A Marker for Neoplastic Progression of Human Melanocytes Is a Cell Surface Ectopeptidase

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Summary

Adenosine deaminase binding protein (ADAbp) is a cell surface glycoprotein that is expressed by normal melanocytes but not by melanoma, the malignant counterpart. ADAbp is specifically downregulated during malignant transformation of melanocytes. Recently, we have developed a system that progressively transforms melanocytes in vitro in defined steps. Transduction with v-Ha-*ras* oncogene followed by long-term culture leads to a cell phenotype and genotype that specifically mimics human melanoma. Loss of ADAbp expression occurred concomitantly with the emergence of growth factor independence and appearance of specific chromosomal abnormalities. The cellular function of ADAbp has not been defined. To characterize ADAbp, the mature 110-kD form was purified from human kidney. Five tryptic peptides from purified human ADAbp revealed 100% homology to a serine protease, human dipeptidyl peptidase IV (DPP IV), also known as CD26. DPP IV activity was detected in lysates from human melanocytes and renal carcinoma cells but not melanoma cells, and DPP IV activity could be specifically isolated from melanocytes by binding to ADA or to S27 monoclonal antibody against ADAbp. These findings show that ADAbp is a cell surface ectopeptidase that is tightly regulated during neoplastic transformation of melanocytes.

Melanoma is a neoplasm derived from cells of the melanocyte lineage. Malignant transformation of melanocytes leads to progressive and profound alterations in cell phenotype, manifested by invasion into surrounding tissues, dysregulated growth, and metastasis. Careful clinical and pathological analyses have led to the description of lesional steps in transformation and progression of human melanoma (1). Because of the accessibility of lesions that represent different stages in melanoma evolution, melanoma has become a useful paradigm to study progression of human cancer.

Some of the best characterized phenotypic changes observed during melanoma progression come from the study of antigen expression. A broad assortment of antigens, including intracellular and cell surface, have been defined on melanocytes and melanoma. Most antigens have been detected on both melanocytes and melanoma cells, and are presumably regulated during melanocyte differentiation. Several molecules or epitopes have been identified that discriminate steps in melanoma progression (2, 3). Despite the profound phenotypic alterations that occur during steps of tumor progression, however, it has been difficult to define qualitative changes (i.e., absence vs. presence) that absolutely distinguish normal cells in the melanocyte lineage from melanoma cells. Adenosine deaminase binding protein (ADAbp),[1] a plasma membrane glycoprotein consisting of 110–120-kD subunits (4, 5), is a candidate for a molecule that is specifically regulated during melanocyte transformation and melanoma progression. We have previously found that ADAbp is consistently expressed by melanocytes in vitro and in vivo but cannot be detected on melanoma cells (6). Furthermore, transformation of melanocytes in vitro extinguishes ADAbp expression (6, 7). The present studies were undertaken to further explore the link between ADAbp expression and transformation of melanocytes. We show that ADAbp expression is lost as melanoma growth becomes independent of exogenous growth factors. ADAbp has been purified and shown to be indistinguishable from the cell surface serine protease, dipeptidyl peptidase IV (EC 3.4.14.5) (DPP IV).

Materials and Methods

Reagents. Con A–Sepharose, wheatgerm agglutinin (WGA)-Sepharose, and protein A–Sepharose were obtained from Pharmacia

[1] *Abbreviations used in this paper:* ADAbp, adenosine deaminase binding protein; DPP IV, dipeptidyl peptidase IV; RAMPS, rabbit anti–mouse IgG protein A–Sepharose.

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Fine Chemicals (Piscataway, NJ). Affigel-10, Affigel-15, and electrophoresis molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Rabbit anti-mouse IgG was obtained from Cappel Laboratories (Durham, NC). Rabbit anti-mouse IgG protein A-Sepharose (RAMPS) was prepared from rabbit anti-mouse IgG and protein A-Sepharose. S27 and S4 mouse mAbs were purified from ascites of hybridoma-bearing mice by binding to protein A-Sepharose. mAbs were >99% pure by SDS-PAGE. Bovine adenosine deaminase (ADA) was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). ADA was >90% pure by SDS-PAGE and was used to prepare Affigel affinity columns without further purification. Cell culture reagents were obtained from Gibco Laboratories (Grand Island, NY). Polyvinylidene difluoride membrane (PVDF, Immobilon-P) was obtained from Millipore (Bedford, MA). Nitrocellulose membrane was obtained from Scheicher & Schuell, Inc. (Keene, NH). [^{3}H]Glucosamine was obtained from New England Nuclear (Boston, MA). Standard reagents and buffers were obtained from Sigma Chemical Co. (St. Louis, MO). Microtest plates were obtained from Robins Scientific (Sunnyvale, CA).

Cell Lines and Tissues. Human melanoma and renal carcinoma cell lines were maintained in Eagle's MEM supplemented with 10% FCS, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Normal human melanocyte cell lines were maintained in Eagle's MEM supplemented with 10% FCS, 0.1 mM nonessential amino acids, 10 ng/ml PMA, 1 μg/ml cholera toxin, and 10 μM isobutylmethylxanthine. 10W *ras*/early and 10W *ras*/late melanocyte cell lines were generated by infection of a human foreskin melanocyte cell line, designated 10W, at passage 3 with murine retrovirus carrying v-Ha-*ras* and pseudotyped with the amphotropic murine leukemia virus 4070A as previously described (8). Cells were passaged with 0.1% trypsin and 0.02% EDTA. Normal human renal tissue was obtained at the time of surgery from patients requiring nephrectomy for renal carcinoma. Tissue was stored at −70°C.

Serologic Assays and Radioimmunoprecipitation. Assay of surface membrane ADAbp was performed by mixed hemadsorption as previously described (6). Indicator cells were prepared by conjugation of rabbit anti-mouse IgG to human red blood cells with 0.01% (wt/vol) chromium chloride. Assays were performed on cells grown in microtest plates. Target cells that were plated 2–3 d earlier were incubated with serial dilutions of mouse mAb S27 for 1 h at room temperature. Target cells were washed, and indicator cells were then added for 30 min. Unbound indicator cells were removed by washing, and the percentage of target cells exhibiting red cell rosettes was scored by light microscopy.

Cell labeling, immunoprecipitation, and gel electrophoresis were carried out as previously described (6). Cells were labeled with [^{3}H]glucosamine over 72 h in glucose-free Eagle's MEM containing 7.5% dialyzed FCS. Lysates were immunoprecipitated with mAb S27 followed by 5 ng/ml protein A-Sepharose. Immunoprecipitates were analyzed by SDS-PAGE.

Affinity Purification of ADAbp. mAb S27 and ADA were coupled to Affigel-10 and Affigel-15, respectively, according to instructions of the manufacturer. BSA was coupled to Affigel-15 for use as a control in experiments with ADA-Affigel. Protein coupling was monitored by the absorbance of acidified aliquots (pH 1) at 280 nm. In preparations used for bulk purification of ADAbp, protein was coupled to Affigel at a concentration of 5–6 mg/ml of gel.

Normal renal tissue was dissected free from fat and connective tissue. Tissue was diced and then forced through a fine sieve in the presence of 6 vol of lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The lysate was incubated with gentle agitation for 1 h. All procedures were carried out at 4°C. Debris was removed by centrifugation for 30 min at 2,000 *g*. Solid NaCl was slowly added to a final concentration of 0.5 M while stirring the lysate. A 10× concentrated stock of $CaCl_2$ and $MnCl_2$ was then added (final concentration, 1 mM Ca^{2+} and Mn^{2+}). High salt conditions were used during incubation with Con A-Sepharose to minimize nonspecific binding to the support matrix. The lysate was incubated with 5 ml of Con A-Sepharose for 4 h with gentle agitation. The Con A-Sepharose was transferred to a column and washed with 20 vol of 50 mM Tris, pH 7.4, containing 0.1% NP-40, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 μg/ml aprotinin. The column was then washed with 10 vol of buffer containing 150 mM NaCl. Glycoproteins were batch eluted by incubation for 1 h with 10 ml of 10% α-methyl-mannopyranoside in 50 mM Tris, pH 7.4, containing 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, and 2 μg/ml aprotinin. The eluted material was incubated with mAb S27-Affigel affinity matrix overnight. The Affigel was transferred to a column and washed with 20 vol of high salt buffer (0.5 M NaCl in 50 mM Tris, pH 7.4, containing 0.1% NP-40, 1 mM EDTA, and 1 μg/ml aprotinin), followed by 20 vol of low-salt buffer (150 mM), then with 10 vol of dilute buffer (5 mM Tris, pH 7.4, containing 0.1% NP-40, 150 mM NaCl, and 1 μg/ml aprotinin). Bound proteins were eluted in 0.5-ml fractions of 100 mM glycine buffer, pH 2.5, containing 150 mM NaCl and 2 μg/ml aprotinin. Fractions were collected in 100 μl of 1 M Tris, pH 8.0 (final pH = 7.2). Aliquots of each fraction were analyzed by 7.5% SDS-PAGE under reducing conditions. Gels were silver stained to visualize protein. ADAbp-containing fractions were pooled. An aliquot was set aside for later purification by ADA-Affigel affinity matrix. The remainder of the ADAbp-containing fractions were precipitated by the addition of an equal volume of ice-cold acetone and stored overnight at −20°C. The precipitate was recovered by centrifugation and dissolved in sample buffer for SDS-PAGE. In parallel experiments, renal tissue was lysed and bound overnight to WGA-Sepharose. Bound glycoproteins were eluted with buffer containing 10% *N*-acetyl-glucosamine. Eluted material was incubated with mAb S27-Affigel and processed as above. An aliquot of ADAbp was further characterized by binding to ADA-Affigel. After binding overnight, the Affigel was washed with 30 vol of 50 mM Tris, pH 7.4, containing 0.1% NP-40, 0.5 M NaCl, and 1 mM EDTA, followed by low-salt buffer (150 mM NaCl). ADAbp was eluted with SDS-containing sample buffer and subjected to electrophoresis on a 7.5% polyacrylamide gel under reducing conditions. Proteins were visualized by silver stain.

Because renal tissue was not easily obtained, it was necessary to maximize the yield of ADAbp for use in amino acid analysis and sequence analysis of tryptic peptides. Material that did not bind to lectin under the stringent high-salt conditions described above was further purified by binding to mAb S27-Affigel affinity matrix and pooled with the ADAbp isolated above.

Amino Acid Analysis of ADAbp. An aliquot, representing 10% of the total mAb S27 affinity-purified ADAbp, was subjected to electrophoresis on a 0.75-mm thick 7.5% polyacrylamide gel under reducing conditions. ADAbp was electrotransferred onto Immobilon-P membrane in 10 mM 3-cyclohexylamino-1-propane sulfonic acid (CAPS) buffer, pH 11.0, containing 10% methanol (9). Transfer was complete after 75 min at 50 V (0.5 A). ADAbp was visualized by staining with 0.2% Ponceau S in 1% acetic acid. The ADAbp band was excised, destained in water, and allowed to air dry. Amino acid analysis was performed at the Harvard

Microchemistry Facility (Boston, MA). Protein was hydrolyzed in in 6 N HCl for 24 h at 110°C. Free amino acids were derivatized with phenylisothiocyanate and analyzed by HPLC (10).

Trypsin Digestion, HPLC Separation, and Microsequencing of ADAbp Peptides. ADAbp, affinity purified with mAb S27, was separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. The band at 110 kD (ADAbp) was excised. Subsequent processing was performed at the Harvard Microchemistry Facility. ADAbp was submitted to in situ digestion with trypsin (11), omitting the NaOH wash. The resulting peptide mixture was separated by narrow-bore HPLC using a Vydac C18 2.1 × 150-mm reverse-phase column on a 1090 HPLC/1040 diode array detector (Hewlett-Packard Co., Palo Alto, CA). Optimum fractions from each peptide chromatogram were chosen based on differential UV absorbance at 210, 277, and 292 nm, peak symmetry, resolution, and predictive column retention. Automated Edman degradation was performed on a protein sequencer (477A; Applied Biosystems, Inc., Foster City, CA) by standard methods except that the reaction cartridge temperature was raised to 53°C during coupling with a commensurate decrease in R2 delivery and drydown time. Details of strategies for the selection of peptide fractions and their microsequencing have been previously described (12).

Demonstration of DPP IV Activity. Cells grown in culture were washed five times with PBS, then removed from the culture flasks with a rubber policeman and resuspended in PBS. An aliquot was diluted into trypan blue and counted on a hemocytometer. Cells were collected by centrifugation at 2,000 *g* for 10 min and lysed by incubation at 4°C for 30 min in 50 mM Tris containing 1% NP-40 and 20 μg/ml aprotinin. The volume of lysis buffer was adjusted so that the number of cells per microliter was equal for all samples. Lysates were incubated for 3 h with either 3.75 μg mAb (S27 or S4) or with no additions. A 20-μl pellet of RAMPS was then added to samples containing mAb and to control samples. Samples were incubated overnight at 4°C with rocking. RAMPS pellets were washed five times with cold 50 mM Tris, pH 7.4, containing 1% NP-40, then washed once with cold H_2O. Antibody-antigen complexes were dissociated by rocking for 10 min at room temperature with 100 mM glycine buffer, pH 3.5, containing 0.1% NP-40. Samples were then subjected to electrophoresis on a 7.5% native polyacrylamide gel (SDS and β-mercaptoethanol omitted). Gels were stained for DPP IV activity by a modification of the method of Yoshimoto and Walter (13). Gels were incubated at room temperature for 30 min in a solution of 0.5 mM glycyl-prolyl-4-methoxy-β-naphthylamide and 1.25 mg/ml tetrazotized *o*-dianisidine in 0.2 M Tris, pH 7.4. Positive staining was indicated by an insoluble precipitate formed by the cleaved 4-methoxy-β-naphthylamine and tetrazotized *o*-dianisidine. In other experiments, lysates also were incubated overnight at 4°C with 40-μl pellets of either ADA-Affigel, BSA-Affigel, or with no additions (ADA and BSA were coupled to Affigel-15 at 5–6 mg/ml of gel). Affigel pellets were washed and eluted as above. Samples were subjected to electrophoresis on a 7.5% native polyacrylamide gel and stained for DPP IV activity.

The ability of mAb S27 to bind to the same protein as that bound by ADA-Affigel was tested as follows. Lysates of SK-RC-28 renal carcinoma cells were incubated with either 20 μg of mAb S27 or with no additions for 3 h at 4°C. A 50-μl pellet of RAMPS was then added to each sample. Samples were incubated overnight at 4°C with rocking. The samples were centrifuged to pellet the RAMPS, and the supernatants were recovered. Supernatants from lysates incubated only with RAMPS were then incubated overnight with either ADA-Affigel or with BSA-Affigel. Supernatant that had been preincubated with mAb S27 and RAMPS was incubated overnight with ADA-Affigel. Proteins bound to the RAMPS or Affigel were dissociated with glycine buffer as described above.

Results

Complete Neoplastic Transformation of Melanocytes by v-Ha-ras In Vitro Induces Loss of ADAbp Expression. We have reported that transduction and expression of v-Ha-*ras* oncogene in cultured normal human diploid melanocytes can induce a subset of traits that distinguish melanoma from melanocytes (6, 8). Long-term expression of v-Ha-*ras* results in complete neoplastic transformation, with melanocytes acquiring all the phenotypic and genotypic characteristics observed in malignant melanoma in vivo (7). Transformation occurred in at least two phases and was associated with spontaneous chromosomal instability. We have found that extinction of ADAbp expression by v-Ha-*ras* expression in melanocytes occurred late in the transformation process, and correlated with a subset of traits that included PMA-independent growth, tumorigenicity in *nu/nu* mice, and the development and retention of specific chromosomal markers.

The v-Ha-*ras* oncogene was introduced into a passage 3 human foreskin melanocyte culture, designated 10W, by a murine retrovirus carrying the v-Ha-*ras* oncogene and pseudotyped with amphotropic murine leukemia virus (MuLV) 4070A. In the early phase of growth, 10W *ras*/early cells changed morphology, were hyperploid, and acquired anchorage-independent growth, but remained dependent on the exogenous growth stimulator PMA for proliferation and survival (8). 10W *ras*/early cells continued to express ADAbp (Fig. 1, *A* and *B*), and the level of cell surface expression of ADAbp was similar to 10W parental cells (data not shown). At ~6 mo, foci of cells with distinct morphology appeared in the 10W *ras*/early culture. The 10W *ras*/late cell line, established from these foci, grew independently of exogenous growth factors in the culture medium and acquired specific

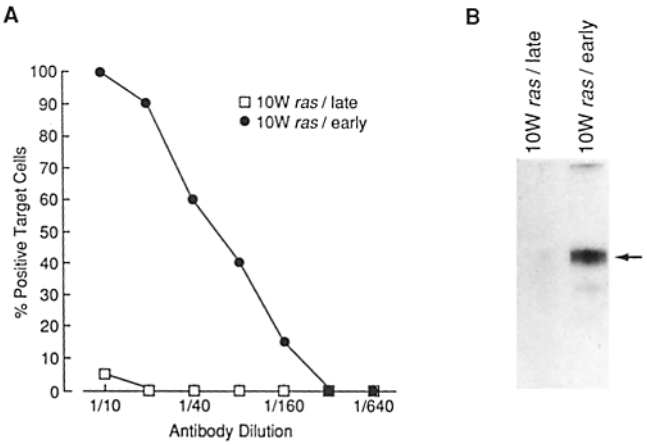

Figure 1. Expression of ADAbp by 10W melanocytes transduced with v-Ha-*ras*. (*A*) Mixed hemadsorption assay of cell surface ADAbp expression using mAb S27 (initial mAb concentration, 30 μg/ml). (*B*) Radioimmunoprecipitation of ADAbp by mAb S27 from lysates of 10W *ras*/early and 10W *ras*/late melanocytes metabolically labeled with [^{3}H]glucosamine.

karyotypic abnormalities frequently detected in human melanomas (i.e., *iso* 6p, *iso* 9q, and *del* 1p) (7). 10W *ras*/late cells did not express ADAbp at the cell surface, and ADAbp synthesis was not detected by metabolic labeling (Fig. 1, *A* and *B*). Thus, in this in vitro model of sequential neoplastic transformation of melanocytes, loss of ADAbp expression corresponded to acquisition of independence from exogenous growth factors and was associated with specific chromosomal alterations.

Purification of Human ADAbp. ADAbp was purified from normal human tissue to determine its identity and possible function. Kidney is the most abundant source of ADAbp, and was used because of difficulty in obtaining large quantities of melanocytes for ADAbp purification. The structure of ADAbp derived from kidney and melanocytes cannot be distinguished: ADAbp from both cell types has identical molecular mass and expresses three distinct epitopes recognized by different mAbs against ADAbp (mAbs S6, S23, and S27) (6, 14, 15).

Initially, an NP-40 lysate was enriched for glycoproteins by lectin affinity purification. This fraction was then affinity purified using mAb S27, directed against ADAbp, coupled to an Affigel support matrix. Proteins eluted from the mAb S27 affinity column included a prominent band present at ~110 kD, corresponding to the known molecular mass of ADAbp (Fig. 2). A second band was present at 132 kD, and a broad region of staining was observed between 97 and 110 kD. Affinity-purified fractions were pooled and further enriched by ADA-Affigel affinity matrix. The 110- and 97–110-kD bands were bound by ADA-Affigel (Fig. 2). Thus, the 110-kD band was characterized as a glycoprotein that bound both to mAb S27 and ADA, and was felt to represent the mature, processed form of ADAbp. The broad band at 97–110 kD also bound to mAb S27 and ADA, and could represent partial breakdown products, intermediate forms, or alternatively processed forms of ADAbp. The 132-kD protein band that copurified with the 110-kD band on mAb S27-Affigel did not bind to ADA. We speculate that the 132-kD band is a distinct protein associated with ADAbp in the cell membrane, although it could represent an alternatively processed form of ADAbp that does not bind to ADA.

Sequences of Tryptic Peptides of ADAbp and Amino Acid Analysis. Five tryptic peptides (totaling 63 amino acids) were sequenced from the 110-kD band of ADAbp. Search of the EMBL/GenBank databases revealed 100% identity (63/63 amino acids) to human CD26, recently reported by Tanaka et al. (16). CD26 is also known as DPP IV (17). There was 87% amino acid homology (55/63 amino acids) to sequences within a cDNA-encoding DPP IV isolated from rat liver (Fig. 3) (18). Peptides corresponded to amino acid positions 259–267, 374–382, 493–522, 598–611, and 659–669 in the putative extracellular domain of human CD26/DPP IV, determined from the deduced polypeptide sequence of human ADAbp described by Tanaka et al. (16).

The 110-kD band was also analyzed for amino acid composition, deduced from the known molecular mass of the ADAbp core polypeptide (85 kD [5]) (Table 1). We compared the amino acid composition of human and rat DPP IV to human ADAbp, revealing nearly identical patterns (Table 1). The 97–110-kD region was also analyzed by excising a band at ~97 kD. The 97-kD band had an amino acid composition that was similar to that of the 110-kD form (Table 1), supporting the identity of core polypeptides for 97- and 110-kD forms. Variations in composition of individual amino acids could be attributed to protein yields. Derivatized amino acids from the 97-kD protein (from 0.2 μg) yielded peaks approximately threefold higher than background noise compared with data generated from higher yields for the 110-kD protein (1.7 μg). For example, glycine yielded a higher value

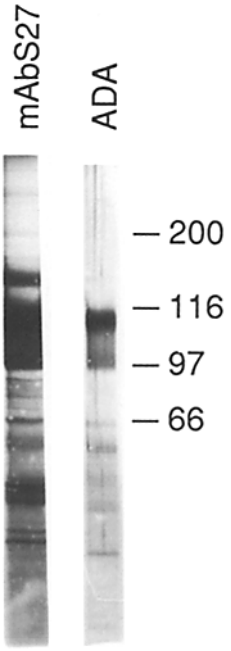

Figure 2. Purification of ADAbp from normal human renal tissue. Lysates, enriched for glycoproteins by lectin affinity purification, were affinity purified using mAb S27 bound to an Affigel support matrix (*left*). Affinity-purified fractions were pooled and further enriched by binding to ADA-Affigel affinity matrix (*right*). Eluted material was subjected to 7.5% SDS-PAGE under reducing conditions. Proteins were visualized by silver stain. ADAbp is seen as a 110-kD band.

1	Human ADAbp:	Ala	Gly	Ala	Val	Asn	Pro	Thr	Val	Lys
	Human DPP IV:	-	-	-	-	-	-	-	-	-
	Rat DPP IV:	-	-	-	-	-	-	-	-	-
2	Human ADAbp:	Ile	Ile	Ser	Asn	Glu	Glu	Gly	Tyr	Arg
	Human DPP IV:	-	-	-	-	-	-	-	-	-
	Rat DPP IV:	Tyr	-	-	-	-	Tyr	Lys	Glu	Met
3	Human ADAbp:	Val	Leu	Glu	Asp	Asn	Ser	Ala	Leu	Asp	Lys-
	Human DPP IV:	-	-	-	-	-	-	-	-	-	-
	Rat DPP IV:	-	-	-	-	-	-	-	-	-	-
	Human ADAbp:	Met	Leu	Gln	Asn	Val	Gln	Met	Pro	Ser	Lys
	Human DPP IV:	-	-	-	-	-	-	-	-	-	-
	Rat DPP IV:	-	-	-	Asp	-	-	-	-	-	-
4	Human ADAbp:	Leu	Gly	Thr	Phe	Glu	Val	Glu	Asp	Gln	Ile
	Human DPP IV:	-	-	-	-	-	-	-	-	-	-
	Rat DPP IV:	-	-	-	Leu	-	-	-	-	Glu	-
	Human ADAbp:	Glu	Ala	Ala	Arg						
	Human DPP IV:	-	-	-	-						
	Rat DPP IV:	-	-	-	-						
5	Human ADAbp:	Trp	Glu	Tyr	Tyr	Asp	Ser	Val	Tyr	Thr	Glu	Arg
	Human DPP IV:	-	-	-	-	-	-	-	-	-	-	-
	Rat DPP IV:	-	-	-	-	-	-	-	-	-	-	-

Figure 3. Amino acid sequence (three-letter code) of five human ADAbp peptides compared with human DPP IV and rat DPP IV. Identical amino acids are indicated by dashes.

Table 1. *Comparison of Amino Acid Composition of Human ADAbp with That of Human 97-kD Band, Human DPP IV, and Rat DPP IV*

	Human ADAbp	Human 97 kD	Human DPP IV	Rat DPP IV
ALA	46	37	40	47
ARG	37	29	30	29
ASN			40	34
ASP			46	46
ASX	113	95		
CYS	ND	ND	12	13
GLN			30	33
GLU			40	41
GLX	82	84		
GLY	53	117	43	40
HIS	23	13	19	21
ILE	39	22	49	46
LEU	57	38	62	63
LYS	48	41	40	44
MET	7	0	15	14
PHE	28	18	31	31
PRO	22	16	29	30
SER	68	79	64	66
THR	60	35	50	50
TRP	ND	ND	21	21
TYR	44	31	56	50
VAL	47	27	49	48

in the 97- than 110-kD protein, presumably due to contamination from glycine in the SDS-PAGE buffer. Methionine, which is only moderately stable during acid hydrolysis, was completely destroyed in the 97-kD protein and >50% was destroyed in the 110-kD protein, based on comparison with derived human DPP IV amino acid sequence.

Purified ADAbp Has Dipeptidyl Dipeptidase IV Activity. DPP IV is a serine protease that cleaves the NH_2-terminal dipeptide X-Pro or X-Ala (19). DPP IV ectopeptidase activity was tested in lysates from human melanocyte and melanoma cell lines and compared to lysates from the human renal carcinoma cell line SK-RC-28. Melanocyte and SK-RC-28 lysates exhibited an identical band of DPP IV activity that could be specifically precipitated by mAb S27 directed against ADAbp (Fig. 4 *A*). No activity was detected in lysates of three melanoma cell lines (Fig. 4 *A*). DPP IV activities from the FS135 melanocyte cell line and the SK-RC-28 renal carcinoma cell line were specifically bound by ADA-Affigel affinity matrix, but not by BSA-Affigel (Fig. 4 *B*). The resultant band of DPP IV activity was identical to that immunoprecipitated by mAb S27 (Fig. 4 *B*). In experiments with SK-RC-28, it was further shown that preincubation of lysates with mAb S27 could deplete DPP IV activity precipitated by ADA-Affigel, establishing that DPP IV activity bound by mAb S27 was

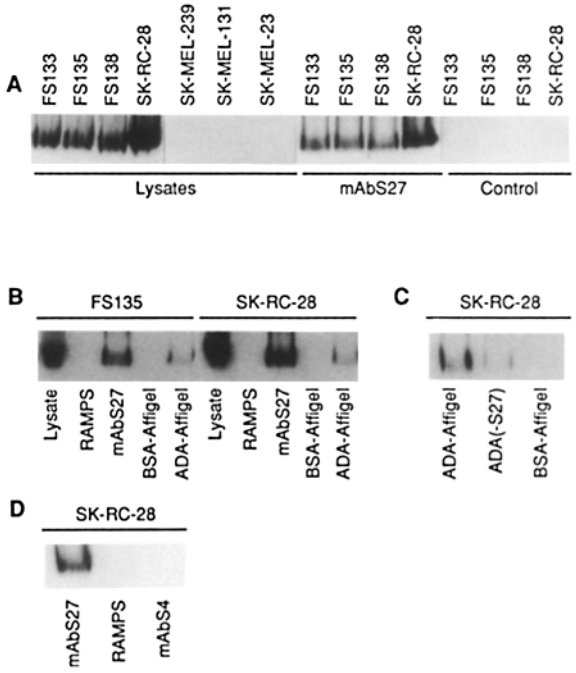

Figure 4. ADAbp possesses DPP IV activity. (*A*) Lysates from three melanocyte cell lines (FS133, FS135, FS138) and a renal carcinoma cell line (SK-RC-28) were immunoprecipitated with mAb S27. Controls were incubated with RAMPS. Lysates or immunoprecipitates were subjected to 7.5% native PAGE and stained for DPP IV activity. Lysates from three melanoma cell lines (SK-MEL-239, SK-MEL-131, SK-MEL-23), melanocytes, and renal carcinoma were analyzed for activity without prior immunoprecipitation. Lysates from melanocyte and renal carcinoma cell lines contained the equivalent of 4.5×10^5 cells/lane. Lysates from melanoma cell lines contained the equivalent of 1.1×10^6 cells/lane. (*B*) Lysates from FS135 and SK-RC-28 were immunoprecipitated with mAb S27 or affinity purified with ADA-Affigel. Controls were incubated with either RAMPS or BSA-Affigel. Lysates contained the equivalent of 6.2×10^5 cells/lane. (*C*) Lysates from SK-RC-28 were preincubated with RAMPS, with or without mAb S27, and then affinity purified with ADA-Affigel. Controls were preincubated with RAMPS and then incubated with BSA-Affigel. Lysates contained the equivalent of 2.0×10^6 cells/lane. *ADA-Affigel*, incubation with RAMPS followed by ADA-Affigel; *ADA(-S27)*, incubation with RAMPS followed by ADA-Affigel; *BSA-Affigel*, incubation with RAMPS followed by BSA-Affigel. (*D*) Lysates from SK-RC-28 were immunoprecipitated with either mAb S27 or S4. Controls were incubated with RAMPS. Lysates contained the equivalent of 6.3×10^5 cells/lane.

identical to DPP IV bound by ADA (Fig. 4 *C*). To control for possible nonspecific binding of DPP IV activity during immunoprecipitation of plasma membrane proteins, SK-RC-28 lysates were incubated with mAb S4 against gp160, a cell surface glycoprotein also present on SK-RC-28 (14). Incubation with mAb S4 did not result in coprecipitation of DPP IV activity (Fig. 4 *D*).

Discussion

ADAbp is a cell marker whose expression is not restricted to a specific lineage or particular stage of differentiation within a lineage. Rather, the regulation and function of ADAbp must be considered within frames of reference, depending on cell type. ADAbp was originally characterized as a surface mem-

brane glycoprotein expressed on a variety of secretory and absorptive epithelia (20, 21), fibroblasts (4), T lymphocytes (22), and melanocytes (6). Our interest in ADAbp has focused on its expression in normal melanocytes and its exquisite regulation during melanocyte transformation.

We have found that ADAbp is identical or analogous to CD26 and DPP IV, based on enzymatic characteristics and peptide sequences. In retrospect, the three markers, ADAbp, CD26, and DPP IV, are indistinguishable with regard to patterns of expression in tissues (23) and biochemical properties (e.g., masses of mature forms [5, 16, 18] and formation of dimers [24, 25]). Human ADAbp and CD26/DPP IV are 100% identical in five peptide regions ranging over approximately two-thirds of the CD26/DPP IV protein. Furthermore, ADAbp and CD26/DPP IV have core polypeptides with the same apparent mass (5, 16, 18), supporting the notion that ADAbp and CD26/DPP IV are encoded by the same gene, although this does not formally rule out the possibility that ADAbp could be encoded by a homologous gene. The derived amino acid sequence of human CD26 has recently been reported, and predicts a type II transmembrane molecule (16). CD26 cDNA encodes a potential signal sequence, followed by a large 738–amino acid extracellular domain (containing 10 possible Asn-linked glycosylation sites and a cysteine-rich region) on the carboxyl side of the putative transmembrane domain, and a short six–amino acid cytoplasmic domain (16). Of particular interest, the peptide sequence Gly-Trp-Ser-Tyr-Gly at amino acid positions 627–631 of human CD26 is a potential catalytic site for serine proteases and esterases (26).

ADAbp, DPP IV, and CD26 have each been characterized within the context of specific cell and tissue types. For instance, ADAbp is expressed on discrete subsets of epithelial cells in kidney tubules (21), and ADAbp in colon carcinoma cells is regulated according to the state of cellular differentiation (27). Within the T lymphocyte lineage, CD26 has been defined as a marker of activated T cells (16, 28). A list of potential functions is equally complex. ADAbp on epithelial cells has been defined by binding to ADA; DPP IV in liver, kidney, bowel, and other tissues has been detected by ectopeptidase activity, and CD26 on T cells plays a putative role in signal transduction (16) but also binds to collagen (29).

Within the melanocyte lineage, ADAbp expression is specifically extinguished during malignant transformation and tumor progression, without regard to stage of melanocyte differentiation (6, 7). ADAbp is a candidate for a gene product that is specifically downregulated or altered during neoplastic transformation. Thus, ADAbp could suppress the neoplastic phenotype in melanoma cells, based on the strong correlation between malignant phenotype and ADAbp regulation. This view fits a paradigm for neoplasia, where losses or defects of cell function have been implicated increasingly in the pathogenesis of many (if not most) human cancers. Gene products of this type stand in contrast to dominant acting oncogenes defined originally in tumor viruses. The tumor suppression phenomenon has been defined genetically by: (*a*) somatic cell hybrids or by transfer of chromosomes, parts of chromosomes, or specific genes into tumor cells (30, 31); (*b*) discovery of homozygous recessive mutations in certain hereditary cancers (e.g., Wilm's tumor [32] and retinoblastoma [33]); and (*c*) nonrandom deletions, alterations, or mutations at defined loci (e.g., p53 [34]) in human cancers, with presumed associated defects in gene function. Genetic approaches have not yet defined recessive oncogenes in human melanoma, although several candidate regions on chromosomes 1, 6, and 9 have been identified (35). Studies using somatic cell hybrids have tentatively assigned the genes encoding both ADAbp (36) and DPP IV (37) to chromosome 2, a chromosome that is altered in ~35% of melanoma specimens (35). Gross alterations in chromosome 2 probably occur during melanoma progression rather than as early events in melanocyte transformation, based on inconsistent alterations in chromosome 2 in different metastases within the same patient and the lack of chromosome 2 changes in potential precursor lesions of melanoma (35). The relationship between melanoma progression and alterations in chromosome 2 are consistent with our observations in vitro that loss of ADAbp expression is correlated with specific steps in melanoma progression. Notably, a specific subset of melanomas derived from the uvea of the eye have shown specific, nonrandom allelic losses in chromosome 2 (38).

We have been able to induce loss of ADAbp expression in melanocytes in vitro in two systems: by transfection with Ha-*ras* followed by long-term culture, and by repeated passage in the presence of isobutylmethylxanthine (6, 7). In both cases, downregulation of ADAbp expression coincided with the emergence of growth factor independence, while other traits characteristic of melanocyte transformation, such as anchorage-independent growth, loss of contact inhibition, induction of class II MHC antigens, and upregulation of G_{D3} ganglioside, did not correlate with the loss of ADAbp expression. These observations suggest that ADAbp might function to regulate cell growth, perhaps through the degradation of crucial growth factors. This proposed function for ADAbp on melanocytes is based on the observed biological properties of DPP IV, which hydrolyzes the carboxy-terminal side of dipeptide sequences, with a preference for proline at the second residue but also recognizing alanine at a lower efficiency. DPP IV is the primary enzyme responsible for cleavage and inactivation of serum growth hormone–releasing hormone (39). Potential cleavage sites exist in the matrix component collagen and the cytokines IL-1β, IL-2, and GM-CSF (28). Interestingly, a possible cleavage site also exists in ADA, which has an amino-terminal Met-Ala (40).

A model relevant to DPP IV expression on melanocytic cells has been proposed for another cell surface peptidase, CD10/neutral endopeptidase 24.11, in small cell lung cancer (SCLC). CD10 is a metalloendopeptidase that hydrolyzes a number of natural peptides, including atrial natriuretic factor, angiotensins 1 and 2, substance P, endothelin, bradykinin, oxytocin, and Leu- and Met-enkephalins (41). CD10, which is expressed at low levels on SCLC, cleaves potential autocrine growth factors for SCLC, including bombesin- and gastrin-releasing peptide (42). CD10 can inhibit proliferation

of growth factor–dependent SCLC cells, and inhibition of CD10 activity can reverse this block (42).

An interesting potential substrate for DPP IV is basic fibroblast growth factor (bFGF), based on reported amino-terminal peptide sequences (43). Although there is no evidence that DPP IV cleaves bFGF, at least one form of purified bFGF has an amino-terminal sequence (i.e., Pro-Ala-Leu-Pro) that provides a substrate for two successive rounds of proteolysis by DPP IV. Basic FGF fulfills criteria as an autocrine growth factor for melanoma: (*a*) exogenous bFGF can stimulate the growth of melanocytes in vitro (44); (*b*) melanoma cells express both bFGF and its receptor (45–47); (*c*) antibodies against bFGF have been used to inhibit melanoma cell growth in vitro (48); (*d*) transfection of melanoma cells with antisense oligonucleotides against bFGF can inhibit melanoma proliferation (49); and (*e*) melanocytes transfected with bFGF cDNA grow independently of exogenous growth factors, although they form only benign, not invasive, tumors when injected into nude mice (50). Thus, expression of bFGF alone is insufficient to confer a full malignant phenotype, but rather appears to represent a critical step in a sequence necessary for full neoplastic transformation. In the 10W melanocyte system, bFGF transcripts were detected in both 10W *ras*/early and 10W *ras*/late cells, but not parental 10W cells (7). Interestingly, both ADAbp and CD10 were expressed by parental 10W cultures and by 10W *ras*/early cells, which maintained only a partially transformed phenotype and required exogenous growth factors for survival. However, expression of both peptidases was extinguished in the fully transformed 10W *ras*/late cell strain (7).

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