still subject to down-regulation via alterations in cell membrane rafts (49). Nevertheless, the possibility to restore the normal regulation of PKB/Akt activity by correction of an H-2K deficiency in B16BL6 melanoma cells may be considered as an attractive approach for cancer therapy, since an aberrant activation of this enzyme is a major characteristic to many apoptosis-resistant malignancies contributing to drug resistance and enhancing genetic instability (28).

In conclusion, our data provide first-time evidence that the inability to express MHC class I glycoproteins in a given tumor cell accentuates its malignant phenotype due to an alteration of cell death-inducing signals as reflected by their ability to survive in the absence of growth factors. Moreover, cell survival-promoting signals provided by the constitutive activation of PKB/Akt, which stem from an MHC class I deficiency, could render tumor cells less susceptible to apoptosis-inducing factors including those originating in immune effector cells. Thus, a deranged expression of MHC class I glyoproteins by malignant cells may contribute to the ability of a given tumor to evade eradication by the immune system by a mode other than a defective Ag presentation capacity. Moreover, our data suggest that MHC class I glycoproteins may be directly involved in the regulation of proliferation, genetic stability, differentiation, and intercellular communication of various cells in multicellular organisms.

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