by Kunkel *et al.* [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)]. The template DNA for mutagenesis was prepared from M13 mp19 containing the cDNA encoding *Drosophila* TFIIB, harboring an Nde I site at the translation initiation site. For NH_2-terminal deletion mutants, deoxyoligonucleotides that introduce an Nde I site (CATATG) at the deletion points were used; mutants were confirmed by digestion of replicative form (RF) DNA with Nde I. The COOH-terminal deletion mutants were made with deoxyoligonucleotides that create the termination codons and Hind III restriction sites (TAAGCTT) at the indicated positions; mutants were detected by digestion of RF DNA with Hind III. For amino acid substitutions, deoxyoligonucleotides that introduce the desired amino acid changes were used, and mutants were confirmed by DNA sequencing. For bacterial expression, RF DNA was digested by Nde I and Eco RI and subcloned into Nde I– and Eco RI–digested 6His-pET-5a [(*16*); F. W. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorff, *ibid.* **185**, 60 (1990)].

9. *Escherichia coli* BL21 harboring the TFIIB expression plasmid were grown in TBG medium to an OD at 600 nm of 0.8; protein synthesis was then induced with 0.5 mM isopropyl β-D-thiogalactoside at 37°C for 3 hours. Cells were collected by centrifugation (6000*g*, 10 min) and resuspended in 1/10 culture volume of buffer B [20 mM tris-HCl (pH 7.9), 0.2 mM EDTA, 10% glycerol, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride] containing 1 mM imidazole, 0.1% Nonidet P-40, and 500 mM KCl. Cell suspensions were incubated on ice for 15 min and then disrupted by sonication. After centrifugation (8000*g*, 20 min), supernatant was applied onto a Ni-agarose column (Qiagen, Studio City, CA), equilibrated with buffer B containing 10 mM imidazole and 100 mM KCl. After washing with the same buffer, protein was eluted with buffer B containing 100 mM imidazole and 100 mM KCl. The COOH-terminal deletion and the second basic repeat mutants, which are mostly insoluble under these conditions, were prepared as follows: The cell pellet was suspended in 1/10 culture volume of buffer B containing 6 M guanidine HCl and 500 mM KCl, and disrupted by a short pulse of sonication. After centrifugation (8000*g*, 20 min), supernatant was applied onto a Ni-agarose column. After washing with buffer B containing 6 M guanidinium-HCl, 500 mM KCl, and 10 mM imidazole, protein was eluted with the same buffer containing 100 mM imidazole. The eluate was then dialyzed against buffer B containing 2 M guanidine-HCl and 500 mM KCl for 2 hours at 4°C and then dialyzed against buffer B containing 1 M guanidine HCl and 100 mM KCl overnight at 4°C.
10. In vitro transcription was carried out as described (*6*), except that the native human TFIID fraction was used. The template plasmids used in each experiment are described in the appropriate figure legends.
11. ^{32}P–end-labeled double-stranded DNA with the adenovirus major late promoter sequence (−40 to +10 of the transcription initiation site) was used as a probe. Each complete system contained 5 × 10^5 dpm of probe (about 50 fmol), poly(dGdC) (10 μg/ml) (Pharmacia), 35 mM Hepes-KOH buffer (pH 8.0), 7.5 mM $MgCl_2$, 6% (v/v) glycerol, 60 mM KCl, 6 mM DTT, 60 μM EDTA, *Drosophila* TFIIDτ (0.5 μg/ml), and TFIIB (1.2 μg/ml) in a total volume of 25 μl. After incubation at 30°C for 40 min, products were analyzed on a 4% polyacrylamide gel (59:1) containing TBE buffer [89 mM tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0)] and 3% (v/v) glycerol at 100 V with the use of TBE buffer as a running buffer.
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Direct Association of Adenosine Deaminase with a T Cell Activation Antigen, CD26

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CD26, the T cell activation molecule dipeptidyl peptidase IV (DPPIV), associates with a 43-kilodalton protein. Amino acid sequence analysis and immunoprecipitation studies demonstrated that this 43-kilodalton protein was adenosine deaminase (ADA). ADA was coexpressed with CD26 on the Jurkat T cell lines, and an in vitro binding assay showed that the binding was through the extracellular domain of CD26. ADA deficiency causes severe combined immunodeficiency disease (SCID) in humans. Thus, ADA and CD26 (DPPIV) interact on the T cell surface, and this interaction may provide a clue to the pathophysiology of SCID caused by ADA deficiency.

CD26, a T cell activation molecule (*1*, *2*), is a 110-kD glycoprotein that is also present on epithelial cells of various tissues, including the liver, kidney, and intestine. CD26 is identical with dipeptidyl peptidase IV (DPPIV) (*3*), which can cleave NH_2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position. No physiological substrates have yet been identified. We isolated cDNA encoding human CD26 and established CD26-transfected Jurkat T cell lines (*4*). Functional analysis of these Jurkat transfectants showed that cross-linking of the CD26 and CD3 antigens with their respective antibodies (Abs) resulted in enhanced intracellular calcium mobilization and interleukin-2 production, providing direct evidence that the CD26 antigen plays an integral role in T cell activation. The cDNA sequence of CD26 predicted a type II membrane protein with only six amino acids in the cytoplasmic region (*4*, *5*), suggesting that other association molecules are involved in CD26-mediated signal transduction. CD26 associates with CD45 (*6*), which might be involved in regulating the $p56^{lck}$ activity through its protein phosphatase activity. Another candidate for the signal transduction molecule was a 43-kD protein, p43, which can be coprecipitated by antibody to CD26 (anti-CD26) from ^{125}I-labeled T cells, phytohemagglutinin (PHA) blast cells, and from CD26-transfected Jurkat cell lines (*4*).

To identify p43, we purified the protein by immunoaffinity chromatography from CD26-transfected Jurkat cells. The purified protein was separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and stained by Ponceau S, resulting in a single band of 43 kD in addition to the CD26 110-kD protein (Fig. 1). The 43-kD protein was then digested with trypsin, separated by reversed-phase high-pressure liquid chromatography (rpHPLC), and subjected to amino acid sequencing. According to the homology search, the amino acid sequences of the two peptides derived from p43 were completely identical to those of residues 35 to 64 and 172 to 206 of the human adenosine deaminase (ADA) (*7*).

ADA is a 41-kD protein, expressed in all tissues (highest expression in lymphocytes), that catalyzes the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. ADA is present on the cell surface, as well as in the cytoplasm, of human fibroblasts, rabbit renal tubular cells, and human mononuclear blood cells (*8*). ADA deficiency causes severe combined immunodeficiency disease (SCID) in humans (*9*), yet no direct interaction between ADA and T cell surface molecules has been identified.

The possibility that CD26 is associated with ADA was investigated by biochemical analysis with polyclonal rabbit Ab to ADA (anti-ADA) (*10*). Immunoblotting with anti-ADA after immunoprecipitation from CD26 transfectants by various Abs demon-

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strated that ADA is coprecipitated by anti-CD26 but not by Ab to CD29 (anti-CD29), CD45 (anti-CD45), or major histocompatibility complex (MHC) class I molecules (anti-MHC) (Fig. 2A). Immunoprecipitation of ^{125}I-labeled CD26 transfectants by anti-ADA showed not only a faint band of 43 kD but also coprecipitation of a 110-kD protein with the same electrophoretic mobility as that of CD26 molecule (Fig. 2B). The association of the 110-kD protein with ADA was also observed in PHA blasts, in which CD26 was less abundantly expressed (*11*). We verified the identity of p43 as ADA and the 110-kD protein as CD26 in two ways, sequential immunodepletion and peptide mapping. In preclearing studies, depletion of ADA resulted in complete loss of p43, as well as marked reduction of CD26 (Fig. 2C). The marked reduction of the CD26 immunoprecipitate implied that nonlabeled ADA originally localized in the cytoplasm may also bind to CD26 during the lysis process. V8 protease–dependent digestion of p43 and ADA yielded the same peptide patterns (Fig. 2D, lanes 1 and 2). Likewise, CD26 and the 110-kD protein associated with ADA also gave the same peptide patterns (Fig. 2D, lanes 3 and 4). It is unlikely that anti-CD26 cross-reacts with ADA, because ADA cannot be detected in immunoblotting by anti-CD26 (*12*). From these results we concluded that the 43-kD protein that coprecipitated with CD26 was ADA.

To examine whether ADA was coexpressed with CD26 on the surface of T cells and T cell lines, we carried out immunofluorescence analysis by flow cytometry. The expression of CD26 and ADA on Jurkat T cell lines was tightly linked (Fig. 3A). ADA was not expressed on vector-only–transfected Jurkat cells (CD26-negative), weakly expressed on CD26 low transfectants, and strongly expressed on CD26 high transfectants. Surface expression of ADA was not detected (<5%) on resting T cells, but became detectable (20 to 30%) after activation by PHA (Fig. 3A). In earlier studies, we showed that anti-CD26 treatment modulated the surface expression of CD26 through internalization, resulting in the enhanced proliferative activity of T cells through the T cell receptor (TCR)–CD3 pathway (*13*). In the present study, we demonstrated that ADA expression was comodulated with CD26 on the surface of CD26-transfected Jurkat cells after anti-CD26 treatment (Fig. 3B). The cell surface ADA appeared to be only a small portion of the total cellular ADA, because the total amount of ADA detected by immunoblotting from the whole lysates showed no difference between vector-only transfectants and CD26 transfectants. The mechanism by which ADA was coexpressed with CD26 on the cell surface is unknown (*14*).

The specificity of the association of ADA with CD26 was confirmed by in vitro

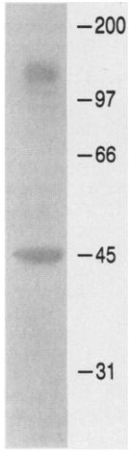

Fig. 1. Ponceau-S–stained nitrocellulose blot of immunoaffinity-purified p43 separated by 8% SDS-PAGE. The purification of p43 was as described in (*28*).

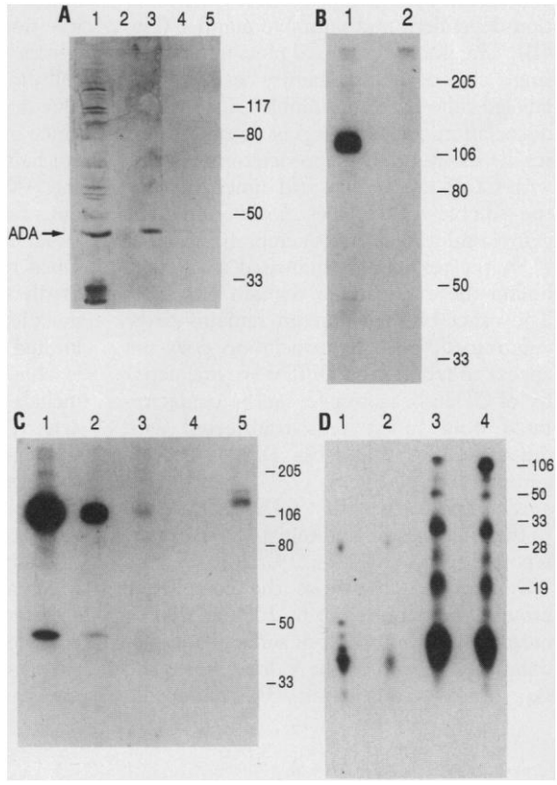

Fig. 2. Physical association of ADA with CD26. Immunoblotting and immunoprecipitation studies were carried out with CD26-transfected Jurkat cells (CD26.11 in Fig. 3) by SDS-PAGE under reducing conditions as described in (*29*). (**A**) Immunoblotting with anti-ADA after immunoprecipitation with various Abs. Lane 1, whole lysate; lane 2, immunoprecipitate with anti-CD29 (4B4); lane 3, anti-CD26 (1F7); lane 4, anti-CD45 (GAP 8.3); lane 5, anti–MHC class I (W6/32). (**B**) Coimmunoprecipitation of a 110-kD molecule with ADA. Lane 1, anti-ADA; lane 2, control rabbit Ab. (**C**) Loss of the 43-kD molecule in the CD26 immunoprecipitate after depletion of ADA. The lysates were precleared by control rabbit Ab (lanes 1 and 2) or anti-ADA (lanes 3 to 5) and then immunoprecipitated with anti-CD26 (lanes 1 and 3), anti-ADA (lanes 2 and 4), and anti-CD29 (4B4, lane 5). (**D**) Comparison of p43 with ADA and of the 110-kD protein with CD26 by peptide mapping with V8 protease as described (*30*). Lane 1, ADA; lane 2, p43; lane 3, 110-kD protein associated with ADA; lane 4, CD26. Molecular sizes are shown on the right (in kilodaltons).

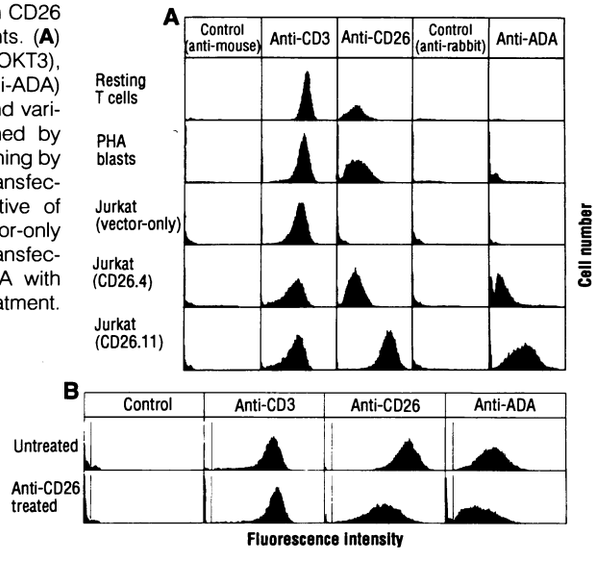

Fig. 3. Coexpression of ADA with CD26 on T cells and Jurkat transfectants. (**A**) Surface expression of CD3 (OKT3), CD26 (1F7), and ADA (rabbit anti-ADA) on resting T cells, PHA blasts, and various Jurkat transfectants examined by indirect immunofluorescence staining by flow cytometry. Three Jurkat transfectants presented are representative of eight clones tested (three vector-only transfectants and five CD26 transfectants). (**B**) Comodulation of ADA with CD26 induced by anti-CD26 treatment. CD26-transfected Jurkat cells (CD26.11) were incubated overnight at 37°C in either medium alone or medium containing anti-CD26 (1F7, 1:100 ascites) as described (*6*). Cells were then stained with anti-CD26 (or second Ab alone), rabbit anti-ADA, and biotinylated anti-CD3.

binding assay. For this purpose, we used purified recombinant soluble CD26 (*15*) and calf spleen ADA (*16*). Binding of ^{125}I-labeled ADA to the soluble CD26-conjugated agarose beads was 80 times greater than that of control beads and was inhibited by unlabeled ADA (Fig. 4A). The binding was also inhibited by rabbit antiserum against whole ADA, but not by the polyclonal Ab against the COOH-terminal peptide of ADA used in Figs. 2 and 3. Binding of ^{125}I-labeled ADA to CD26-conjugated beads occurred in a concentration-dependent and saturable manner (Fig. 4B). The linear Scatchard plots indicated a single class of binding affinity between ^{125}I-labeled calf ADA and soluble CD26 with a dissociation constant (K_d) of 65 nM. These results showed that the association of ADA with CD26 was specific and direct. Because the soluble CD26 does not contain the cytoplasmic and transmembrane regions (*15*), the results also indicated that ADA bound the extracellular domain of CD26. The exact binding domain remains to be determined, but this association does not appear to require the DPPIV enzyme activity of CD26 because p43 can be coprecipitated from Jurkat cells transfected with mutant CD26 that lacks DPPIV enzyme activity (*17*).

Our data suggest that CD26 is identical to the ADA binding protein (ADABP) (*18*) reported previously (*8, 19, 20*). Human ADABP, also known as the complexing protein, was reported to be 108 and 95 kD, mostly located on the cell surface, and predominantly found in the kidney, liver, and skin fibroblasts. It binds ADA extracellularly, because the binding occurs on intact fibroblasts. The function of the ADABP is still unknown, although some functional roles have been proposed, such as positioning of ADA on the plasma membrane or acting as a cell surface receptor for ADA. The characteristics of ADABP are compatible with those of CD26. The genes encoding human ADABP and human DPPIV were both assigned to chromosome 2 (*21, 22*). Rabbit ADABP binds ADA from various species, except rodents (*19, 20*). This is also in agreement with our finding that p43 was not coprecipitated by CD26 Ab from lysates of human CD26–transfected 300.19 cell line, a mouse pre-B cell line (*23*). Questions have been raised about the importance of the ADABP because of the apparent failure to bind rodent ADA (*19*), but if the ADABP is CD26, the association warrants further investigation.

ADA deficiency (*9*), inherited as an autosomal recessive trait, constitutes about one-fourth of human SCID, primarily as a result of mutations or deletions of the ADA gene located on chromosome 20. The immunologic abnormalities common in ADA$^-$ SCID include a reduced number of T cells and defective responses of T cells to mitogens or antigens in in vitro assays. The mechanism underlying T cell dysfunction has been attributed to the accumulation of toxic purine substrates and metabolites, particularly deoxyadenosine triphosphate and deoxyadenosine, which are considered to interfere with T cell activation and proliferation by inhibiting ribonucleotide reductase activity. Yet, some patients with ADA$^-$ SCID respond poorly to the enzyme replacement therapy by polyethylene glycol–modified ADA even though the concentration of deoxyadenosine is decreased as effectively as in patients who experience greater immune reconstitution (*24*). In addition, the inhibition of ADA activity by deoxycoformycin results in reduction of TCR CD3-mediated inositol phospholipid hydrolysis and calcium flux, implying the possible involvement of ADA in early events of T cell activation (*25*). Because CD26 appears to be important as an accessory molecule for T cell activation, an association of ADA with CD26 suggests that ADA may be directly involved in T cell activation through interaction with CD26. In contrast to the prevailing concept that ADA is a simple housekeeping gene that does not require any regulation, we propose that ADA, at least ADA on the T cell surface, might be regulated during the process of T cell activation. Extracellular adenosine has been proposed as a regulator of many processes, such as mediating electrophysiologic action on cardiac supraventricular tissues (*26*); adenosine may also serve as a signal transducer between B and T cells (*27*). Perhaps CD26 may be involved in regulating the extracellular concentration of ADA, which may in turn regulate the extracellular concentration of adenosine.

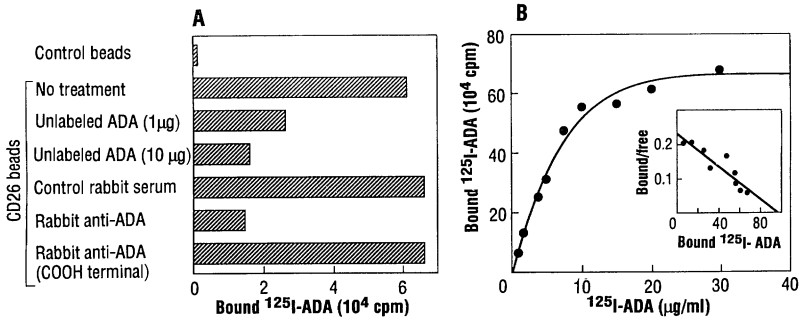

Fig. 4. In vitro binding assay between human soluble CD26-conjugated beads and ^{125}I-labeled calf ADA. One milligram of the soluble CD26 (*15*) was coupled to 2.5 ml of agarose beads (Affi-Gel). Fifty micrograms of ADA [Sigma; type X, from calf spleen (*16*)] was labeled with ^{125}I to a specific activity of 1×10^4 cpm per nanogram of protein with Iodobeads (Pierce Chemical Co., Rockford, Illinois). (**A**) The ^{125}I-labeled ADA (3 μg/ml) was incubated with 50 μl of soluble CD26-conjugated agarose beads or control beads in the binding buffer [50 mM tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 0.05% NaN_3] at a final reaction volume of 150 μl at 4°C for 4 hours in the presence of 25 μl of binding buffer alone, unlabeled ADA (1 or 10 μg), control rabbit serum (1:50 dilution), rabbit anti-ADA (1:50 dilution), and rabbit anti-ADA (against COOH-terminal peptide of ADA used in Figs. 2 and 3, 1:50 dilution). After binding, the beads were washed extensively in washing buffer [200 mM tris-HCl (pH 8.0), 500 mM NaCl, 0.1% NP-40], and bead-bound radioactivity was counted. (**B**) Various concentrations (1 to 30 μg/ml) of ^{125}I-labeled ADA were incubated with 50 μl of soluble CD26-conjugated agarose beads as described in (A). Direct plot analysis and Scatchard plot analysis are presented. The apparent K_d for ^{125}I-labeled calf ADA binding to CD26 was 65 nM. Data are representative of results from four separate experiments.

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12. Further evidence against cross-reactivity was as follows: (i) ADA can be coprecipitated by other monoclonal antibodies to CD26, such as Ta1, which recognizes an epitope on CD26 different than that recognized by 1F7; (ii) the predicted amino acid sequences from the cDNAs of CD26 and ADA showed no homology; and (iii) the positive results from the in vitro binding assay described below.
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14. Because the predicted amino acid sequence from the cDNA of ADA contains no hydrophobic

domains (*7*), there may be another cDNA that encodes a secreted form of ADA. If the ADA on the cell surface is encoded by the same cDNA as the internal ADA, then (i) ADA may be expressed on the surface after binding to CD26 inside the cells; (ii) membrane ADA may be expressed by a yet-to-be defined mechanism not requiring hydrophobic domains, as is predicted from the secretions of the interleukin-1s and the fibroblast growth factors; or (iii) the ADA expressed on the cell surface may be derived from dead cells.

15. T. Tanaka *et al.*, unpublished data. The soluble form of CD26 was purified by concanavalin-A and anti-CD26 (1F7) affinity chromatography from culture supernatants of CHO cells transfected with mutant CD26 lacking the codons for amino acids 3 to 9. SDS-PAGE followed by a staining with Coomassie brilliant blue revealed one single band of 110 kD.
16. The purity of the calf spleen ADA was greater than 90% by SDS-PAGE. Calf spleen ADA was used because human ADA was not available, but we presume that the positive results with calf ADA were highly suggestive of the similar interaction between human CD26 and human ADA. The use of calf ADA was also suggested on the basis of evidence that characteristics of the binding between ADA and the ADA binding protein are well conserved among various species (*20*).
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28. Purified monoclonal antibody to CD26 (1F7) (*2*) was coupled to an immunoaffinity support (Affi-Gel 10, Bio-Rad) and transferred to a column. CD26-transfected Jurkat cells (CD26.11 in Fig. 3, total of 5×10^9 cells) were lysed in lysis buffer [0.5% NP-40, 50 mM tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were applied to the column and washed with 50 mM tris-HCl buffer (pH 8.0) containing 0.5% NP-40. Bound proteins were eluted with 0.2 M glycine (pH 2.5) and neutralized with one-quarter volume of 1 M tris-HCl (pH 8.0). Peak fractions were collected after estimation by absorbance at 280 nm. The samples were concentrated with a Centricon 10 (Amicon, Beverly, MA), electrophoresed in 8% SDS-PAGE, transferred to nitrocellulose membranes, stained with Ponceau S (Sigma), and the bands cut out. The proteins on the nitrocellulose pieces were digested with trypsin, and the products were resolved by narrow-bore rpHPLC as described [W. S. Lane, A. Galat, M. W. Harding, S. L. Schreiber, *J. Prot. Chem.* **10**, 151 (1991)]. Two optimum fractions were selected after the molecular size of the proteins was confirmed by matrix-assisted laser desorption mass spectrometry. Sequence analysis of eluted peptides was done on a protein sequencer (Applied Biosystems, Model 477A).
29. Immunoprecipitation was done as described (*6*). For immunoblotting studies, after SDS-PAGE and transfer to nitrocellulose membranes the blots were blocked for 2 hours with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), probed with anti-ADA (1:200 dilution) in PBS containing 1% BSA overnight at 4°C, washed, and visualized with alkaline phosphatase–conjugated goat Ab to rabbit immunoglobulin G (1:2000 dilution) and nitro blue tetrazolium and bromochloroindolyl phosphate (Promega Biotec.).
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Atherosclerosis in Transgenic Mice Overexpressing Apolipoprotein A-II

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Concentrations of plasma high density lipoprotein (HDL) are inversely correlated with atherosclerotic coronary artery disease. The two most abundant protein constituents of HDL are apolipoproteins A-I and A-II (apoA-I and apoA-II). ApoA-I is required for assembly of HDL and, when overexpressed in transgenic mice, confers resistance to early atherosclerosis. The present studies reveal that transgenic mice that overexpress mouse apoA-II had elevated HDL-cholesterol concentrations but, nevertheless, exhibited increased atherosclerotic lesion development as compared to normal mice. The HDL in the transgenic mice was larger and had an increased ratio of apoA-II to apoA-I. Thus, both the composition and amount of HDL appear to be important determinants of atherosclerosis.

High density lipoproteins (HDL) are particles found in the blood plasma that contain approximately equal parts lipid and protein. The most abundant proteins of HDL are apolipoproteins A-I and A-II (apoA-I and apoA-II). Low concentrations of HDL in the plasma are associated with increased risk of atherosclerotic coronary artery disease (CAD), but the mechanisms responsible have remained elusive (*1–5*). HDL may protect against CAD by removing excess cholesterol from peripheral tissues and transporting it to the liver, a process termed "reverse cholesterol transport". Alternatively, the association of atherosclerosis with low HDL levels may reflect the metabolism of triglyceride-rich and other atherogenic lipoproteins, as suggested by the observation that rare genetic syndromes resulting in less HDL are not always associated with increased CAD risk (*2, 5, 6*). Fractionation of HDL by electrophoresis and immunochemical procedures has revealed several subpopulations differing in size and apolipoprotein composition (*7–9*). Several, but not all, studies have shown that HDL containing both apoA-I and apoA-II are less effective at promoting cholesterol efflux from tissue culture cells than are HDL containing apoA-I alone (*10–14*). We report here that overexpression of mouse apoA-II in transgenic mice results in an increase in the number and size of fatty streak atherosclerotic lesions, even though total plasma HDL-cholesterol levels are significantly elevated.

Transgenic mice overexpressing human apoA-I show elevated plasma HDL-cholesterol (*15, 16*) in association with reduced aortic fatty streak development when maintained on atherogenic diets high in fat and cholesterol (*17*). Whether this is a result of alterations in HDL subpopulations or increased HDL levels is unclear. This observation suggests that HDL is directly involved in the pathogenesis of atherosclerosis. Studies of naturally occurring variations of apoA-II gene expression in mice suggest that apoA-II can influence both HDL amounts and apolipoprotein compositions (*18, 19*). To examine the contribution of HDL subpopulations to the early stages of atherosclerosis, we have constructed transgenic mice overexpressing mouse apoA-II.

Transgenic mice were constructed using a genomic clone of the mouse apoA-II gene containing several kilobases of 5′ flanking and 3′ flanking DNA (*20*). Founder transgenic mice were backcrosses derived from a cross of C57BL/6J with C57BL/6J × DBA/2J heterozygotes. All of the mice used in this study were derived from a line developed from one of the founder mice by backcrossing for two generations to strain C57BL/6J mice, such that on average 93% of the genetic back-

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