A Role for Dipeptidyl Peptidase IV in Suppressing the Malignant Phenotype of Melanocytic Cells

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Summary

Dipeptidyl peptidase IV (DPPIV) is a cell surface peptidase expressed by normal melanocytes, epithelial cells, and other cells. Malignant cells, including melanomas and carcinomas, frequently lose or alter DPPIV cell surface expression. Loss of DPPIV expression occurs during melanoma progression at a stage where transformed melanocytes become independent of exogenous growth factors for survival. Tetracycline-inducible expression vectors were constructed to express DPPIV in human melanoma cells. Reexpressing DPPIV in melanoma cells at or below levels expressed by normal melanocytes induced a profound change in phenotype that was characteristic of normal melanocytes. DPPIV expression led to a loss of tumorigenicity, anchorage-independent growth, a reversal in a block in differentiation, and an acquired dependence on exogenous growth factors for cell survival. Suppression of tumorigenicity and reversal of a block in differentiation were dependent on serine protease activity, assessed using mutant DPPIV molecules containing serine→alanine substitutions. Surprisingly, dependence on exogenous growth factors was not dependent on serine protease activity. Reexpression of either wild-type or mutant DPPIV rescued expression of a second putative cell surface serine peptidase, fibroblast activation protein α, which can form a heterodimer with DPPIV. This observation suggests that rescue of fibroblast activation protein α may play a role in regulating growth of melanocytic cells. These results support the view that downregulation of DPPIV is an important early event in the pathogenesis of melanoma.

Key words: melanoma • fibroblast activating protein α • serine protease • tumorigenicity • differentiation

Dipeptidyl peptidase IV (DPPIV)[1] is a cell surface peptidase that is expressed on epithelia, melanocytes, and T cells (1–3). It is a type II membrane glycoprotein that has multiple properties, including serine protease activity and the ability to bind adenosine deaminase and extracellular matrix components (2–4). Because it binds to adenosine deaminase, DPPIV has also been called adenosine deaminase binding protein or adenosine deaminase complexing protein (4, 5). DPPIV expression on T cells has been designated CD26 (3).

It has been recognized for several decades that expression of DPPIV can be extinguished or altered on cancer cells (6–8). Loss or alteration of membrane expression of DPPIV has been reported in prostate, colorectal, lung, and hepatocellular carcinomas and melanomas (2, 6–13). DPPIV expression during malignant transformation has been best characterized in melanocytic cells. DPPIV is expressed in vitro and in vivo by normal melanocytes but not by melanoma, the malignant counterpart (2, 13). Previous studies have shown that expression of DPPIV is lost as melanocytes are transformed into melanoma cells (2, 13, 14). Loss of DPPIV expression probably occurs at an early stage of melanoma progression as melanocytes transform into melanoma cells (2). Specifically, DPPIV is expressed by cutaneous melanocytes and common nevi but is not detected in vivo or in vitro on cells from primary or metastatic melanomas. In an in vitro system that sequentially transformed melanocytes in defined steps, loss of DPPIV expression occurred concomitantly with the emergence of growth factor independence (13, 14).

Despite these correlative observations both in vivo and in vitro, a role for DPPIV in regulating the malignant phenotype has not been shown. We report that reexpression of DPPIV in human melanoma cells at levels comparable to those found in normal melanocytes produced profound phenotypic changes. These included abrogation of tumorigenicity, reemergence of requirements for exogenous growth

[1]*Abbreviations used in this paper:* dox, doxycycline; DPPIV, dipeptidyl peptidase IV; FAPα, fibroblast activating protein α; TRP, tyrosinase-related protein; TUNEL, TdT-mediated dUTP-biotin nick-end labeling.

factors to maintain cell survival, and removal of a block in cell differentiation. Using a point mutation in the active serine protease domain of DPPIV, we observed that serine peptidase activity was required for most effects but not for cell survival. Reexpression of DPPIV rescued expression of a second putative surface peptidase, fibroblast activation protein α (FAPα) (15), suggesting that expression of this second molecule contributes to effects on cell survival in malignant cells.

Materials and Methods

Wild-Type and Mutant Plasmid Constructs. Tetracycline-inducible expression vectors pUHG16-3 and pUHD172-1neo were provided by Hermann Bujard (Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany) (16). The plasmid pUHG16-3 has a cytomegalovirus minimal promoter fused to a tetracycline operator (teto). Transcription is activated by the reverse tet repressor in the presence of tetracycline or doxycycline (dox). pUHD172-1neo has a neomycin resistance gene and reverse tetracycline-controlled transactivator (16). Full length cDNA (2.3 kb) of human DPPIV was amplified by PCR and subcloned into the XbaI site of pUHG16-3 to create pDPPIV. The DNA sequence was identical to the human DPPIV sequences, available from EMBL/GenBank/DDBJ under accession number M74777. The orientation of the insert was confirmed by DNA sequencing and restriction enzyme digests. Mutant DPPIV (pmuDPPIV, producing amino acid substitution of alanine for serine at codon 630, was constructed using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, Inc.). The oligonucleotide primers used for site-directed mutagenesis were 5′-GCA ATT TGG GGC TGG GCA TAT GGA GGG TAC-3′ and 5′-GTA CCC TCC ATA TGC CCA GCC CCA AAT TGC-3′. Mutants were identified by DNA sequencing.

Establishment of Human Melanoma Cells Expressing DPPIV. Human melanoma cells and melanocytes were established and cultured as described (17–19). Human melanoma cell lines MEL-22a, SK-MEL-28, and SK-MEL-29 were cotransfected with plasmid pUHD 172-1neo and empty vector pUHG16-3, pDPPIV, or pmuDPPIV. Lipofectamine reagent was used for transfections as described by the manufacturer (GIBCO BRL Life Technologies).

Immunofluorescence Microscopy and Flow Cytometry. Cells were grown on chamber slides (Nunc, Inc.) and then stained with mAb S27 (4 μg/ml) against DPPIV or mAb TA99 against $gp75^{TRP\text{-}1}$ and incubated with FITC-conjugated rabbit anti–mouse IgG (DAKO Corp.). Stained cells were viewed with a Nikon Optiphot microscope. Flow cytometry was performed using FACScan™ (Becton Dickinson). Cells were stained with S27 mAb or F19 mAb (anti-FAPα) (20) and FITC-conjugated rabbit anti–mouse IgG.

Immunoprecipitation and Western Blot Analysis. For immunoprecipitation assays (21), cells were cultured in medium containing [^{35}S]methionine (NEN Dupont) for 18 h, and cell lysates were precipitated with anti-DPPIV mAb S27. Western blot analysis was performed as described (21) using rabbit PEP7H antibody against human tyrosinase (a gift of Vincent Hearing, National Institutes of Health [NIH], Bethesda, MD).

Dipeptidyl Peptidase Enzyme Activity, In Situ Apoptotic Cell Detection by TdT-mediated dUTP-Biotin Nick-End Labeling Assay, Cell Cycle Analysis, Anchorage-independent Studies, and Growth Curves. DPPIV peptidase activity was measured by colorimetric assay (22). In brief, cells expressing DPPIV in the presence (2 μg/ml for 2–4 d) or absence of dox were suspended in lysis buffer containing 0.5% CHAPS (3-[3-cholamidopropyl]dimethyl-ammonio-1-propanesulfonate). Untransfected and vector-transfected cells were used as controls. 30 μl of cell lysates was incubated with 10 μl of 10 mM substrate, Gly-Pro *p*-nitroanilide (Sigma Chemical Co.), at 37°C for 30 min. Reactions were stopped with 250 μl of 10% TCA, and the supernatants were mixed with 250 μl of 0.1% $NaNO_2$ and incubated at room temperature for 3 min followed by addition of 250 μl 0.5% ammonium sulfamate. At the end of a 2-min incubation, 500 μl of 0.05% *N*-(1-naphthyl)ethylenediamine was added, and *p*-nitroaniline release was measured at 540 nm. Peptidase activities were standardized based on protein concentration and also on cell number. Protein concentrations were measured by the Bradford assay using the BioRad DC protein assay kit. Specific activities were expressed as picomoles per microgram protein per minute.

For apoptosis assays, cells were grown in plain RPMI medium without serum for 3, 8, or 15 d. TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay was performed using the APOPTAG kit (Oncor, Inc.). Percent apoptosis was calculated by FACScan™ (Becton Dickinson). Evidence of apoptosis and percent of cells in each phase of the cell cycle was analyzed by CellFIT and PC-LYSIS™ software (Becton Dickinson).

Growth curves were determined as described (18). In brief, cells were plated at a density of 10^4 cells per well in triplicate in 24-well plates. Every 3 d, cultures were refed with fresh media. Cells were trypsinized daily for 10–12 d and stained with trypan blue, and viable cells were counted. Time of doubling was determined from a least squares regression fit of cell number versus time during the logarithmic growth phase.

Colony formation was performed in soft agar. In brief, the top layer, consisting of 5,000 viable cells suspended in 0.3% agarose and RPMI 1640 with 20% FCS, was overlaid on a 1% agarose layer in 35-mm culture plates. 14 d after seeding, colonies ≥200 μm in diameter were counted under a light microscope. The data are presented as the mean of triplicate plates.

Tumor Growth In Vivo. Nude mice (*nu/nu*, BALB/c) were injected subcutaneously with 3×10^6 cells (either MEL-22a or SK-MEL-29) expressing mutant or wild-type DPPIV and control cells. Five to six animals were used for each group. The tumors were measured every 2–3 d along the greatest diameter. All mouse experiments were performed under protocols approved by the Institutional Animal Care and Utilization Committee of Memorial Sloan-Kettering Cancer Center according to NIH animal care guidelines.

Results

Establishment and Characterization of Melanoma Cells Expressing DPPIV. To define a possible functional role of DPPIV in melanocytic cells, we established melanoma cells that expressed DPPIV in an inducible manner using tetracycline-inducible vectors. Three human melanoma cell lines, MEL-22a, SK-MEL-28, and SK-MEL-29, derived from metastatic lesions of different patients, were selected for study. These melanoma cell lines are representative of more than 150 melanoma cell lines that we have tested that do not express detectable DPPIV glycoprotein (reference 2 and our unpublished data). In addition, the growth and differentiation of these three melanoma lines have been well characterized (17, 18). These cell lines represent different

Table I. *Characteristics of Human Melanoma Cells Expressing Wild-Type and Mutant DPPIV*

Cell line	No. clones tested	Morphology	Pigmentation
MEL-22a			
Parental	−	Short spindle/epithelioid	−
Vector	6	Short spindle/epithelioid	−
wtDPPIVhi	3	Long spindle/bipolar	+++
wtDPPIVmed	2	Long spindle/bipolar	++
wtDPPIVlow	2	Short spindle	−
mut DPPIV	3	Short spindle/epithelioid	−
SK-MEL-28			
Parental	−	Long spindle/bipolar	−
Vector	4	Long spindle/bipolar	−
wtDPPIV	3	Polydendritic	+++
mutDPPIV	3	Long spindle/bipolar	−
SK-MEL-29			
Parental	−	Long spindle/bipolar	−
Vector	4	Long spindle/bipolar	−
wtDPPIV	2	Polydendritic	++
mutDPPIV	3	Long spindle/bipolar	−

Morphology nomenclature is described in the text (references 17, 18). Pigmentation: −, no detectable pigmentation observed in cell pellet (SK-MEL-28 and SK-MEL-29 had pigment detectable only by melanin assays; 18). +, ++, and +++, lighter, medium, and darker pigmentation, respectively (with +++ equivalent to pellet of wtDPPIV in Fig. 4 A and ++++ equivalent to the deeply pigmented pellet observed for melanocytes in Fig. 4 B).

stages of melanocyte/melanoma differentiation (17). They are either completely nonpigmented with a phenotype that corresponds to an immature stage of melanocyte differentiation (MEL-22a) or minimally pigmented with a phenotype of an intermediate stage of melanocyte differentiation (SK-MEL-28 and SK-MEL-29; reference 17 and 18; Table I).

Because we had trouble isolating stable transfectants expressing DPPIV using constitutive vectors, we used an inducible vector system. Melanoma cells were cotransfected with a neomycin-resistant regulator plasmid and tetracycline-inducible vector carrying: (a) the full length wild-type (wt)DPPIV cDNA, (b) a mutant (mut)DPPIV having minimal serine protease activity (substitution of alanine for serine at codon 630 altering the catalytic domain), or (c) a control empty vector (16). Multiple clones that expressed each cDNA construct were isolated for each cell line (Tables I and II). Transfected clones expressing empty vector and mutDPPIV were always relatively easy to derive, and at least three clones were established for each melanoma cell line. Transfected clones expressing wtDPPIV were more dif-

Table II. *Range of DPPIV Enzyme Activity*

	Parental		Vector		WtDPPIV		MutDPPIV	
dox:	−	+	−	+	−	+	−	+
Melanoma line								
	pM/min/μg protein (no. clones tested)							
MEL-22a	10–30	10–30	10–30	10–30 (6)	20–60	60–310 (7)	20–40	20–60 (3)
SK-MEL-28	10–30	10–30	10–30	10–30 (4)	20–60	240–300 (3)	20–40	20–60 (2)
SK-MEL-29	10–20	10–20	10–20	10–20 (4)	20–40	180–220 (2)	20–60	20–60 (3)

Parental or transfected melanoma cell lysates were tested for DPPIV activity as described in Materials and Methods.

ficult to establish, suggesting that DPPIV expression affected cell survival or growth. Despite this difficulty, seven different clones expressing low, medium, and high levels of DPPIV were selected from MEL-22a transfectants, and two clones each were isolated for SK-MEL-28 and SK-MEL-29.

DPPIV expression was assessed by three methods: (a) immunofluorescence staining, (b) immunoprecipitation from metabolically labeled cells, and (c) enzymatic activity. Fig. 1 shows DPPIV expression in representative clones of MEL-22a transfectants, with or without induction by dox. DPPIV cell surface expression was substantially induced by dox (Fig. 1 A). Melanoma cells transfected with wtDPPIV and mutDPPIV expressed the expected 110–120-kD glycoprotein, showing that both the wild-type and mutant polypeptides were processed and expressed appropriately (Fig. 1 B). Furthermore, these results showed that mutDPPIV was stable and expressed at levels comparable to those of wtDPPI. A weak 110-kD band was detected in parental cells and cells transfected with empty vector upon long exposure of autoradiographs (Fig. 1 B), suggesting that melanoma cells can express very low levels of endogenous DPPIV.

Transfected clones and parental melanoma cells were assessed for DPPIV enzymatic activity (Table II and Fig. 1 C). Parental and vector control–transfected cells expressed $\leq$30 pM/min/μg protein of DPPIV activity, which we believe represents very low endogenous DPPIV activity. In

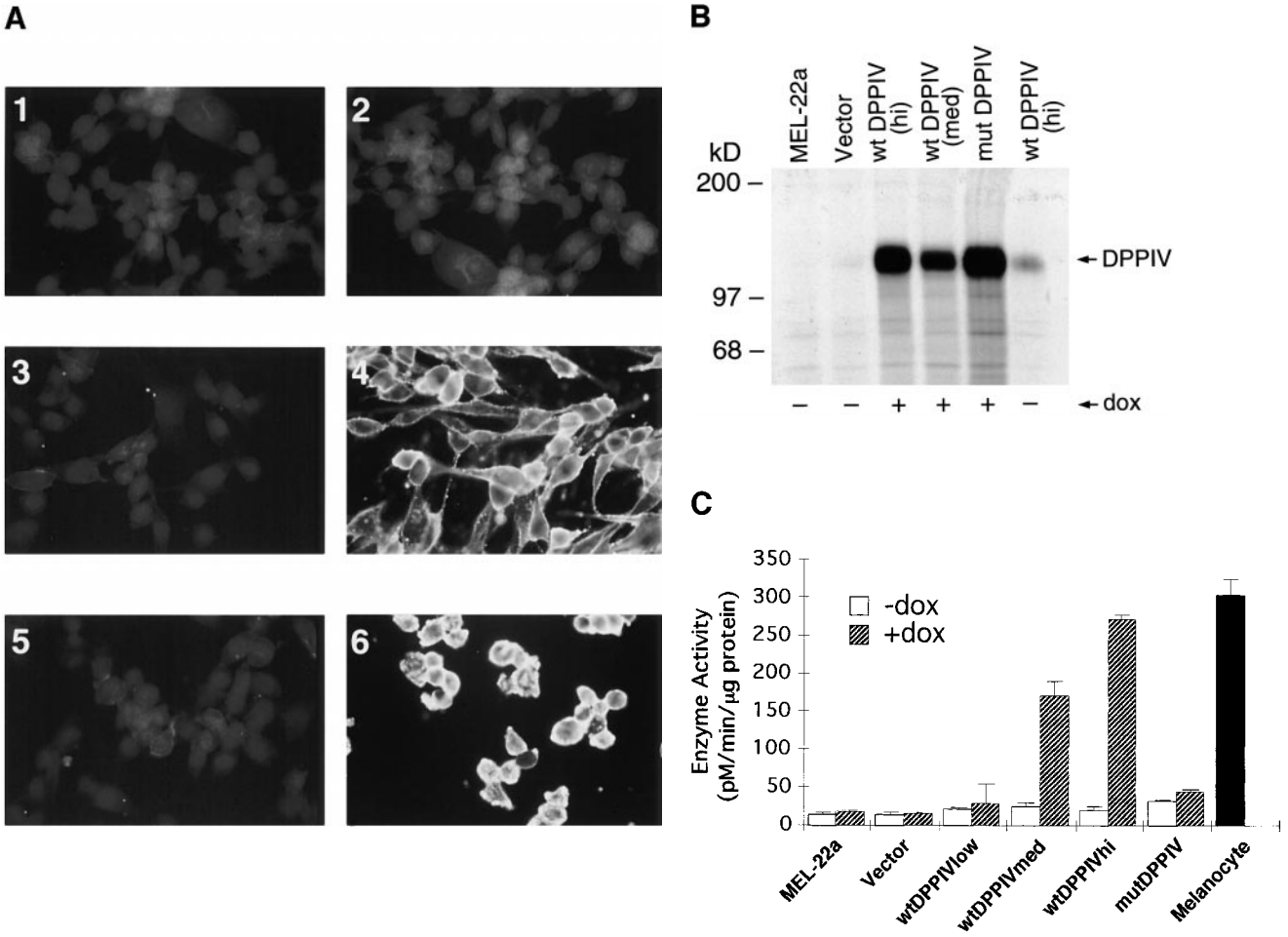

Figure 1. Expression of DPPIV in transfected melanoma cells. (A) Immunofluorescence microscopy showing expression of DPPIV. MEL-22a cells were cultured in the presence or absence of dox (2 μg/ml) for 48 h and stained with S27 mAb against DPPIV. Groups include untransfected MEL-22a cells (panel 1) and control vector–transfected cells (panel 2); cells transfected with wtDPPIV and grown in the absence of dox (panel 3) or induced with dox for 48 h (panel 4); cells transfected with mutDPPIV grown in the absence of dox (panel 5) or induced with dox for 48 h (panel 6). Original magnification 400. (B) Immunoprecipitation analysis of DPPIV expression in MEL-22a cells transfected with wt- or mutDPPIV. Cells were labeled with [^{35}S]methionine for 18 h, lysed with 1% NP-40, immunoprecipitated with mAb S27 against DPPIV, analyzed by 9% SDS-PAGE, and visualized by autoradiography. The first and second lanes (from left) are untransfected MEL-22a and vector-transfected MEL-22a controls, respectively. The third and fourth lanes are two separate clones of transfected MEL-22a cells expressing either high (hi) or intermediate (med) levels of wtDPPIV when grown in the presence of dox. The fifth lane is MEL-22a cells transfected with mutDPPIV grown in the presence of dox. The sixth lane shows wtDPPIVhi-transfected MEL-22a clone grown in the absence of dox. Arrow, 110–120 kD band of DPPIV. −, not induced or +, induced with dox. (C) DPPIV activity in MEL-22a clones transfected with wtDPPIV, mutDPPIV, or control vector and in cultured normal foreskin melanocytes. Three different clones of MEL-22a cells transfected with wtDPPIV were analyzed expressing low (wtDPPIVlow), intermediate (wtDPPIVmed), and high (wtDPPIVhi) levels of enzyme activity. Open bars (−dox), enzyme activities in absence of dox; hatched bars (+dox), enzyme activity after induction with dox. Results shown are mean values ± 1 SD of triplicates.

the absence of dox, DPPIV activity in DPPIV-transfected melanoma cells was ≤60 pM/min/μg protein. Peptidase activity of melanoma cells induced to express high levels of wtDPPIV in the presence of dox was 220–310 pM/min/μg protein (Table II and Fig. 1 C). This level was comparable to that of melanocytes (300–350 pM/min/μg protein; range from three distinct assays; Fig. 1 C). Despite high expression of mutDPPIV protein (Fig. 1, A and B), melanoma cells expressing mutDPPIV exhibited low levels of enzyme activity even in the presence of dox (≤60 pM/min/μg protein; Table II and Fig. 1 C). Transfected MEL-22a clones were isolated that expressed high (hi), medium (med), and low DPPIV activity for more detailed studies to compare phenotype and level of DPPIV expression (Fig. 1, B and C).

In summary, levels of DPPIV expression were consistent across the three assays, showing that steady-state level of protein expression corresponded to wtDPPIV enzymatic activity. As expected, there was low DPPIV enzyme activity in cells expressing mutDPPIV. Results of DPPIV activity in transfected melanoma lines SK-MEL-28 and SK-MEL-29 showed levels similar to those of MEL-22a (Table II). These results showed that: (a) the maximum level of DPPIV activity in transfected melanoma cells did not exceed levels expressed by cultured normal melanocytes (either normalized to protein concentration or when calculated on a per-cell basis), (b) dox induced DPPIV expression fivefold or more, and (c) mutDPPIV expressed minimal or no enzyme activity.

Inhibition of Tumorigenicity by Expression of DPPIV. Tumorigenicity of melanoma cells expressing wtDPPIV or mutDPPIV was compared with that of control melanoma cells. Nude mice were injected subcutaneously with transfected and control MEL-22a or SK-MEL-29 melanoma cells (parental SK-MEL-28 melanoma cells do not form tumors in immune-compromised mice). Parental and control vector melanoma cells formed progressive tumors in all mice. Fig. 2, A and B shows results from two different experiments for MEL-22a, and Fig. 2 C shows results for SK-MEL-29. Tumorigenicity was essentially ablated in MEL-22a cells when DPPIV was induced to levels expressed by normal melanocytes (wtDPPIVhi). Mice showed no progression of tumors over 100 d (Fig. 2, A and B), although viable tumor cells remained after 100 d (data not shown). Similar results were observed with SK-MEL-29 expressing wtDPPIV (Fig. 2 C). Tumor growth was also reduced in melanoma cells expressing medium levels of DPPIV, although not as profoundly as for high levels of DPPIV (Fig. 2, A and C). Transfected MEL-22a melanoma cells expressing low levels of DPPIV, either in the absence of induction of DPPIV (wtDPPIVhi [−dox], Fig. 2 A) or constitutively (DPPIVlow [+dox], Fig. 2 B), showed slightly reduced tumor growth, perhaps due to either low levels of DPPIV activity or recruitment of FAPα, which forms a heterodimer with DPPIV (as discussed below). Melanoma cells expressing high levels of mutDPPIV formed tumors at variable rates, with some mice showing inhibition of tumor growth (note error bars in Fig. 2, A and C for mutDPPIV). These results were consistent with a requirement of DPPIV serine peptidase activity for complete inhibition of tumorigenicity. However, inconsistent inhibition of tumorigenicity in melanoma cells expressing mutDPPIV suggested that some effects of DPPIV on in vivo tumor growth were possibly independent of DPPIV serine peptidase activity.

Inhibition of Anchorage-independent Growth by Expression of DPPIV. Another characteristic of malignant cells is anchorage-independent growth. Expression of DPPIV led to a marked decrease in the ability of MEL-22a melanoma cells to grow in soft agar. DPPIV expression inhibited colony-forming ability by ~75% in MEL-22a cells, with little inhibition of MEL-22a cells expressing mutDPPIV compared with parental and vector control cells (Fig. 3). Thus, serine peptidase activity was required to decrease anchorage-independent growth.

Phenotypic Changes of Melanoma Cells Expressing DPPIV. Marked morphological changes were observed in melanoma cells expressing wtDPPIV (Fig. 4 and Table I). Parental MEL-22a melanoma cells and cells transfected with control vector or mutDPPIV were a disorganized array of epithelioid, polygonal, and short bipolar spindle-shaped cells and grew in piled colonies without any apparent organization (Fig. 4 A). Cells expressing medium or high levels of wtDPPIV were consistently long bipolar spindle shaped, with organized growth behavior and sheet-like appearance, suggesting organization by cell–cell contact (Fig. 4 A). SK-MEL-28 and SK-MEL-29 cells also changed morphology from the long spindle shape of parental-, control vector–, and mutDPPIV-transfected cells to a more mature polydendritic shape of cells expressing wtDPPIV (Table I). The polydendritic shape is characteristic of well-differentiated melanoma cells (17, 18).

We have previously shown that MEL-22a cells have a block in differentiation associated with a nonpigmented, immature melanocytic phenotype (18). Five MEL-22a clones expressing medium and high levels of DPPIV were pigmented when grown to confluence (Fig. 4 B and Table I). Three clones expressing mutDPPIV had no pigment. None of the six clones with control vector nor parental cells were pigmented (Fig. 4 B and Table I). Differentiation of melanocytic cells is characterized not only by appearance of pigmentation but by expression of melanosome membrane glycoproteins involved in melanin metabolism. The best characterized glycoproteins are members of the tyrosinase family, including tyrosinase and tyrosinase-related proteins (TRP). Expression of wtDPPIV, but not mutDPPIV, correlated with a markedly increased expression of human tyrosinase (Fig. 4 C). Expression of wtDPPIV (but not mutDPPIV) was also associated with de novo expression of the *brown* locus protein, $gp75^{TRP\text{-}1}$ (23), measured by indirect immunofluorescence staining (data not shown). Expression of $gp75^{TRP\text{-}1}$ and upregulation of tyrosinase protein occur at a later stage in melanocyte differentiation, confirming that the tyrosinase low/$gp75^{TRP\text{-}1}$–negative MEL-22a had differentiated. Induction of pigmentation associated with expression of DPPIV was also observed in the two other melanoma cell lines, SK-MEL-28 and -29 (Table I). These observations show that DPPIV expression is associated with a relief of the block in differentiation of melanoma cells.

Growth Characteristics of Melanoma Cells Expressing DPPIV. Expression of DPPIV did not affect growth of MEL-22a

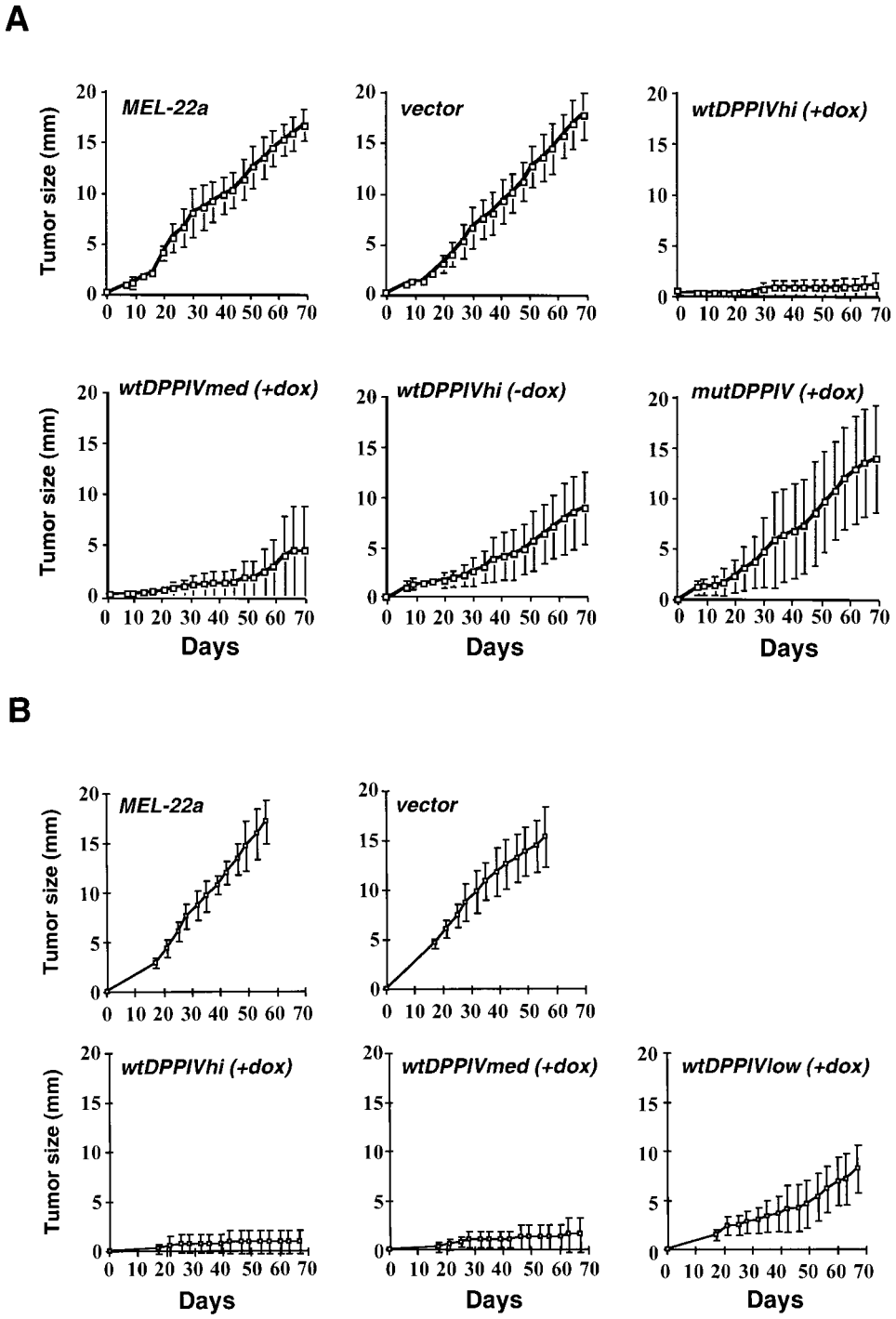

Figure 2.

cells during the logarithmic growth phase. The doubling time of cells expressing high wtDPPIV, mutDPPIV, and control vectors was 36–38 h and was exactly the same as for parental MEL-22a in culture media containing serum (36 h). However, melanoma cells expressing medium and high levels of wtDPPIV had a much longer lag period after plating before they entered the logarithmic growth phase (4–5 d) compared with parental cells and melanoma cells expressing mutDPPIV and control vectors (1–2 d). Also, growth of wtDPPIV cells was inhibited when cells reached a confluent state, whereas parental melanoma cells and cells expressing mutDPPIV and control vector continued to grow and pile up after reaching confluency (Fig. 4 A). Thus, the total cell number of wtDPPIV cells was decreased by 40% compared with control melanoma cells or mutDPPIV cells on days 10–14 after plating. This was due to the delay be-

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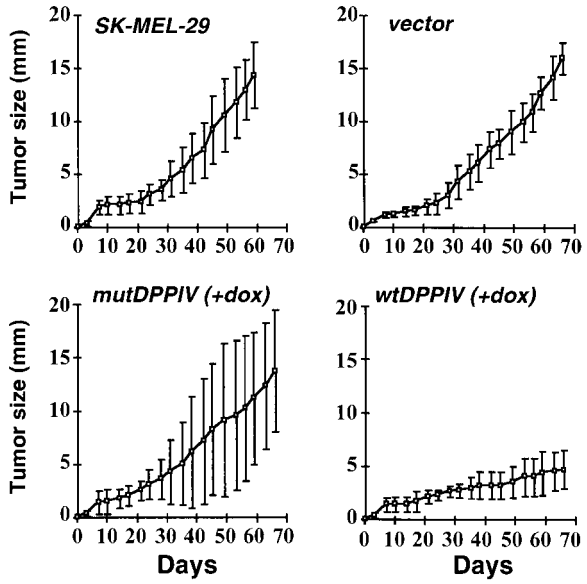


Figure 2. Effects of DPPIV expression on tumorigenicity. (A) Expression of wtDPPIV was associated with inhibition of tumor growth of melanoma cells MEL-22a in nude mice. Six different sets of nude mice (BALB/C *nu/nu*, $n = 5$–6 for each group) were challenged subcutaneously on day 0 with 3×10^6 cells, either parental MEL-22a or transfected with wtDPPIV (with medium or high levels of expression induced by dox as presented in Fig. 1), mutDPPIV, or control vector. Cells were induced (+dox) or not induced (−dox) with dox for 7 d before tumor challenge. Untransfected (MEL-22a) or vector-transfected cells were used as controls. Tumor diameters were measured every 2–3 d. Results are presented as mean tumor diameter ± 1 SD. (B) A repeat of the experiment shown in A with 5–6 mice per group challenged with MEL-22a melanoma cells. The procedure was the same as in A. (C) Tumorigenicity of SK-MEL-29 melanoma cells, including parental SK-MEL-29 cells and cells transfected with wtDPPIV, mutDPPIV, or control vector. 5×10^6 tumor cells were injected subcutaneously on day 0.

fore entering logarithmic growth but also perhaps to inhibited growth upon reaching confluency. Thus, wtDPPIV expression did not affect log growth of MEL-22a cells but did slow entry into the rapid growth phase and appeared to induce some level of growth inhibition at cell confluency. The difficulty in initiating growth might explain in part the difficulty in establishing transfected clones of melanoma cells expressing wtDPPIV.

Apoptosis and Block of Cell Cycle in Serum-Free Conditions Induced by DPPIV Expression. Transformed cells are typically released from dependence on exogenous growth factors for survival during tumor progression (24). This characteristic applies to melanoma cells, which have been shown to survive and grow in serum-free culture medium without addition of exogenous growth factors, whereas normal melanocytes die over 7–14 d when serum is withdrawn (25, 26). We had previously shown that loss of DPPIV expression was associated with acquisition of growth factor independence during in vitro transformation of melanocytes (14, 13). This observation demonstrated a correlation between DPPIV expression and a requirement for exogenous growth factors for survival. We investigated this possible link by growing transfected and parental melanoma cells in serum-free conditions.

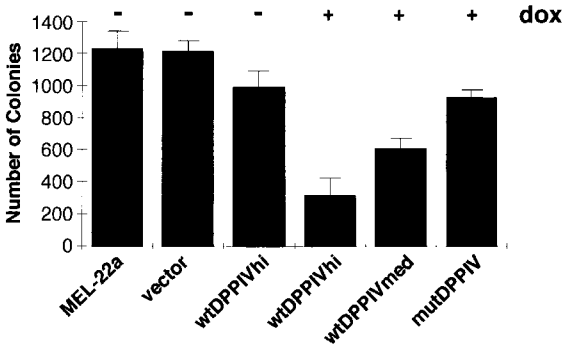
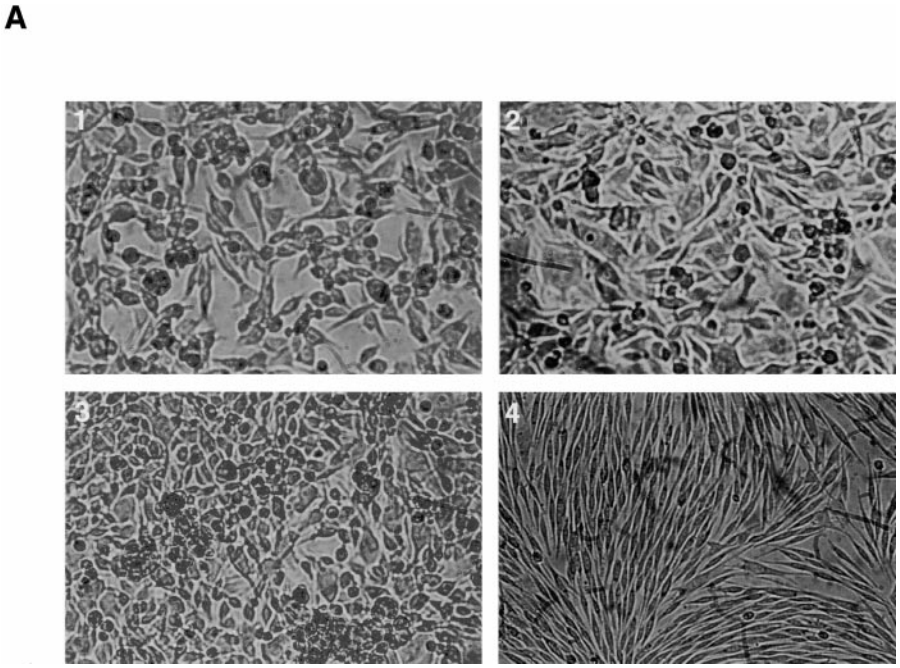
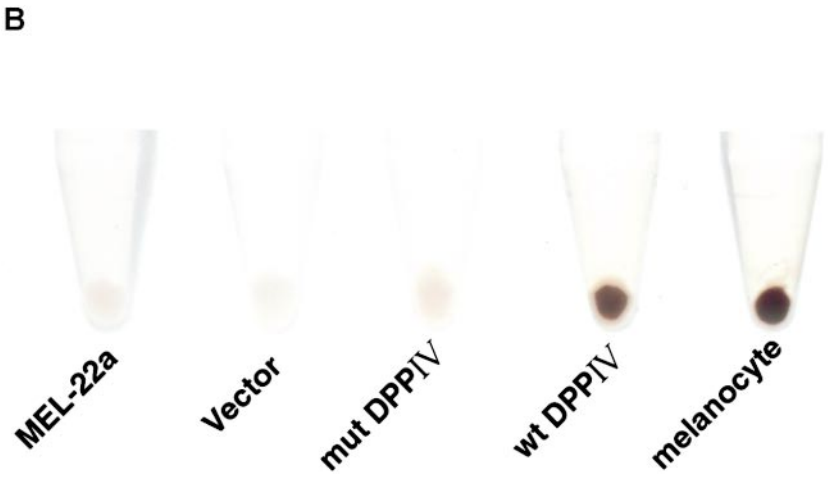


Figure 3. Anchorage-independent growth in soft agar. MEL-22a cells described in Fig. 2 A were cultured in the absence (−) or presence (+) of dox for 48 h, and 5,000 viable cells were plated in agar in triplicate as described in Materials and Methods. Results are mean number of colonies ± 1 SD at 14 d. Results are pooled from two separate experiments.

WtDPPIV, mutDPPIV, and control MEL-22a cells were serum starved with or without induction of DPPIV by dox. Parental and vector control cells grew in serum-free media with only low levels of detectable apoptotic cell death (~2–3% of cells showed DNA fragmentation by TUNEL assay over 15 d) (Table III). A minor population of transfected melanoma cells not induced for wtDPPIV cells demonstrated cell death in serum-free conditions (21% of cells at 15 d; (Table III). However, cells induced to express either wtDPPIV or mutDPPIV with dox showed a marked, progressive loss of cell viability; the proportion of apoptotic cells was 15–18% at day 3, 45–53% at day 8, and 62–78% at day 15 (Table III). Similar results were observed with SK-MEL-29 cells. Only 8% of control vector–transfected cells were apoptotic 8 d after serum withdrawal compared with 52% of cells expressing wtDPPIV.

The same set of cells was analyzed for cell cycle progression in serum-free conditions (Fig. 5). wtDPPIVhi expression induced a cell cycle arrest at the G0/G1 phase, with 62–76% of the cells present in the G0/G1 stage by day 8 compared with only 5–12% of control vector and parental cells in the G0/G1 (range of percentages from duplicates of two experiments). Interestingly, cell cycle arrest at the G0/G1 stage was detected in 32% of cells expressing mutDPPIV, intermediate between wtDPPIV and melanoma cells not expressing DPPIV. These results with mutDPPIV suggest ei-





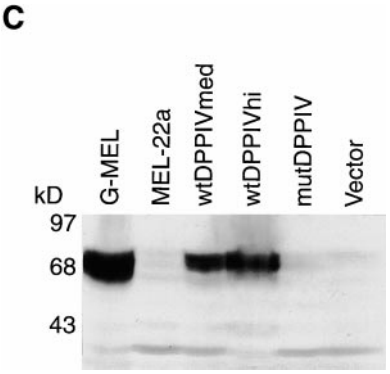


Figure 4. Phenotypic changes associated with DPPIV expression. (A) Morphology of MEL-22a clones. Untransfected (panel 1) and control vector–transfected cells (panel 2) showed short spindle-shaped and polygonal morphology and grew in unorganized clusters. MutDPPIV-transfected cells (panel 3) were morphologically similar to control cells. WtDPPIV-transfected cells (panel 4) showed long spindle bipolar morphology with more organized growth and sheet-like appearance. Original magnification 200. (B) Pigmentation of MEL-22a clones in cell pellets. Untransfected and control vector–transfected MEL-22a cells were not melanotic (first and second pellets from left). Minimal pigmentation was observed in mutDPPIV-transfected MEL-22a cells (third pellet). Expression of wtDPPIV led to brown pigmentation (fourth pellet). Dark brown pigmentation of normal foreskin melanocytes is shown in the fifth pellet. (C) Expression of human tyrosinase detected by Western blot analysis. Lysates (1% NP-40) of human melanoma cell line G-MEL or MEL-22a cells, either parental or transfected with wtDPPIV (med and hi), mutDPPIV, or control vector were separated on a 9% SDS-PAGE. After transfer to membrane, tyrosinase was detected as a broad, ~75-kD band using rabbit anti-PEP7H antibody against human tyrosinase. Molecular mass markers are shown, left.

ther that some other function than serine protease activity of DPPIV is involved in apoptosis and cell cycle arrest induced by serum withdrawal or that DPPIV interacts with other molecules that mediate survival and cell cycle effects.

DPPIV Rescues Expression of FAPα. FAPα is a potential cell surface serine protease that is coexpressed with DPPIV by melanocytes. Loss of FAPα expression occurs concomitantly with loss of DPPIV expression during in vitro transformation of melanocytes, and expression is also lost in primary and metastatic melanoma cell lines (14, 15). DPPIV and FAPα can form heterodimers in addition to homodimers formed by DPPIV (15). Reexpression of either wt- or mutDPPIV by MEL-22a melanoma cells induced the cell surface expression of FAPα (Fig. 6). The relative level of surface expression of FAPα corresponded to the level of DPPIV expression, irrespective of wild-type or mutant forms. Thus, DPPIV rescued surface expression of FAPα.

Discussion

Cell surface proteases are generally thought to participate in malignant transformation and cancer progression by facilitating invasion and metastasis. Inhibition of surface proteases can block tumor progression, and aberrant expression can facilitate tumorigenesis (27). However, cell surface proteases may also have the opposite effect, suppressing the malignant phenotype (27). How cell surface proteases play a role in suppressing the malignant phenotype of human cancer without interacting with the extracellular matrix is not well characterized.

Our results show that loss of DPPIV expression is directly implicated in suppressing the malignant phenotype of melanoma cells. A crucial question arising from these studies is how a cell surface peptidase might have such pleiotropic effects on the malignant phenotype of melanoma cells, revers-

Table III. *Apoptosis of MEL-22a Human Melanoma Cells Expressing DPPIV in Serum-free Conditions*

Melanoma line	dox	Day 3	Day 8	Day 15
			% Apoptosis	
MEL-22a parental	−	3.3	2	3.4
Vector	−	2.1	3	2.5
wtDPPIVhi	−	4	18	21
wtDPPIVhi	+	17.5	53	78
wtDPPIVmed	+	15	45	62
mutDPPIV	+	17	50	68

Control (parental and vector-transfected) MEL-22a cells or MEL-22a cells transfected with DPPIV constructs were assessed for apoptosis by TUNEL assay as described in Materials and Methods. Percent apoptotic cells was measured by flow cytometry. dox, induction with doxycycline.

ing tumorigenicity, affecting the differentiation program, and changing decisions about survival without exogenous growth factors. DPPIV has several functions, including serine peptidase activity, binding to extracellular matrix components, and complexing adenosine deaminase (3). Thus, each of these particular functions, presumably handled by different domains of the protein, could contribute to suppression of the malignant phenotype. Serine→alanine mutation did not suppress tumorigenicity or anchorage-independent growth, nor did it reverse the block in differentiation, showing that serine peptidase activity is required for these phenotypic changes. The different contributions of other domains and functions of DPPIV and recruitment of FAPα is yet uncertain. Biochemical and enzymatic studies may give clues to their potential functions.

Reexpression of DPPIV led to apoptotic cell death upon serum withdrawal and cell cycle arrest. Unexpectedly, apoptosis was also observed in cells expressing mutDPPIV, which suggests that the rescue of FAPα as a heterodimer with DPPIV could explain at least part of these proapoptotic effects. At this point, we have no data to support this notion other than this correlative observation. However, consistent with this view, a paralogue of FAPα is induced during tadpole tail resorption, which is essentially a massive program of cell death (28), suggesting that a proapoptotic role of FAPα might be conserved throughout vertebrate evolution. FAPα contains a potential serine protease site, but the functions of FAPα protein, including peptidase activity, are not well characterized. It will be important to determine whether FAPα has serine protease activity and if this function might be important for proapoptotic effects induced by expression of DPPIV. It will also be important to identify downstream components that are involved in decisions about melanoma cell survival and how DPPIV and FAPα participate in these decisions. Pathways for cell survival in melanocytes are not well understood. However, bcl-2 is probably a central mediator of resistance to apoptotic death in melanocytic cells (29, 30), and one speculation is that DPPIV expression might ultimately intersect with bcl-2.

DPPIV expression may play a crucial role in checking cell growth of normal melanocytes. This idea is supported by our observations that loss of DPPIV correlates with growth factor–independent proliferation of melanoma cells (13, 14), as well as the experiments described above. One explanation is that DPPIV degrades growth factors required for survival of melanocytic cells. As our experiments were performed in strict serum-free conditions, the most likely

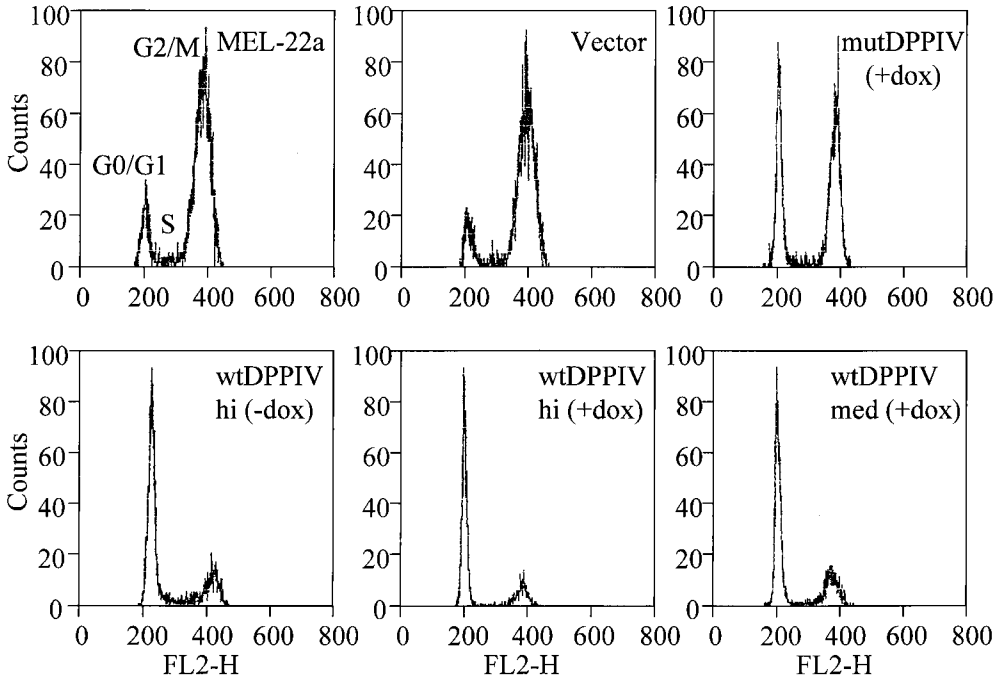


Figure 5. Cell cycle analysis of melanoma cells expressing DPPIV and FAPα. Expression of both wt- and mutDPPIV in MEL-22a cells was associated with changes in cell cycle. Parental MEL-22a cells and cells transfected with control vector, mutDPPIV, and wtDPPIV were assessed for cell cycle progression after 8 d of culture in serum-free media. Number of cells at each stage of the cell cycle were measured by flow cytometry (first peak was G0/G1, the intervening trough was S phase, and the second peak represented cells in G2/M) and analyzed by CellFIT and PC-LYSIS™ software.

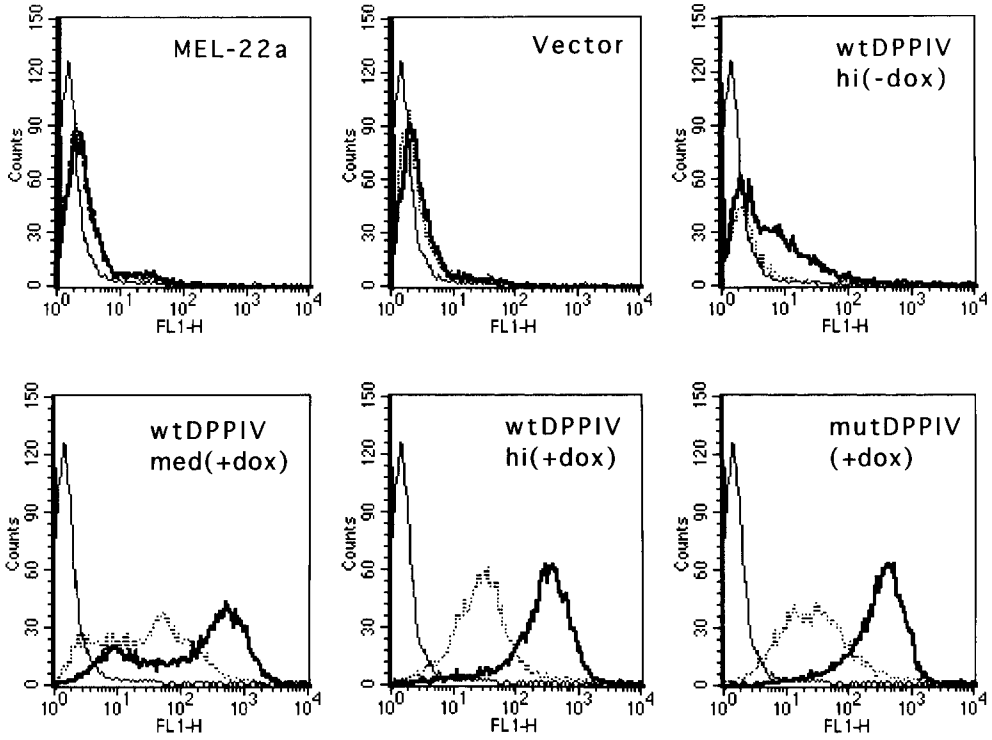


Figure 6. Expression of DPPIV and FAPα. Expression of either wt- or mutDPPIV in MEL-22a cells rescued the expression of FAPα. Immunofluorescence staining and flow cytometry analysis of DPPIV-transfected cells was performed using mAbs against DPPIV and FAPα. Cell clones are described in the legends of Figs. 1 and 2. Y-axis, relative cell number; x-axis, log fluorescence intensity. Shaded curve, control IgG1 antibody; solid line, DPPIV expression; dotted line, FAPα expression.

source of growth factors is autocrine factors secreted by melanoma cells. In prostate cancer, autocrine neuropeptides such as bombesin and endothelin-1 can stimulate the growth of prostate carcinoma cells, and these growth factors are inactivated by the cell surface metallopeptidase, neutral endopeptidase 24.11 (31). Chemokines are potential substrates for DPPIV, including RANTES (regulated on activation, normal T cell expressed and secreted), stromal cell–derived factors 1α and 1β, IP-10 (IFN-γ–inducible protein 10), monocyte chemotactic proteins 1, -2, and -3, and GCP-2 (granulocyte chemotactic protein 2) (32–36). In addition, regulatory peptides, including glucagon-like peptide 1 and 2, neuropeptide Y, and peptide YY are DPPIV substrates (37, 38). It is uncertain whether chemokines or regulatory peptides could be involved in maintaining the malignant phenotype of melanoma or whether other substrates of DPPIV are involved. It will be important to identify substrates of DPPIV that are made as autocrine factors by melanoma cells.

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