Adenosine deaminase (ADA), which converts adenosine and 2′-deoxyadenosine to inosine and 2′-deoxynosine, has a wide phylogenetic distribution from bacteria to humans (1). Humans with inherited ADA deficiency have a profound lymphopenia. Phagocytes that occur when Pg binds to the cell surface and activates human T lymphocytes and epithelial cells of many organs because of the association of ADA with a multifunctional cell membrane glycoprotein, CD26/dipeptidyl peptidase IV (2). By modulating extracellular adenosine, this “ecto-ADA” may regulate adenosine receptor signaling implicated in various cellular functions. CD26 is expressed on the surface of human prostate cancer 1-LN cells acting as a receptor for plasminogen (Pg). Since ADA and Pg bind to CD26 at distinct but nearby sites, we investigated a possible interaction between these two proteins on the surface of 1-LN cells. Human ADA (α20648) binds to CD26 (22). ADA binding occurs at a region between amino acids 340 and 345 on CD26, and the Thr345 residue is phosphorylated in the presence of ADA. In the absence of CD26 via the Pg kringle 4 (K4) domain. Exogenous ADA enhances conversion of Pg 2 to plasmin by 1-LN endogenous urinary plasminogen activator (u-PA), as well as by added tissue Pg activator (t-PA), suggesting that ADA and Pg bind simultaneously to CD26 in a ternary complex that stimulates the Pg activation by its physiologic activators. Consistent with this, in melanoma A375 cells that bind Pg, but do not express CD26, the rate of Pg activation was not affected by ADA. Thus, ADA may be a factor regulating events in prostate cancer cells that occur when Pg binds to the cell surface and is activated.

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ing a connection between the Pg activation system and the protein complex CD26-ADA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture media were purchased from Invitrogen. 125I-labeled Bolton-Hunter reagent for protein iodination was obtained from PerkinElmer Life Sciences. All other reagents were of the highest grade available.

**Proteins**—ADA was purified from human monocytic leukemia U937 cells grown in 10 liters of RPMI 1640 culture medium containing 10% fetal bovine serum. The protein was purified to homogeneity using a combination of ion-exchange and affinity chromatographies as described by Aran et al. (24).

Human Pg was purified and separated into its two classes of isoforms, Pg 1 and Pg 2, as described previously (25, 26). CD 26 from 1-LN cell membranes was purified as described previously (17). Pg kringle 1–3 (K1–3) and krl 4 (K4) were prepared by limited proteolysis of Pg with porcine pancreatic elastase and purified as described by Slotrup-Dersen et al. (27). Pg kringle 5 (K5) was obtained by digestion of mini-Pg with pepsin and purified as described by Cao et al. (28). Iodination of ADA was carried out using 125I-labeled Bolton-Hunter reagent according to the manufacturer’s protocol. The specific activity varied from 500 to 700 cpm/μg.

**Construction and Expression of ADA Mutants**—Wild type human and mouse ADA cDNAs and the hybrid human ADA cDNA in which the amino acid sequence 126–143 was substituted by the corresponding segment from mouse cDNA (H1–125/M126–143/H144–363) were constructed and expressed as described previously (14, 29). To prepare ADA for CD26 binding studies, the cells from 100-ml overnight cultures were sonicated in 3 ml of lysis buffer (29). ADA enzymatic activity and concentration in these lysates was determined as described previously (14, 29).

**Antibodies**—Antibodies to CD26 were raised in rabbits according to standard protocols, using purified CD26 (17) as the antigen, by Research Genetics (Huntsville, AL). The IgG fraction specific against CD26 was isolated from a column containing CD26 covalently attached to Sepharose 4B. The mouse monoclonal antibody 1C5 was prepared against ADA purified from human T cell leukemia cells as described previously.

**Cell Cultures**—The human prostate tumor cell line 1-LN, a generous gift of Dr. Philip Walther of the Department of Surgery, Duke University Medical Center, Durham, NC, was grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. The human melanoma A375 cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s Modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 10 μg/ml penicillin G, and 100 μg/ml streptomycin.

**Ligand Binding Analysis**—Cells were grown in tissue culture plates until the monolayers were confluent. The cells were washed in Hank’s balanced salt solution. All binding assays were performed at 4 °C in RPMI 1640 containing 2% bovine serum albumin (BSA). Increasing concentrations of 125I-labeled ADA were incubated with cells for 60 min in 96-well strip plates. Free ligand was separated from bound ligand by aspirating the incubation mixture and washing the cell monolayers rapidly three times with RPMI 1640 containing 2% BSA. Wells were stripped from the plates, and radioactivity was determined in an Amersham Biosciences LKB Biotechnology® 1272-γ counter. Molecules of ligand bound were calculated after subtraction of nonspecific binding measured in the presence of 100 μM unlabeled ADA. Estimates of dissociation constants (Kd) and maximal binding of ADA (Bmax) were determined by fitting data directly to a nonlinear regression program using the statistical program SYStat® for Windows 98.

**Solid-phase Radioligand Binding Studies**—To study specific binding of ADA to immobilized CD26 purified from 1-LN cells, 96-well strip plates were coated with CD26 (1 μg/ml in 0.1 M sodium carbonate, pH 9.6; 200 μl/well; 37 °C; 2 h). After coating, plates were washed with 200 μl of 10 mM sodium phosphate/100 mM NaCl, pH 7.4, containing 0.05% Tween 80 (PBS-Tween) to remove unbound protein. Non specific sites were blocked by incubation with PBS-Tween containing 2% (w/v) BSA at room temperature for 1 h. Plates were then washed twice with 200 μl of PBS-Tween, air-dried, and stored at 4 °C. For assays, increasing concentrations of 125I-labeled ADA, with or without a 50-fold excess of unlabeled ligand, were added to triplicate wells and incubated at 22 °C for 1 h. Following incubation, the supernatants were removed, and the plates were rinsed three times with 200 μl of PBS-Tween. Wells were stripped from the plates, and radioactivity was measured in an Aristar Labtec® kinetic plate reader. Bound ADA was expressed as ΔA405 nm/min.

**Plasmin Generation on the Surface of 1-LN Cells or Immobilized CD26**—Plasmin generation by endogenous u-PA was measured on confluent 1-LN cell monolayers grown in 96-well plates. Cells were incu-
Adenosine Deaminase Stimulates Plasminogen Activation

RESULTS

Binding of ADA to 1-LN Cells and Immobilized CD26 Isolated from 1-LN Cell Membranes—Analyses of the binding isotherm resulting from incubation of 1-LN cells with increasing concentrations of 125I-labeled human ADA (Fig. 1A), purified from U937 cells (Fig. 1A, inset), suggest that ADA binds to these cells in a dose-dependent manner with high affinity (Kd of 20 ± 3.7 nM) and to a large number of sites (Bmax of 28 ± 4.3 × 105 sites/cell). Analyses of the binding isotherm of ADA to CD26 immobilized on cell culture plates (Fig. 1B) indicates a single class of binding sites (a Kd of 18 ± 5.4 nM and a Bmax of 0.8 nmol of bound ADA/nmol CD26). This Kd value is within the same order of magnitude to previous estimates of Kd determined for the binding of recombinant human ADA to purified rabbit CD26 (15).

Binding of Recombinant ADAs to Purified CD26 and to CD26-expressing versus CD26-nonexpressing Cells—We examined the capacity of recombinant ADA constructs expressed in lysates of Escherichia coli St3834 to compete for binding of purified, 125I-labeled human ADA to human CD26 immobilized on culture plates. These experiments (Fig. 2) show that only wild type recombinant human ADA competed significantly for

CD26 binding. Lysates expressing mouse ADA or the human ADA mutant H1–125/M126–143/H144–363, in which the mouse α2 helix is substituted for the human segment, showed no more evidence of binding to human CD26 than did a lysate of E. coli St3834 transformed with the carrier plasmid pZ alone, used as a negative control. These data confirm that the amino acid segment 126–143 of human ADA comprises the functional epitope for binding to CD26.

The binding of recombinant ADAs to 1-LN cells was compared with their binding to human melanoma A375 cells, which do not express CD26, using a CELISA assay as described under “Experimental Procedures.” As was found with purified CD26, wild type recombinant human ADA, but neither mouse ADA nor the H1–125/M126–143/H144–363 mutant, showed binding to 1-LN cells (Fig. 3A), indicating that ADA binding to 1-LN cells was exclusively via cell surface CD26. Consistent with this conclusion, and with previous studies of 125I-labeled ADA binding to several other melanoma cell lines (23), neither wild type nor mutant recombinant human ADA constructs showed any specific binding to A375 cells (Fig. 3B).

Binding of ADA to Immobilized Human Pg 2—A close examination of the human and murine CD26 segment containing amino acids 310–350 (19, 20) reveals a conserved Pg2 binding region (Fig. 4) (18). Confirming our previous finding (18), Fig. 5 shows that 125I-labeled ADA binds to Pg 2 immobilized on cell culture plates in a dose-dependent, saturable manner. 125I-labeled ADA also binds to Pg 2 electroblotted to a nitrocellulose
membrane after electrophoresis (Fig. 5, inset, lane 2). Analysis of the binding isotherm demonstrates that ADA binds to Pg 2 with high affinity ($K_d$ of 0.28 μM and $B_{max}$ of 0.93 nmol of bound ADA per nmol Pg).

Since both Pg 2 and ADA bind to CD26, and ADA binds directly to Pg 2, we investigated the capacity of Pg 2 to influence the binding of ADA to CD26 (Fig. 6). Increasing concentrations of Pg enhanced the binding of ADA to the complex CD26-Pg 2 (Fig. 6A). Furthermore, the binding of 125I-labeled ADA to immobilized Pg 2 was not affected by either K1–3 or K5 domains of Pg, whereas the K4 domain was an effective inhibitor (Fig. 6B). These results suggest that the interaction of ADA and Pg 2 is mediated by K4.

**Effect of the Interaction between Pg 2 and ADA on Pg Activation or ADA Enzymatic Activity**—Pg 2 binds to CD26 on 1-LN cells, where it is rapidly activated by u-PA, the primary Pg activator at the surface of these cells (17). We studied the effect of increasing concentrations of ADA on Pg 2 activation by 1-LN cell monolayers at a single Pg 2 concentration (0.2 μM) (Fig. 7A) and also on Pg 2 activation by t-PA on plates containing immobilized CD26 isolated from 1-LN cell membranes (Fig. 7B). In both cases, ADA stimulated Pg 2 activation in a dose-dependent manner, when either u-PA or t-PA was the activator. The enzymatic activity of ADA (0.1 μM) was not affected by increasing concentrations of Pg 2 (values of ΔA$_{265}$nm/min of 0.15 and 0.14 in the absence or presence of 1 μM Pg 2, respectively). These experiments suggest that ADA facilitates Pg 2 activation, possibly by inducing a conformational change in Pg 2.

We next assessed Pg 2 activation by t-PA in the presence of ADA and poly-D-lysine, which mimics a fibrin surface, a physiological substrate for Pg activation by t-PA. In this system, we studied the effect of Pg K1–3, K4, and the anti-fibrinolytic amino acid 6-AHA, which inhibits binding of Pg or t-PA to fibrin. The results (Table I) demonstrate that poly-D-lysine enhances the effects of ADA on Pg 2 activation by t-PA. Furthermore, these experiments demonstrate that K4 effectively suppresses the ADA effect, suggesting again that K4 is the region in Pg responsible for the interaction with ADA. 6-AHA was an effective inhibitor of ADA-induced Pg activation. This raises the possibility that Pg and its activators must be closely localized by substrate molecules, such as fibrin or CD26, in order for

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**Fig. 4.** Alignment of amino acid residues of human CD26 and ADA involved in the interaction between these two proteins. Amino acids 310–350 of human CD26 showing regions involved in Pg 2 and ADA binding are illustrated.

**Fig. 5.** Binding of ADA to immobilized human Pg 2. Increasing concentrations of 125I-labeled ADA were added to Pg 2-coated 96-well culture plates and were incubated at 22 °C for 1 h. Bound ADA was calculated as described under "Experimental Procedures." Inset, SDS/10% PAGE of purified Pg 2 (10 μg) which was examined under reducing conditions and then transferred to a nitrocellulose membrane by the Western blot method. Lane 1, Coomassie Brilliant Blue R-250-stained gel. Lane 2, reaction of Pg 2 in a blot with 125I-labeled ADA. Data represent means ± S.D. from three experiments.

**Fig. 6.** Binding of ADA to Pg attached to CD26 and inhibition of its binding to Pg by Pg 2 structural modules. A, increasing concentrations of Pg 2 were added to CD26-coated 96-well culture plates and incubated with a single concentration of 125I-labeled ADA (0.2 μM) at 22 °C for 1 h. Bound ADA was calculated as described under "Experimental Procedures." B, a single concentration of 125I-labeled ADA was added to Pg 2 coated 96-well culture plates and incubated with increasing concentrations of K1–3 (■), K5 (△), or K4 (○). Bound ADA was calculated as described under "Experimental Procedures." Data represent means ± S.D. from three experiments.
of a single concentration of Pg 2 (0.2 μM) and 

S-2251 (0.2 mM), t-PA (0.5 nM), poly-D-Lys (1 mg/ml), K1/H9262/3.

We have shown that ADA binds to CD26 on 1-LN cells and that ADA binds to Pg 2, either alone or when Pg 2 is bound to immobilized CD26. The enzymatic activity of ADA was not affected by binding to Pg 2. ADA stimulated the activation of Pg by u-PA on the surface of 1-LN cells or by t-PA when Pg 2 was bound to immobilized CD26. ADA also stimulated Pg 2 activation by t-PA in the presence of poly-D-lysine, which mimics a fibrin surface, thereby suggesting that extracellular ADA may act as a pro-fibrinolytic factor. The interaction between ADA and Pg is mediated by K4. ADA appears to mimic the effect of tetranectin, a specific K4-binding protein occurring in plasma at 10 mg/liter, which enhances Pg activation by t-PA in the presence of poly-D-lysine (34). This effect of tetranectin on Pg activation is inhibited by 6-AHA, suggesting a mechanism mediated by the L-lysine binding site on K4 (34). All these effects are similar to those described above for the interaction between ADA and Pg 2.

Our data suggest a mechanism that permits acceleration of the rate of Pg activation when both Pg and ADA are bound to CD26 in prostate carcinoma 1-LN cells, which is not available in A375 melanoma cells, which do not express CD26. However, Pg is found colocalized with tetranectin at the invasive front of malignant melanoma lesions, suggesting a coordinated role for tetranectin and Pg, mediated by the L-lysine binding site on K4 (35); this Pg 2 activation may facilitate the migration of melanoma tumor cells (35). Therefore, both the evolution of melanoma and prostate cancer may depend on the interaction of ligands specific for Pg K4 to regulate the rate of Pg activation at their invasive fronts.

In this context, the levels of CD26 and ADA have been evaluated in prostate cancer. The specific activity of ADA in cancerous human prostate has been reported to be similar or slightly less than in normal prostatic tissue (36, 37). However, ADA levels in benign prostatic hypertrophy (BPH) tissue were significantly higher than in normal prostate (36). CD26 levels are significantly elevated in prostate cancers relative to BPH tissues, but CD26 levels were similar to those of the tumor in BPH tissue adjacent to the tumor (38). These studies, along with our observations, suggest that one of the local factors influencing growth of epithelial cells in prostate cancer may be high levels of the protease plasmin generated by BPH tissue in

ADA to enhance the rate of Pg activation. To test this hypothesis, we examined the effect of ADA on Pg activation by A375 cells, which do not express CD26 on their surface (Table II). In contrast to results with 1-LN cells, Pg activation was not substantially changed in the presence of two concentrations of ADA. Taken together, these results suggest that ADA-facili-

Table II

Plasminogen activation on the surface of A375 melanoma cells

| Substrate | Plasmin activity (ΔA405nm/min) |
|-----------|-------------------------------|
| Pg 2      | 2.4 ± 0.32                    |
| Pg 2 + ADA (0.1 μM) | 2.3 ± 0.41               |
| Pg 2 + ADA (0.2 μM) | 2.6 ± 0.53               |

Stimulation of Pg activation by ADA when Pg is bound to 1-LN cells or immobilized CD26. A, increasing concentrations of ADA were added to 1-LN cell monolayers (1 × 10^6 cells/well) incubated with a single concentration of Pg 2 (0.2 μM) at 37 °C for 1 h. The rate of plasmin generation was monitored with the plasmin substrate S-2251 using an Anthos Labtec® kinetic plate reader. B, increasing concentrations of ADA were added to CD26 coated 96-well plates in the presence of a single concentration of Pg 2 (0.2 μM) and t-PA (0.5 nM). Plasmin generation was monitored as described above. Data shown represent means ± S.D. from three experiments.

**FIG. 7.** Stimulation of Pg activation by ADA when Pg is bound to 1-LN cells or immobilized CD26. A, increasing concentrations of ADA were added to 1-LN cell monolayers (1 × 10^6 cells/well) incubated with a single concentration of Pg 2 (0.2 μM) at 37 °C for 1 h. The rate of plasmin generation was monitored with the plasmin substrate S-2251 using an Anthos Labtec® kinetic plate reader. B, increasing concentrations of ADA were added to CD26 coated 96-well plates in the presence of a single concentration of Pg 2 (0.2 μM) and t-PA (0.5 nM). Plasmin generation was monitored as described above. Data shown represent means ± S.D. from three experiments.

**Table I**

Effect of ADA on poly-D-lysine enhanced t-PA activity

| Stimulator   | Plasmin activity (ΔA405nm/min) |
|--------------|-------------------------------|
| None         | 2.8 ± 0.61                    |
| ADA          | 4.3 ± 1.03                    |
| Poly-D-Lys   | 5.4 ± 1.43                    |
| ADA + poly-D-Lys | 8.7 ± 2.31               |
| ADA + K4 + poly-D-Lys | 8.1 ± 2.07               |
| ADA + K4     | 3.1 ± 1.06                    |
| ADA + 6-AHA  | 2.6 ± 0.83                    |
the immediate growth environment of the tumor, resulting from high levels of CD26 and ADA, which facilitate Pg localization and activation on the surface of these cells.

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