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Effect of Deoxycoformycin and Val-boroPro on the Associated Catalytic Activities of Lymphocyte CD26 and Ecto-Adenosine Deaminase

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ABSTRACT. CD26 and ecto-adenosine deaminase (ADA) are found associated on the plasma membrane of T lymphocytes and each possess distinct catalytic activities. CD26 has a proteolytic activity identical to dipeptidylpeptidase IV (DPPIV; E.C. 3.4.14.5), and ecto-ADA (E.C. 3.5.4.4) degrades extracellular adenosine. The cell surface expression of CD26 and ecto-adenosine deaminase (ecto-ADA) is regulated on stimulated T lymphocytes, and ADA binding to CD26 produces a synergistic costimulatory response with T cell receptor activation. This study addresses the potential regulation by allosteric interactions of the catalytic activities of CD26 associated with ecto-ADA, which could define the mechanism of the synergism observed in T cell signaling. Cell lines genetically deficient in ADA, ligands for ADA such as adenosine, and a specific inhibitor of ADA, deoxycoformycin, were used to define the effect of ADA activity on CD26 DPPIV activity and affinity for dipeptide substrate. Conversely, a recombinant Chinese hamster ovary cell line expressing human CD26 with or without a mutation in the DPPIV catalytic domain, and the boronic acid inhibitor Val-boroPro, were used to determine the effect of DPPIV activity on ecto-ADA activity and association with CD26. These studies found no significant allosteric interaction between the catalytic activities of CD26 and ecto-ADA when associated. Therefore, signaling events in T cells involving costimulation with CD26 and ecto-ADA and the synergism observed upon ADA binding to CD26 occur independently of the catalytic activities of these cell surface molecules. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1757–1765, 1996.

KEY WORDS. adenosine deaminase; adenosine; deoxycoformycin; dipeptidylpeptidase IV; lymphocyte activation; boronic acid inhibitors

ADA¶ (adenosine aminohydrolase, E.C. 3.5.4.4) catalyzes the degradation of adenosine. ADA is expressed in all tissues but most highly in lymphocytes, where it appears to play a fundamental role in lymphocyte development and function [1]. Deficiencies in ADA activity are associated with one kind of severe combined immunodeficiency disease characterized by the absence of functional mature T and B lymphocytes [2–4] Although most ADA is found in the cytosol, a high molecular weight isoform is found as ecto-ADA in plasma membrane fractions, associated with an integral membrane protein termed ADA-binding protein [5–7].

The ADA-binding protein has been defined to be identical with the lymphocyte activation antigen CD26 [1, 8, 9]. CD26 is an ectoenzyme identical with DPP IV (E.C. 3.4.14.5), cleaving Xaa-Pro (or Xaa-Ala) dipeptides from the N-termini of polypeptides [10, 11]. Binding of ADA to CD26 from several species shows that the characteristics required for binding have been conserved by enzymes from a diverse group of animals [6]. This provides evidence for the physiological significance of the interaction of CD26 and ecto-ADA.

The mechanism by which ADA is coexpressed with CD26 on the cell surface is unknown. The association of these molecules on lymphocytes is particularly intriguing given their potential roles in immune development and function. The expression of both ecto-ADA and CD26 increases significantly upon T lymphocyte activation [12]. Several lines of evidence have implicated CD26 as a costimulatory molecule for T lymphocyte activation in coordination with anti-CD3, CD2, protein kinase C and tyro-

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¶ *Abbreviations:* ADA, adenosine deaminase; DPPIV, dipeptidylpeptidase IV; CHO, Chinese hamster ovary cells; MNA, 4-methoxy-2-napthylamine; SCID, severe combined immunodeficiency disease; mAb, monoclonal antibody; PBL, peripheral blood leukocytes; sPBL, stimulated peripheral blood leukocytes; and PMSF, phenylmethyl sulfonyl fluoride.

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sine kinases [13–16]. CD26 expression is requisite to elicit a memory T cell response or to trigger cytotoxic T cells [14, 17, 18]. Recently, the binding of CD26 to exogenously added ADA has also been shown to potentiate activation of T lymphocytes stimulated through the T cell receptor/CD3 pathway, suggesting that association with ADA can modulate CD26 function [12]. In this regard, the distinct intrinsic catalytic activities of CD26 and ecto-ADA may play a role regulating their association and may also induce allosteric regulation of each molecule's enzymatic activity, which could effect T cell signal potentiation.

The aim of this study was to determine the effect of modulation of either ecto-ADA or CD26 DPP IV activity in an associated complex by chemical and molecular techniques. CD26/ecto-ADA complexes were isolated by immunoprecipitation from peripheral blood lymphocytes or lymphoid cell lines and assayed for DPPIV and ADA activity in the presence of chemical modifiers of both enzymatic activities. The activities of either CD26 or ADA were also evaluated in cell lines derived from patients genetically deficient in ADA or recombinant cell lines expressing CD26 with or without an active catalytic site. The association of ecto-ADA with CD26, the activity of both enzymes in the complex, and the affinity of CD26 for dipeptide substrate were analyzed under these conditions for potential allosteric regulation.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Chemicals

The human lymphoid cell lines YT and Jurkat were provided by Dr. J. Yodoi, Kyoto University (Kyoto, Japan) and Dr. Peter Lipsky, Baylor University, (Houston, TX), respectively. The CHO DUXK cell line was obtained from Genetics Institute (Cambridge, MA). The ADA-deficient cell lines were purchased from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Polyclonal antibody recognizing ADA was provided by Dr. William Osborne, University of Washington School of Medicine (Seattle, WA). Deoxycoformycin was provided by Parke Davis Pharmaceutics, Inc., Detroit MI. The boronic acid inhibitor ValboroPro and an inactive diastereomer were synthesized and analyzed as described [19].

Cell Lysates, Membranes, and Immunoprecipitation

Peripheral blood lymphocytes were isolated and mitogen-stimulated by standard procedures [20]. Cells or cell lines (1 to 1.5×10^7 cells/0.4 mL) were lysed with 0.1 M Tris, pH 7.8 containing 1% Triton X-100 and 0.01% NaN_3 (Buffer A) and centrifuged at 3000 *g* for 2 min at 4° with the resulting supernatant used as the enzyme source. For cell membrane preparation, cells were lysed by sonication in 25 mM HEPES, pH 7.4 containing 14 mM 2-mercaptoethanol, 1 mM benzamidine, 1 mM PMSF, 1 μg/mL pepstatin and 5 γg/mL leupeptin. The homogenate was underlaid with 41% (wt/vol.) sucrose and separated from soluble proteins and cellular debris by centrifugation at 95,000 *g* for 1 hr. The membrane fraction was collected and resuspended in lysis buffer as above (Buffer A). For immunoprecipitation of CD26/ecto-ADA complexes, protein G beads (Pierce, Rockford, IL) were bound to monoclonal antibody directed against CD26 (Coulter Immunology, Hialeah, FL) or an IgG1 isotype control, R3.1 [21] and incubated with equal volumes of cell supernatants. An aliquot (100 μL) of beads was then assayed for DPPIV and ADA enzyme activity. For inhibitor studies, immunoprecipitates were incubated with chemicals as noted, washed and then assayed for DPPIV and ADA activity.

DPPIV Enzyme Assay and Kinetic Analysis

Cell lysates (10^7 cells/sample) or immunoprecipitates were incubated with 1 mM substrate dipeptide L-alanyl-L-proline attached to MNA (Enzyme System Products, Livermore, CA) for 1 hr at 37°. Liberated MNA was detected by the addition of 50 μL of 4-dimethylaminocinnamaldehyde (3.3 mg/mL) and the optical density measured at 570 nm. DPPIV kinetic activity was determined by monitoring the change in fluorescence associated with the cleavage of the same substrate in a SLM Aminco Bowman (Urbana, IL) series 2 Model SQ-340 fluorescence detector [19]. Reactions were run at 23° in a final volume of 2.0 mL containing 100 mM Tris, pH 8.0, 0.5% dimethylformamide and 0.5% DMSO. The reaction was initiated by the addition of substrate to final concentrations varying from 9.75×10^{-6} M to 2.50×10^{-4} M. Data acquisition was begun within 5 sec after the addition of substrate. The kinetic constant K_m was determined from a non-linear regression fit of the initial velocity and substrate concentration data to a rectangular hyperbola.

Adenosine Deaminase Enzyme Assay

ADA enzymatic activity was measured as previously described [11]. Briefly, 10 μl of lysate or antibody-coated protein G immunoprecipitates were incubated at 35° with 1.5 mM adenosine, 20 mM potassium phosphate, pH 7.2, 0.12 U of nucleoside phosphorylase and 0.1 U of xanthine oxidase (the Sigma Chemical Co., St. Louis, MO). ADA enzymatic activity was measured by monitoring the optical density at 293 nm.

cDNA Cloning, Mutagenesis, and Transfection

A cDNA encoding for human CD26 was isolated from YT cell mRNA by traditional polymerase chain reaction cloning techniques [22]. A catalytic site deletion of CD26 was generated by polymerase chain reaction mutagenesis techniques [23]. The resulting mutant clone has a deletion in the catalytic site of Trp^{632}, Ser^{633}, Tyr^{634} and Gly^{635}. Full length CD26 or Ser^{633}-negative CD26 cDNA was subcloned into the *Eco* RI site of pMT2 (Genetics Institute, Cambridge, MA) and co-transfected with pSV2-*neo* into

CHO DUXK cells by calcium phosphate precipitation. The resulting transfectants were selected with G418 (Gibco) and amplified with methotrexate (Sigma). Cells expressing CD26 were isolated by flow cytometry on a FACS Vantage (Becton Dickinson) after labeling with anti-human CD26 mAb.

Sucrose Gradient Sedimentation

Linear sucrose density gradients (11 mL, 5–30% with a 0.5-mL 50% cushion) were made with 20 mM Iso-KCl, 5 mM N_2HPO_2, 0.5 mM EDTA, 0.5 mM 2-mercaptoethanol and 0.1 mM PMSF. Cells to be analyzed were lysed in 0.2 mL buffer containing 0.1M Tris, pH 7.8, with 0.1% NaN_3 and 1% Triton-X 100 for 5 min, 4°. The cell lysate was centrifuged for 45 sec at 3000 g at 4° and applied to gradients. Gradients were centrifuged 16 hr, 111,000 g 4°. Fractions were pumped from the top of the gradient using a Buchler Auto-Densiflow (Buchler, Lenexa, KA) and collected in 0.25 mL fractions, yielding approximately 47 samples/gradient. Sedimentation profiles of reference standards (BSA, 4.3 S; apoferritin, 16.6 S; thyroglobulin, 19 S) were determined by standard biochemical protein quantitation.

RESULTS

Characterization and Isolation of CD26 and Associated Ecto-ADA

The YT lymphoid dell line initially was characterized for plasma membrane CD26 and ecto-ADA expression by flow cytometry after surface labeling with either anti-CD26 or anti-ADA. Figure 1 shows that the YT cell line expressed significant plasma membrane levels of both CD26 and ecto-ADA. Intact cells or isolated plasma membranes from YT cells were prepared with non-ionic detergent and immunoprecipitated with a mAb specific for CD26. The resulting immunoprecipitates were washed and analyzed for DPPIV and coprecipitated ADA activities (Table 1). Table 1 shows that anti-CD26 but not an isotype control antibody was able to directly immunoprecipitate CD26 with DPPIV activity from either YT cell lysates or plasma membranes. Furthermore, specifically associated ADA activity was coprecipitated with CD26. Freshly isolated peripheral blood lymphocytes stimulated with mitogen also demonstrated that immunoprecipitated CD26 had associated ADA activity similar to the YT cell line. In contrast, the Jurkat cell line, which does not express CD26 had negligible DPPIV and ADA activity in CD26 precipitates. Therefore, coimmunoprecipitation from either YT cells or peripheral blood lymphocytes was determined to be a good source for the isolation of active enzymatically CD26/ecto-ADA complexes.

Adenosine Deaminase Association with Recombinant CD26

Expression of enzymatically active CD26 in stably transfected CHO cells provided a system for more detailed biochemical study of DPPIV activity and its relationship to

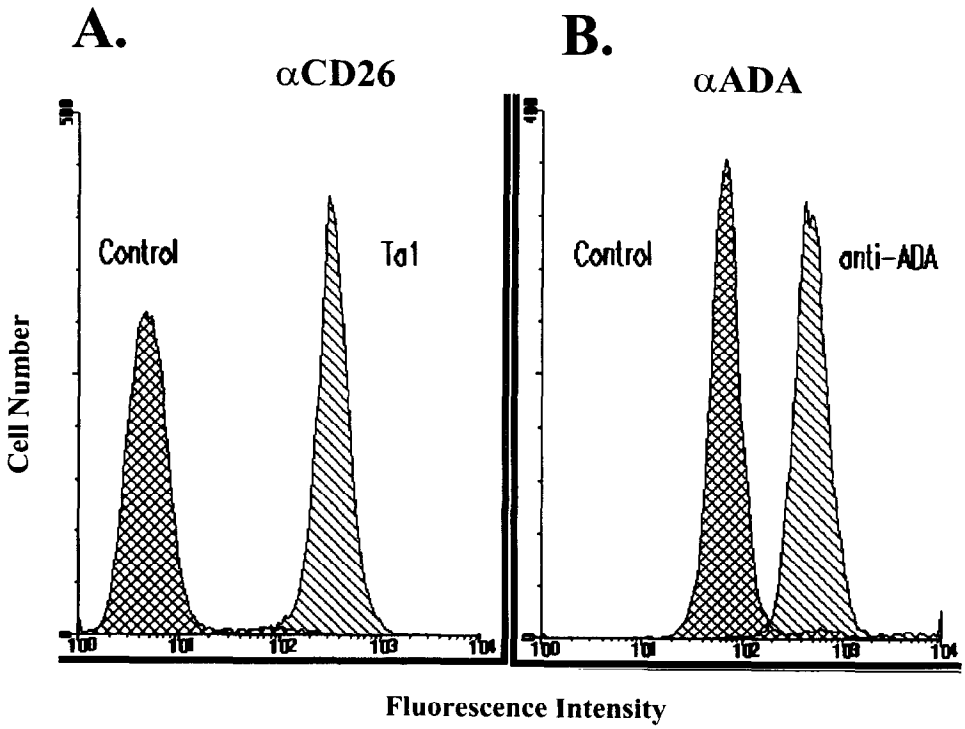

FIG. 1. YT cell surface expression of CD26 and ecto-ADA. YT cells were incubated with phycoerythrin-conjugated goat anti-mouse (Panel A, control) or anti-rabbit antibody alone (Panel B, control) or secondary to incubation with mouse anti-human CD26 antibody (Panel A, Ta1) or rabbit anti-human ADA (Panel B, anti-ADA). Analysis of the flow cytometry profile is shown as cell number vs fluorescence intensity.

TABLE 1. Co-immunoprecipitation and enzymatic activity of DPP IV and ADA

Cell source	Antibody	DPP IV Activity*	ADA Activity†
YT Cell lysate	Anti-CD26	109.5 ± 16.1	23.43 ± 5.75
	control mAb‡	13.4 ± 10.1	2.34 ± 2.0
YT cell membranes	Anti-CD26	31.1 ± 6.2	6.2 ± 1.2
	control mAb	3.48 ± 0.7	1.28 ± 0.26
Jurkat cell lysate	Anti-CD26	0.36 ± 0.7	§
	control mAb	0.24 ± 0.05	§
Stimulated PBL's	Anti-CD26	7.5 ± 0.082	5.06 ± 0.45
	control mAb	1.06 ± 0.11	0.9 ± 0.17

Values shown are averages ± SD (N = 3–4) except for YT cell membranes where values shown are averages ± range (N = 2).

* nmol of Ala-Pro-MNA cleaved/10^6 cells.

† nmol of uric acid produced/10^6 cells.

‡ Control mAb is an anti-CD11a, an isotype control.

§ Negligible activity measured.

ADA. CHO DUKX cells that had been transfected with human lymphoid CD26 or catalytic site deletions of CD26 were selected and amplified. The highest expressing population for each was isolated by flow cytometry and used in the experiments that follow. The top panel of Fig. 2 (Panel A) shows the relative levels of immunoprecipitated DPPIV activity of the host cell line, CHO DUKX, the CD26 transfectants (CHO CD26), and the CD26 transfectants with a deletion of the active serine residue in the catalytic site (CHO CD26 Ser-). As shown, the CD26 transfectants had 25-fold higher DPPIV activity than either the host cell line or the CD26 mutants, which do not demonstrate enzymatic activity. ADA activity did not coprecipitate with CHO CD26 or CHO CD26Ser- recombinantly expressed protein (Panels B, C; no ADA added) consistent with reports that rodent ADA does not associate with human CD26 [24]. However, upon addition of exogenous calf spleen ADA, both recombinantly expressed CD26 proteins were able to specifically associate with ADA as seen by CD26 mAb-dependent coprecipitation (Fig. 2, Panels B, C; 5 and 10 μg ADA). These data demonstrate that CD26 DPPIV activity is not dependent on ADA association, that free CD26 is capable of being reconstituted with the addition of exogenous ADA from a non-rodent source, and that the enzymatic activity of CD26 is not requisite for the binding of ADA.

CD26 DPPIV Activity in ADA-Deficient SCID Cell Lines

Given that the DPPIV activity of CD26 was not dependent on association with ADA it was of interest to test if ADA activity affected the catalytic function of CD26 by regulating the affinity of DPPIV for dipeptide substrate. In humans, a genetic mutation in ADA, rendering the molecule functionally inactive, results in one kind of SCID, characterized by dysfunctional lymphocytes. However, the severity of the clinical symptoms of this disease can vary broadly. Four cell lines, immortalized from SCID patients, all with a genetic mutation in ADA and less than 5% normal ADA activity, were analyzed for DPPIV affinity for dipeptide substrate. Two of the SCID lymphoblast lines were isolated from patients with a clinical SCID phenotype, and two patients had a normal phenotype. The DPPIV activities of these cell lines were directly compared to the DPPIV activity in YT cell and peripheral blood lymphocytes, which, as shown in Table 1, is associated with enzymatically active ADA. Regardless of whether lymphocytes or lymphoblast cell lines were immunodeficient, ADA deficient or normal, there was no significant difference in the affinity of DPPIV for dipeptide substrate (Table 2). These data demonstrate that ADA activity or association does not regulate the kinetic properties of CD26 DPPIV catalytic activity.

Allosteric Interactions of DPPIV and ADA Activities

To directly characterize the effect of chemical modulation on either DPPIV or ADA activity while associated, CD26/ecto-ADA complexes were immunoprecipitated with anti-CD26 from YT cell lysates. Aliquots of immunoprecipitated lysates were incubated with agonists or antagonists of DPPIV and ADA, washed and assayed for enzymatic activity. To inactive DPPIV activity, a potent boronic acid inhibitor of DPPIV, Val-boroPro, was tested with the diastereomer of this molecule as a negative control. While Val-boroPro, but not the diastereomer, eliminated measurable DPPIV activity, the coprecipitation of ADA activity was not affected (Table 3). The addition of exogenous ADA to the coprecipitated complexes also did not significantly enhance either DPPIV or associated ADA activity. Potential agonists of ecto-ADA including ATP, deoxy ATP and diadenosine tetraphosphate [25] when incubated with the complexes had no significant effect on either DPPIV or associated ADA activities. Finally, a potent inhibitor of ADA, deoxycoformycin [5, 26], that completely inhibited ADA activity had no effect on DPPIV activity. Taken together, clearly there is no allosteric regulation between the

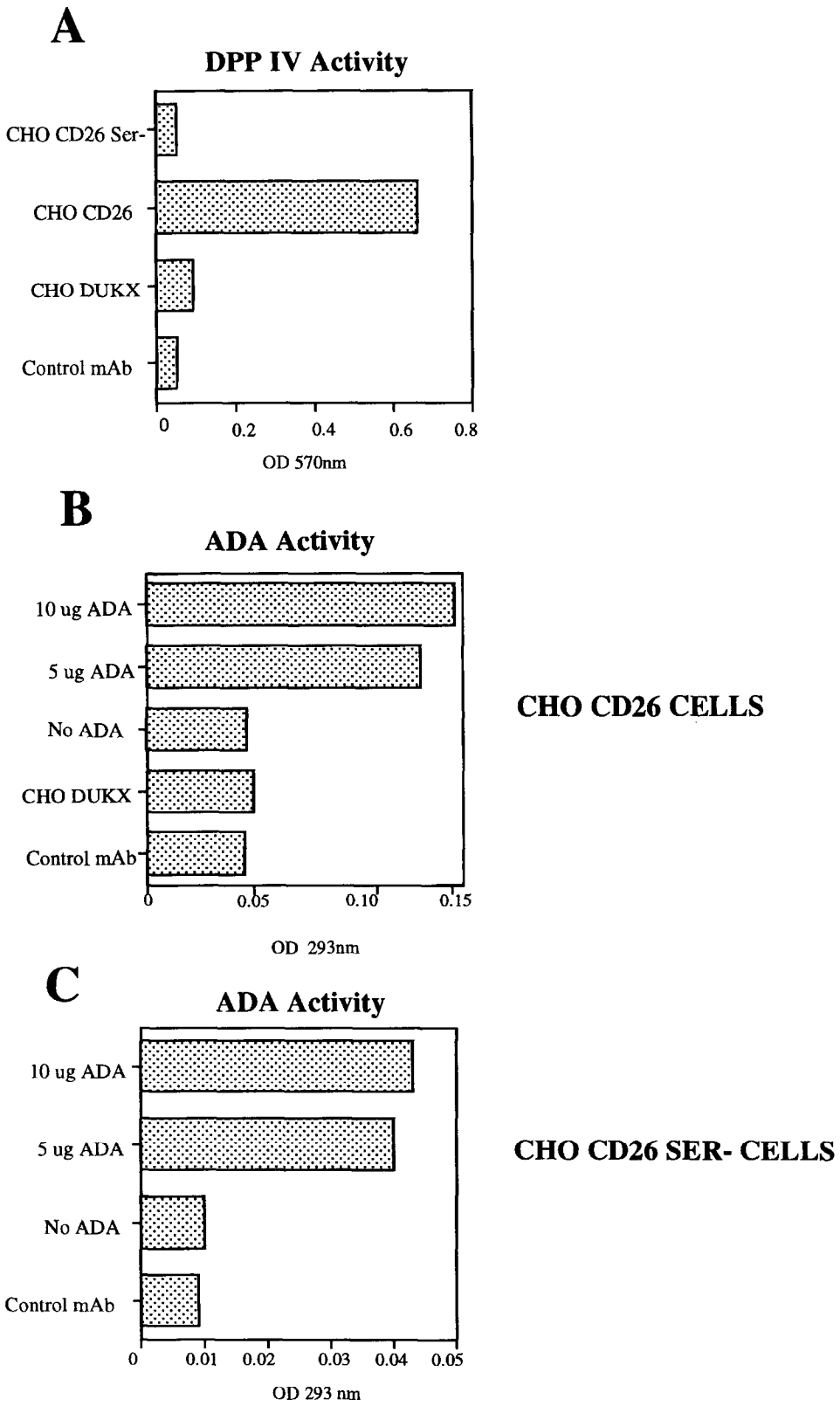

FIG. 2. Co-precipitation of CD26 mutants with exogenous ADA. CHO cells (CHO DUKX), CHO cells transfected with human CD26 (CHO CD26) or CD26 lacking the catalytic site serine residue (CHO CD26 Ser-) were lysed and immunoprecipitated with anti-CD26 mAb or an isotype control antibody (Anti-CD11a). Anti-CD26 immunoprecipitates from the CHO cell lines were assayed directly for DPPIV (Panel A) and ADA activity (Panel B, C; No ADA added) or preincubated with calf spleen ADA (5 or 10 μg/mL, Panels B, C). Control mAb samples for ADA determination indicate the amount of ADA activity non-specifically immunoprecipitated upon the addition of 10 μg of exogenously added ADA to CHO cell lysates. The standard error for each experimental group (n = 3) is less than 5%.

TABLE 2. Genetic ADA deficiency and DPP IV enzymatic activity

Cell lysate	Clinical phenotype	DPP IV K_m (μM)
Peripheral blood lymphocytes	NA*	48.05 ± 5.0
YT Cell line	NA	41.41 ± 1.48
SCID Lymphoblast GM01715	Immunodeficient	49.77 ± 3.4
SCID Lymphoblast GM04258A	Immunodeficient	47.69 ± 4.54
SCID Lymphoblast GM04396A	Normal	52.09 ± 2.0
SCID Lymphoblast GM05816	Normal	50.3 ± 4.59

Data shown are calculated mean K_m ± range from a non-linear regression fit and are representative of 2–3 separate experiments with similar results. NA, not applicable.

catalytic activities of co-associated CD26 and ecto-ADA. These results are consistent with the apparent lack of catalytic regulation observed in the SCID and CHO CD26 catalysis-deficient cell lines.

Sedimentation Velocity Profiles of Free and Complexed CD26 and ADA

In the previous sections, it was determined that the CD26/ecto-ADA complex demonstrated no evidence for allosteric regulation. However, it has been shown previously in T cells that exogenous ADA can potentiate T cell receptor mediated signaling. If catalytic activity is not requisite for function in the CD26/ecto-ADA complex, then it is possible that the CD26/ecto-ADA complex participates in T cell signaling by association with other proteins. To determine if these was any evidence for additional proteins co-associating with CD26 and ADA, cell lysates were layered onto linear sucrose gradients, centrifuged, fractionated and analyzed for DPPIV and ADA activity. The migration profile of DPPIV and ADA activity from recombinant CD26 CHO cells, SCID and YT lymphoid cell lines and stimulated peripheral blood lymphocytes were directly compared (Fig. 3). In both immunodeficient SCID cell lines and the CHO CD26 cell line, the DPPIV activity resides in a single major peak (Fractions 5–15, approximately), and in the CHO CD26 cell line the ADA activity is clearly dissociated from the DPPIV activity (Fig. 3, panels A and B). However, the YT cell line and stimulated peripheral blood lymphocytes had significantly more complex profiles of DPPIV and ADA activity (Fig. 3, panels C and D). Both these cell populations coprecipitate CD26 with ADA, in contrast to the CHO CD26 and SCID cell lines, and there are several peaks of comigratory DPPIV and ADA activity along the entire range of the sucrose gradient. In addition, there was routinely a large peak of comigratory DPPIV and ADA activity far into the gradient, fractions 42–48. This is consistent with larger complexes of proteins in the sucrose gradient, which contain comigrating CD26 and ADA activity and may indicate a functional complex with as yet unidentified proteins involved in signaling.

The affinity of DPPIV for dipeptide substrate was directly compared from the unassociated DPPIV activity (fractions 7–14) and the DPPIV that comigrated with ADA in the large molecular complexes (fraction 42–48). As shown in Table 4, there was a four-fold enhancement of DPPIV affinity when the complexed material was tested vs. the unassociated or free DPPIV. This was seen consistently with YT cells, unstimulated and stimulated peripheral blood lymphocytes. Furthermore, the kinetics of the complexed DPPIV was not modified by deoxycoformycin, nor was the free DPPIV altered by the addition of saturating doses of ex-

TABLE 3. Allosteric interactions of DPP IV and ADA enzymatic activities

Pharmacological agent	DPP IV Activity*	ADA Activity†
No inhibitor	209 ± 42	4.40 ± 0.88
Val-boroPro	‡	3.69 ± 0.72
Inactive diastereomer	210 ± 42	4.99 ± 0.98
Exogenous ADA	236 ± 47	5.77 ± 1.14
ATP	211 ± 42	5.71 ± 1.14
Deoxy ATP	204 ± 40	6.13 ± 1.23
Diadenosine-tetraphosphate	220 ± 44	4.70 ± 0.94
Deoxycoformycin	207 ± 41	‡

Inhibitors were added to immunoprecipitates containing CD26 and ecto-ADA at the following final concentrations: Val-boroPro, 5 ng/mL; inactive diastereomer, 5 ng/mL; Exogenous calf spleen ADA, 100 ng/mL; ATP, 10 μM; Deoxy ATP, 10 μM; Diadenosine tetraphosphate, 10 μM; deoxycoformycin, 100 nM. Data shown are averages in duplicate ± range (N = 2–4).

* nmoles of Ala-Pro-MNA cleaved/10^6 cells.

‡ nmol of uric acid produced/10^6 cells.

‡ Negligible activity measured.

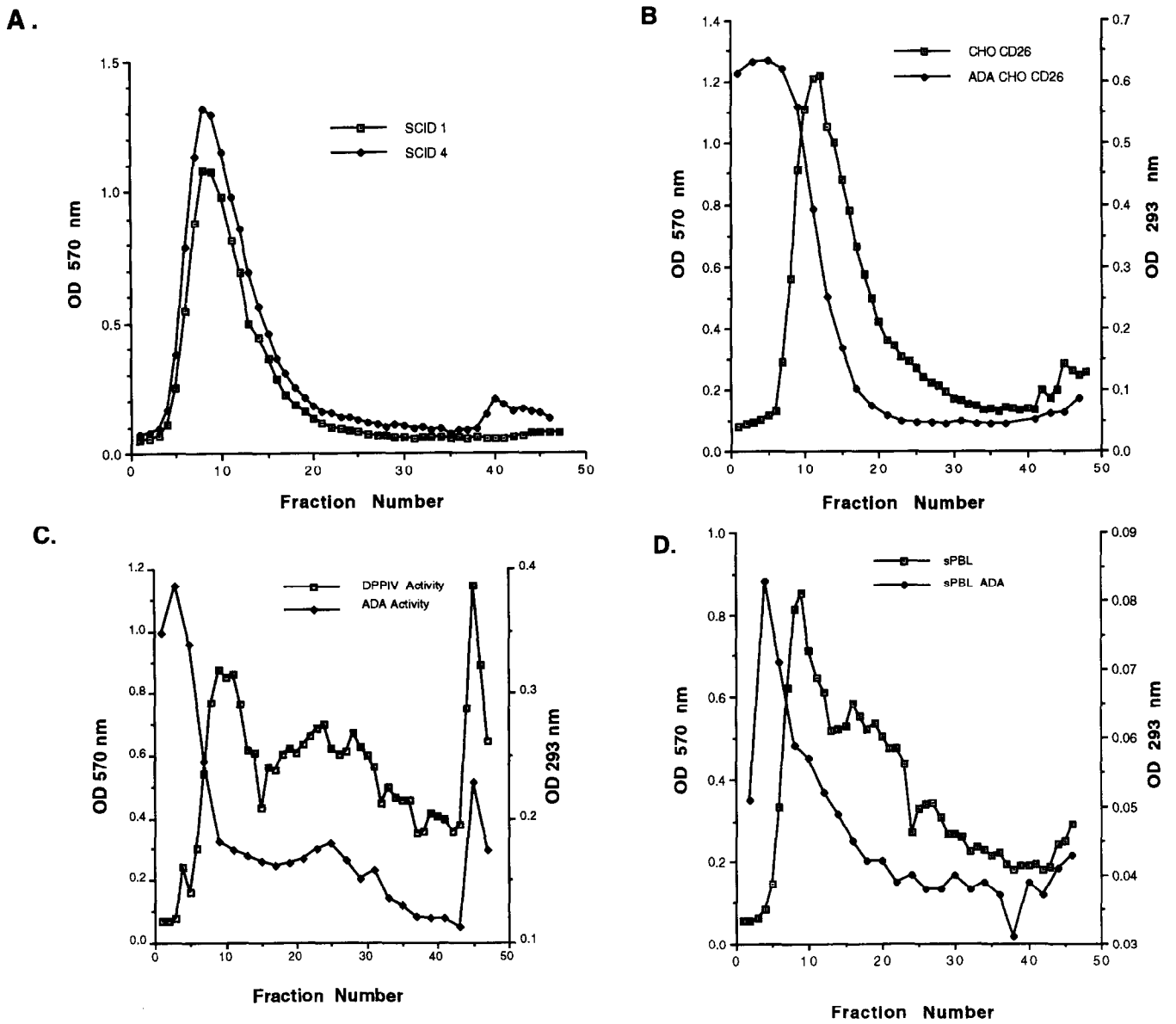

FIG. 3. Sedimentation velocity profiles of CD26 and ADA. Cells or cell lines, were lysed and sedimented on a linear sucrose gradient and fractionated. Each fraction was assayed for DPPIV and ADA activity shown with fraction 1 at the top of the gradient and fraction 48 at the bottom of the gradient. The migration of protein standards, run in parallel with each experimental set, determined the approximate sedimentation value of the peaks shown. Panel A, profile of DPPIV activity from severe combined immunodeficiency disease lymphoblasts with genetic ADA deficiency; Panel B, profile of DPPIV (CHO CD26) and ADA activity (ADA CHO CD26) in CHO CD26 transfected cell line; Panel C, profile of DPPIV and ADA activity in YT lymphoid cell line; and Panel D, DPPIV (sPBL) and ADA (sPBL ADA) profile in mitogen stimulated peripheral blood lymphocytes. The experiments shown are all representative of 2–5 similar runs. OD, optical density.

ogenously added ADA from either calf spleen or human lymphocyte (Table 4). Therefore, the association of CD26 and ecto-ADA with additional proteins may be required for signaling potentiation seen in T cells, and this association can directly modulate the affinity of DPPIV for dipeptide substrate.

DISCUSSION

The functions of adenosine deaminase and CD26 and their individual roles in immune function have been well characterized. However, the physiologic role and regulation of the association of CD26 with ecto-ADA on the plasma membrane of lymphocytes is not well defined. It has been suggested that the increased cell surface expression of CD26 upon lymphocyte stimulation may orient the ecto-ADA to enable it to degrade extracellular adenosine [27]. For lymphocytes there may be a protective role in regulating local serum concentrations of adenosine generated by neighboring cell death or ecto-ATPases. In addition to the role of adenosine as a neurohormone and agonist of many cardiovascular processes [28], adenosine may also serve as a

TABLE 4. DPPIV and ADA complexes: affinity for DPPIV substrate

Sample source	K_m (μM)
YT lysate, free	41.41 ± 1.48
YT lysate, complex	10.42 ± 0.87
PBL's, free	48.05 ± 5.0
PBL's, complex	10.89 ± 1.6
Stimulated PBL's, free	21.19 ± 1.35
Stimulated PBL's, complex	10.87 ± 2.28
YT lysate, complex with deoxycoformycin	9.692 ± 0.8
YT lysate, free with calf ADA	40.81 ± 1.66
YT lysate, free with lymphocyte ADA	40.88 ± 1.28

Lysates from various cell sources were sedimented on linear sucrose gradients, fractionated and subjected to kinetic analysis to determine affinity for DPPIV substrate. Free peaks, with no association of ADA activity, corresponded to fractions 7–14 and complexed fractions, corresponding to high molecular weight complexes containing both DPPIV and ADA activity, corresponded to fractions 42–48. Data shown are calculated mean K_m ± range from a non-linear regression fit and are representative of 2–3 separate experiments with similar results.

signal transducer between B and T cells and an inhibitor of early T cell activation events such as the hydrolysis of PIP_2 [29] and the mobilization of free calcium [30]. Therefore, the sequestration of local adenosine by ecto-ADA may ensure efficient early activation events in T cell response.

Recently, the regulation of the cell surface expression of CD26 molecules on T cells was surveyed with and without stimulation. It is of interest that exogenous ADA was unable to modulate CD26 expression on the lymphocyte surface [31]. CD26 molecules that were not associated with ecto-ADA were found on both resting and activated cell states, and these free molecules could be bound with exogenously added ADA. Exogenously added ADA synergized [^{3}H]-thymidine uptake produced by anti-CD3 mAb. This synergism was not affected by deoxycoformycin [12] and, therefore, required only the binding of ADA, not its catalytic activity. This is consistent with the lack of allosteric interaction that we have observed by inhibiting ADA with deoxycoformycin and measuring no effect on the catalytic function of CD26. These data clearly indicate that the binding of ADA to CD26 on the T cell surface signals CD26 directly, not involving the catalytic activity of ADA.

However, the role of DPPIV enzymatic function as required for T cell signaling is less certain. There are conflicting reports as to the requirement for DPPIV activity of CD26 for its stimulatory and costimulatory role in T cell signaling [32, 33]. At the cellular level inhibitors of DPPIV, including Val-boroPro, are clearly immunosuppressive; however, the exact mechanism of the immune effect is not known [17, 34]. Our data clearly indicate that CD26 enzymatic activity is not requisite for ADA binding, nor does ADA activity have any effect on CD26 DPPIV activity. We have also shown that deglycosylation of CD26 with tunicamycin or deoxymannojirimycin does not effect either DPPIV enzymatic function or affinity for substrate (data not shown). In addition, ADA binding to fixed rabbit membranes expressing CD26 is not dependent on sulfhydryls, divalent cations, EDTA or deoxycoformycin [6].

Taken together, there clearly is no indication that the catalytic activities of CD26 and ecto-ADA regulate the synergism noted in T cell signaling. This suggests that signaling of the complex may require the association of alternate proteins. As CD26 has a tiny cytoplasmic tail, six amino acids, the association of CD26 and ecto-ADA with alternate, presently unidentified protein(s) could mediate signaling, and these interactions may involve the association with the extracellular domains of alternate proteins with the CD26 and ADA molecules. In this regard, CD26 has been reported to associate with CD45, an integral membrane phosphatase [35]. Signaling and cosignaling in T cells via the CD26 complex may therefore involve an induced conformation in an associated molecule which can directly signal T cells, such as CD45.

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