Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity

Andrey V. ZAVIALOV*¹ and Åke ENGSTRÖM†

*Institute of Immunological Engineering, 142380 Lyubuchany, Moscow Region, Russia, and †Department of Medical Biochemistry and Microbiology, Uppsala Biomedical Centre, Uppsala University, Box 582, SE-751 23 Uppsala, Sweden

Two distinct isoenzymes of ADA (adenosine deaminase), ADA1 and ADA2, have been found in humans. Inherited mutations in ADA1 result in SCID (severe combined immunodeficiency). This observation has led to extensive studies of the structure and function of this enzyme that have revealed an important role for it in lymphocyte activation. In contrast, the physiological role of ADA2 is unknown. ADA2 is found in negligible quantities in serum and may be produced by monocytes/macrophages. ADA2 activity in the serum is increased in various diseases in which monocyte/macrophage cells are activated. In the present study, we report that ADA2 is a heparin-binding protein. This allowed us to obtain a highly purified enzyme and to study its biochemistry. ADA2 was identified as a member of a new class of ADGFs

(ADA-related growth factors), which is present in almost all organisms from flies to humans. Our results suggest that ADA2 may be active in sites of inflammation during hypoxia and in areas of tumour growth where the adenosine concentration is significantly elevated and the extracellular pH is acidic. Our finding that ADA2 co-purified and concentrated together with IgG in commercially available preparations offers an intriguing explanation for the observation that treatment with such preparations leads to non-specific immune-system stimulation.

Key words: adenosine deaminase (ADA), cat eye syndrome critical region candidate 1 (CECR1), growth factor, heparin, immunoglobulin G (IgG), macrophage.

INTRODUCTION

Adenosine deaminase (ADA; EC 3.5.4.4) catalyses the deamination of adenosine and 2'-deoxyadenosine to inosine and deoxyinosine. Two different isoenzymes of ADA designated as ADA1 and ADA2 were found in mammals and lower vertebrates [1]. In humans, almost all ADA activity is attributed to a single-chain Zn-binding protein ADA1 [1]. This 35 kDa protein can form a 280 kDa complex [2] with a dimer of DPPIV (dipeptidyl peptidase IV) or CD26, which cleaves N-terminal dipeptides from a variety of biologically active peptides related to immune function [3]. ADA1 is present in all human tissues and in erythrocytes, and a small amount of the enzyme circulates in plasma [4]. Genetic absence of ADA1 in infants leads to SCID (severe combined immunodeficiency) [5]. This discovery stimulated intense investigation of ADA1 biochemistry, structure and function [6,7]. The crucial role of ADA1 in adaptive immune system development and, in particular, in T-cell proliferation was explained by its catalytic activity, which is required to reduce the local concentration of adenosine, a powerful immunosuppressant [5]. In lymphocytes, cell-surface ADA1 is associated with adenosine receptors A₁ [8] and A_{2B} [9] and thus can regulate those actions of adenosine mediated by these receptors. Considerable efforts have been made to study the specific binding properties of ADA1 to the T-cell surface-localized CD26 receptor. ADA1 binding to human CD26 is inhibited by the HIV-1 glycoprotein gp120, providing one possible explanation for the compromised CD4 cell function in this disease [10].

A significant component of overall ADA activity in plasma and liver of lower vertebrates comes from another ADA isoenzyme, named ADA2, with a molecular mass of approx. 100 kDa [1]. In

contrast with ADA1, the biochemical properties and physiological role of ADA2 have not been determined. In chicken liver, ADA2 and ADA1 have almost equal ADA activity, although the catalytic parameters of ADA1 and ADA2 drastically differ [11]. An isoenzyme of similar molecular mass and catalytic parameters to the ADA2 of lower vertebrates is present in small amounts in livers of SCID patients with ADA1 deficiency [12] and is abundant in human plasma. Recently, it was shown that activated rat macrophages produce extracellular ADA2 [13]. HIV-1-infected T-cells [14] and B-cells [12] might also be a source of ADA2 in plasma. ADA2 activity is profoundly elevated in plasma from patients suffering from liver diseases, such as chronic hepatitis and cirrhosis, AIDS, adult T-cell leukaemia, acute lymphoblastic leukaemia, tuberculosis and diabetes mellitus [4,15–17]. However, it is puzzling that ADA2, an enzyme more abundant in plasma than in ADA1, has a Michaelis constant $K_m = 2$ mM, which is several orders higher than the concentration of adenosine in plasma (0.1 μ M) [18], suggesting that the rate of adenosine deamination catalysed by ADA2 is close to zero at physiological adenosine concentrations. This fact might indicate that adenosine is not the main substrate of ADA2. However, the analysis of ADA2 interactions with a wide spectrum of adenosine analogues and derivatives established that adenosine is the only substrate for this enzyme [19]. Another explanation for the high K_m value of ADA2 was that ADA2 is a derivative of an ADA1 isoform, which has lost its affinity for adenosine due to mutations in its catalytic centre. This hypothesis was also at variance with several observations. First, antibodies specific to ADA1 are not cross-reactive with partially purified ADA2 [12]. Secondly, ADA2 failed to form a complex with DPPIV (CD26) [12]. Thirdly, the level of ADA2 in some animals is

Abbreviations used: ADA, adenosine deaminase; ADGF, ADA-related growth factors; CES, cat eye syndrome; CECR1, CES critical region candidate 1; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; HSPG, heparan sulphate proteoglycan.

¹ To whom correspondence should be addressed (email andrey_zavialov@mail.ru).

comparable with ADA1 [1]. Fourthly, only certain cell types express ADA2 in humans, whereas ADA1 is present in all cells [4]. Fifth, the optimum pH for ADA2 activity is different from ADA2 (pH 6.5 and 7.5 for ADA2 and ADA1 respectively); ADA2 displays lower sensitivity to many specific inhibitors of ADA1 and is more stable at high temperatures than ADA1 [12]. Taken together, these observations argue strongly against the hypothesis that ADA2 is a simple derivative of ADA1. A third hypothesis to explain the existence of two different isoenzymes was based on evolutionary selection resulting in ADA1 dominance in mammals [12]. However, the persistent presence and regulated expression of ADA2 in the setting of human disease argues against this explanation.

Despite several attempts to purify ADA2 from different organisms [20,21], the ADA2 gene has not been identified. In the present study, we report the purification of human ADA2 and identify it as a product of the CECR1 (CES critical region candidate 1, where CES stands for cat eye syndrome) gene, a member of a novel family of ADGFs (ADA-related growth factors). Our work should facilitate the investigation of the physiological function of ADA2 and may lead to its use as an informative biomarker for human diseases.

EXPERIMENTAL

Materials

All nucleotides and ADA1 inhibitor EHNA [erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride] were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). All other chemicals were from Merck Chemicals (Darmstadt, Germany). IgG preparations for intramuscular injection [1, Immunopreparat (Ufa); 2, State Unitary Enterprise of Bacterial Preparations (Omsk); 3, State Enterprise Virion (Tomsk); 4, Biomed (Perm); 5, State Enterprise of Bacterial Preparations (Irkutsk); 6, Pasteur State Enterprise of Bacterial Preparations (S-Peterburg); 7, State Enterprise of Bacterial Preparations (Kazan); 8, Gabrichevsky State Unitary Enterprise of Bacterial Preparations (Moscow); 9, State Enterprise of Bacterial Preparations (Khabarovsk); 10, Mechnikov Biomed (Moscow)] were obtained from L. A. Tarasevich State Institute of Standardization and Control of Biomedical Preparations (Moscow, Russia). A probe was obtained from Gabrichevsky State Enterprise of Bacterial Preparations (Moscow, Russia) that contains different plasma pools from more than 1000 healthy donors.

Enzymatic activity of ADA

To determine ADA activity, 20 μ l of IgG preparation and 10 μ l of plasma were incubated at 37 °C with 34 mM adenosine in 400 and 100 μ l of 0.1 M Pipes (pH 6.5) respectively in microcentrifuge tubes (1.5 ml). The incubation time (5 h for IgG preparation and 15 h for plasma) was selected to get less than 10 % conversion of adenosine into inosine. To separate ADA2 and ADA1 activities, the ADA1 inhibitor EHNA was added into the incubation mixture to a final concentration of 0.1 mM and the reaction mixture was diluted to 1 ml with cold buffer A (0.01 M sodium phosphate buffer, pH 7.3) and kept on ice. To remove excess proteins, the samples were boiled for 2 min and the protein precipitate was separated by centrifugation at 10000 *g* for 5 min. The supernatant (400 μ l) was applied to an XK 16/20 column (GE Healthcare, Piscataway, NJ, U.S.A.) with 10 ml of Toyopearl TSK HW-40 S gel (Sigma–Aldrich) equilibrated with buffer A. The peaks of inosine and adenosine were subsequently eluted from the column at a flow rate of 2.5 ml/min. The chromatography and the peak area

analysis were performed using either FPLC/ÅKTA explorer (GE Healthcare) or Hitachi 7000 Series HPLC (Hitachi, Tokyo, Japan) purification systems. For routine ADA analysis, the samples were injected using an L-7200 autosampler (Hitachi).

The ADA activity in units/l was determined using the formula:

$$1 \text{ unit/l} = S_{\text{inosine}} / (S_{\text{inosine}} + S_{\text{adenosine}}/f) k_{\text{dilution}} C_M k_{\text{correction}} \\ \times 10^6 / \tau \text{ (}\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}\text{)}$$

where S_{inosine} and $S_{\text{adenosine}}$ are the areas of inosine and adenosine peaks; $f = 1.347$ is the ratio between adenosine and inosine molar absorbance coefficients at 254 nm; k_{dilution} is a dilution coefficient ($V_{\text{sample}}/V_{\text{reaction mix}}$); C_M is the adenosine concentration in the reaction mixture (M); $k_{\text{correction}} = 1 + K_m/C_M$ is the difference between the maximal reaction rate at a saturating adenosine concentration and the observed reaction rate at the given adenosine concentration in the reaction mixture ($V_{\text{max}}/V_{\text{experimental}}$); and τ is the incubation time (min).

Determination of the optimum pH and catalytic parameters for ADA2

The pH dependence of ADA2 activity was determined in 0.1 M sodium phosphate buffer with pH ranging from 5.6 to 8.2. To determine catalytic parameters, 10 nM ADA2 was incubated with adenosine dilutions (0.5–12.8 mM) in buffer A (200 μ l) for 26 min at 37 °C. K_m and k_{cat} values were derived from a Lineweaver–Burk plot. To determine the K_m for ADA2 in the IgG preparations, 100 μ l of IgG preparation was diluted 6-fold with 0.05 M sodium phosphate buffer (pH 6.5) and incubated with 0.8–1.8 mM adenosine for 2–2.5 h at 37 °C.

ADA2 purification and identification

All purification steps were performed in the cold room using the ÅKTA explorer purification system (GE Healthcare). As a source of ADA2, we used commercial IgG preparations (100 mg/ml IgG in PBS; Gabrichevsky State Unitary Enterprise of Bacterial Preparations) obtained by cold ethanol fractionation [22] with modifications. Alternatively, a protein fraction containing IgG and ADA2 activity was isolated from fresh human donor plasma using ion-exchange chromatography. Human plasma was dialysed against buffer B (0.02 M phosphate buffer, pH 8.0) and passed through a column with TSK EMD DEAE-650 (M) gel (Merck) equilibrated with buffer B (750 ml of the gel/200 ml of plasma). The flowthrough contained 95 % pure IgG (SDS/PAGE) and almost 100 % of the plasma ADA2 activity. The other purification steps were the same as for ADA2 purified from IgG preparations.

To purify ADA2 from IgG preparations, 135 ml of IgG solution was diluted to 250 ml with buffer C (0.02 Mops, pH 6.7, and 0.1 M NaCl). A 60 ml aliquot of the diluted preparation was applied to an XK16/20 column packed with 40 ml of heparin–Sepharose 6 fast flow (GE Healthcare) and equilibrated with buffer C. After extensive washing with buffer C, a fraction possessing ADA2 activity was eluted by increasing the NaCl concentration to 0.5 M. The fractions containing ADA2 activity were pooled together and the resulting solution was passed through the HiTrap protein G column (GE Healthcare) equilibrated with buffer C. The flowthrough was collected, diluted three times with buffer C and re-applied to a heparin–Sepharose column. The protein fraction containing ADA2 activity was eluted from the heparin–Sepharose column with a 0.1–0.5 M NaCl gradient (300 ml) and then concentrated to 400 μ l using Vivaspin-20 centrifugal concentrators (Vivascience, Hanover, Germany). The

Figure 1 Characterization of ADA from IgG preparations

(A) Typical elution profile showing separation of the substrates and products of three reactions of purine metabolism on a column with Toyopearl TSK HW-40 S gel. PNP, purine nucleotide phosphorylase; HX, hypoxanthine. A 400 μ l mixture containing 5'-AMP, adenosine, inosine and hypoxanthine was injected into an XK 16/20 column packed with 10 ml of Toyopearl TSK HW-40 S gel and equilibrated with 0.01 M sodium phosphate buffer. The nucleotides and nucleosides, which are specifically retained by the gel matrix, were subsequently eluted in the same buffer. The ratio between the peak areas was used to calculate the rates of the corresponding enzymatic reactions. (B) ADA2 activity in ten commercial IgG preparations in comparison with the ADA2 activity in human plasma. ADA2 is co-purified and concentrated together with the immunoglobulin fraction during industrial processing of human plasma using cold ethanol precipitation. (C) pH dependence of ADA activity in IgG preparations. The acid optimum pH of the reaction is a characteristic feature of the ADA2 isoenzyme. (D) Determination of K_m value for ADA in IgG preparations using a Lineweaver-Burk plot.

protein solution was passed through the High Load Superdex 200 preparation grade column (GE Healthcare) equilibrated with buffer C. The fractions with ADA2 activity were pooled together and concentrated using Vivaspinn-6 centrifugal concentrator. The protein concentration was determined by the Bradford method. For SDS/PAGE analysis, 10 μ l of 0.25 mg/ml protein solution was used. The R-250 Coomassie Blue-stained band corresponding to a 57 kDa protein was cut from the gel and the protein was reduced, alkylated with iodoacetamide and digested with trypsin. The peptide digest was analysed by MALDI-TOF (matrix-assisted laser-desorption ionization-time of flight) using an Ultraflex TOF/TOF instrument (Bruker Daltonics, Bremen, Germany).

RESULTS

ADA2 is co-purified with IgG during cold ethanol fractionation of human plasma

Our initial study of the binding of different nucleotides to IgG revealed that commercial IgG preparations, obtained by the modified Cohn method [23] for cold ethanol fractionation of

plasma from healthy donors, contained several enzymes that catalysed the reactions of purine metabolism. To analyse these reactions, we combined a novel chromatographic method based on the unique properties of Toyopearl HW-40 gel to separate proteins, nucleotides and nucleosides in a single run [24] with a previously established method to measure the rate of enzymatic reactions using the peak area ratio between the product and the substrate of the reaction [23,25] (Figure 1A). This method allowed us to monitor substrate transformation in several simultaneously ongoing reactions catalysed by different enzymes of the purine nucleotide salvage pathway (A.V. Zavialov, unpublished work). As an example, AMP degradation in the presence of three different enzymes present in human plasma is shown in Figure 1(A). Using this novel method, we found high ADA activity in the IgG preparations (Figure 1B). The ADA activity appeared to be solely due to the ADA2 isoenzyme contamination (Figures 1C and 1D) because EHNA, a selective ADA1 inhibitor, did not affect the enzymatic activity. Other parameters such as a slightly acidic optimum pH for the reaction (Figure 1C) and a high K_m value (Figure 1D) were also distinct features of ADA2 as compared with the ADA1 isoenzyme [12]. The examination of ADA2 activity in

Figure 2 Purification of ADA2 from IgG preparations

(A) Elution of ADA2 from heparin-Sepharose using a linear NaCl gradient. (B) ADA2 purification using a High Load Superdex 200 gel-filtration column. Inset: SDS/PAGE analysis of ADA2 after gel filtration. (C) A table summarizing the purification steps of ADA2.

IgG preparations from 10 different manufacturers revealed that ADA2 is co-purified with IgG and that ADA2 concentration in the final product increased approx. 8–10-fold relative to its normal concentration in plasma (Figure 1C). We therefore used commercial IgG preparations as a source to further purify and characterize ADA2.

Purification and identification of ADA2

Furthermore, we found that ADA2 binds to heparin (Figure 2A). This allowed us to isolate ADA2 without significant loss of enzymatic activity (Figure 2C). In the first step, ADA2 was separated from the majority of IgG molecules by passing the Ig preparations through a heparin-Sepharose column. The protein fraction containing ADA2 was eluted with an NaCl step gradient and further purified from the remaining IgG by passage through a Protein G column, which specifically adsorbs IgG. To concentrate and further purify ADA2, the flowthrough from the Protein G column was re-applied on to the heparin-Sepharose column and the ADA2 fraction was eluted with an NaCl gradient (Figure 2A). To separate ADA2 from other heparin-binding proteins, we used gel-filtration chromatography on a Superdex 200 column (Figure 2B). The fraction corresponding to a 110 kDa protein and displaying ADA2 activity was collected, concentrated and analysed using SDS/PAGE (12% polyacrylamide) (Figure 2B, inset). A band corresponding to a 57 kDa protein was cut from the gel, digested with trypsin and the digest was analysed using MS (Table 1).

ADA2 was identified as a product of the CECR1 gene (Entrez Protein database accession no. AAF65941) [26] by searching the

NCBI nr (NCBI non-redundant) database (released on 11 May 2004) using Mascot software [27], which yielded a score of 233 compared with the statistical limit of 95% at a score of 76. As shown in Figure 3(A), the peptides derived from ADA2, which were identical with those from a predicted CECR1 protein [26], covered more than 60% of the CECR1 sequence exclusive of the signal sequence. To show further that the ADA2 purified recently from chicken liver [20] is also the product of a corresponding chicken CECR1 gene, we used a protein–protein BLAST analysis [28] to find a protein similar to CECR1 in the chicken genome (Figure 3A). The best score was found for a hypothetical protein having 59% identity and 76% similarity to CECR1 (Entrez Protein database accession no. CAG31985). In the second step, we used the N-terminal sequence, determined on the purified chicken ADA2 protein by Iwaki-Egawa et al. [20], to identify a protein that would have nearly exact matches with that sequence. As expected, the best score was obtained with the chicken analogue of the CECR1 protein (Entrez Protein database accession no. CAG31985; Figure 3B). The discrepancy between the N-terminal protein sequence of ADA2 and the cDNA could be possibly explained by the presence of several cDNA variants in chicken. From these results, we conclude that both human and chicken ADA2 are encoded by the CECR1 gene in humans and chicken respectively. It has recently been shown that the product of the CECR1 gene belongs to a novel family of ADGFs [29].

As is shown in Figure 4(A), a human member of this family possesses a very high turnover rate ($k_{\text{cat}} = 88 \text{ s}^{-1}$) for the reaction of deamination. However, the concentration of adenosine must be very high to saturate the enzyme ($K_m = 2.53 \text{ mM}$).

Figure 4 Catalytic parameters and a possible physiological role of ADA2

(A) Determination of the catalytic parameters of purified ADA2 using a Lineweaver-Burk plot. (B) ADA2 expressed by activated macrophages might be involved in signal-transduction pathways via adenosine receptor A₃. *M.t.*, *M. tuberculosis*.

CECR1 gene [26]. We further demonstrated that chicken ADA2 is also encoded by a homologous gene. BLAST search of the chicken proteome revealed a hypothetical protein similar to the product of the CECR1 gene (Figure 4A). The protein amino acid sequence starting after the predicted signal sequence matched the N-terminal sequence obtained from chicken liver ADA2 [20] (Figure 4B). This observation provides additional support for our conclusion that human ADA2 is encoded by the CECR1 gene, a candidate gene for CES, which is caused by a duplication of chromosome 22q11.2 and is associated with multiple congenital anomalies in organ development [32].

The ADA2 amino acid sequence analysis indicated that ADA2 is synthesized as a preprotein with a signal peptide, which is probably required for ADA2 secretion [26]. As shown in Figure 3(B), the signal peptide is absent from the mature chicken ADA2 protein. The polypeptide chain of chicken ADA2 is glycosylated [20]. The latter may explain why several peptides of the human ADA2 analogue, which have potential glycosylation sites, were not identified by MS (Figure 3A). According to the gel-filtration and SDS/PAGE analyses, both human and chicken [20] ADA2 are secreted as a homodimer. Homodimer formation is probably required for ADA2 interaction with HSPGs and/or receptors on the cell surface, resembling fibroblast growth factors

[31] (Figure 4B). In contrast with ADA2, ADA1 does not bind to heparin (results not shown). Whereas ADA2 has the potential to bind directly to the cell surface via HSPG (Figure 4B), the binding of ADA1 to the cell surface requires a complex interaction with the CD26 receptor [3].

Gene comparison analysis revealed that ADA2 belongs to a novel family of ADGFs, found in many organisms from fly to human [29]. Although the function of ADGFs is not known, their growth factor activity is coupled with ADA activity, which was shown for the *Drosophila* ADGF member both *in vitro* [33] and *in vivo* [34]. Recently, it was discovered that deficiency of the ADGF-A gene in *Drosophila* leads to a fly phenotype with abnormal haemocyte development, formation of melanotic tumour, fat body degeneration and delay in development [34]. The mutant phenotype could be rescued by expression of ADGF-A only in the lymph gland, suggesting a crucial role for haemocytes in the expression of ADGF-A [34]. This finding is in line with the observation that ADA2, a human ADGF member and a product of the CECR1 gene, is expressed by human blood cells in response to bacterial or viral infection. Similar to its *Drosophila* homologue, human ADA2 might stimulate blood cell differentiation by regulating the level of adenosine and interacting with the Toll signalling pathway [34], which controls innate immunity in both the organisms [35].

To date, no experimental results exist that demonstrate that the human member of the ADGF family has ADA activity [29]. In the present study, we show that ADA2, a product of the CECR1 gene, catalyses adenosine deamination. However, in contrast with other ADGF family members [29], the enzyme has a very high K_m for adenosine ($K_m = 2.53$ mM; Figure 4A). The fact that ADA2 has a very high K_m for adenosine and reaches maximal activity at slightly acidic pH further indicates that ADA2 is active in sites with high adenosine concentration and low pH (Figures 1C and 4A). We suggest that macrophage (and other cell) ADA2 [13] may stimulate the proliferation and activation of different immune cells and tumour cells via cell-surface adenosine receptors [36] (Figure 4B) in sites of inflammation or tumourigenesis, where the concentration of adenosine is significantly elevated [36] and the extracellular fluid has an acidic pH due to hypoxia [37–39]. It is possible that adenosine receptor A₃, which is activated at high inosine concentrations [36], is involved in the signal transmission (Figure 4B). For instance, ADA2 bound to the mast cell may stimulate the cell degranulation via adenosine receptor A₃ [39]. Monocytes/macrophages infected with *Mycobacterium tuberculosis* are thought to be the main source of ADA2 in human pleural fluid [15,21]. Therefore ADA2 produced by macrophages in response to pathogen invasion may lead to the activation of immune cells at the site of inflammation (Figure 4B). The CECR1 gene is also highly expressed in human B-cells transformed with Epstein-Barr virus [26], raising the interesting possibility that activated B-cells are responsible for the high ADA2 activity in plasma of patients with infectious mononucleosis [4].

Although the high K_m value for the reaction of deamination catalysed by ADA2 suggests that this enzyme requires a high adenosine concentration for its activity, we cannot exclude the possibility that ADA2 may act at physiological concentrations of adenosine. In this case, it would act in an analogous fashion to ADA1, which was shown to have a co-stimulatory role in T-cell activation via binding to the CD26 receptor [40]. The function of ADA2 *in vivo* deserves further investigation.

We thank L. Glimcher for her help in preparing this paper. This work was supported by the George Soros Foundation (New York, NY, U.S.A.; Open Society Institute and Soros Foundation Network Scholarship to A. V. Z.) and the Wallenberg Consortium North (Stockholm, Sweden; to Å. E.).

REFERENCES

- 1 Ratech, H., Thorbecke, G. J., Meredith, G. and Hirschhorn, R. (1981) Comparison and possible homology of isozymes of adenosine deaminase in Aves and humans. *Enzyme* **26**, 74–84
- 2 Schrader, W. P., Woodward, F. J. and Pollara, B. (1979) Purification of an adenosine deaminase complexing protein from human plasma. *J. Biol. Chem.* **254**, 11964–11968
- 3 Weihofen, W. A., Liu, J., Reutter, W., Saenger, W. and Fan, H. (2004) Crystal structure of CD26/dipeptidyl-peptidase IV in complex with adenosine deaminase reveals a highly amphiphilic interface. *J. Biol. Chem.* **279**, 43330–43335
- 4 Ungerer, J. P., Oosthuizen, H. M., Bissbort, S. H. and Vermaak, W. J. (1992) Serum adenosine deaminase: isoenzymes and diagnostic application. *Clin. Chem.* **38**, 1322–1326
- 5 Thompson, L. F. and Seegmiller, J. E. (1980) Adenosine deaminase deficiency and severe combined immunodeficiency disease. *Adv. Enzymol. Relat. Areas Mol. Biol.* **51**, 167–210
- 6 Wilson, D. K., Rudolph, F. B. and Quijcho, F. A. (1991) Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. *Science* **252**, 1278–1284
- 7 Daddona, P. E. and Kelley, W. N. (1977) Human adenosine deaminase. Purification and subunit structure. *J. Biol. Chem.* **252**, 110–115
- 8 Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluís, C. and Franco, R. (1996) Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. *FEBS Lett.* **380**, 219–223
- 9 Herrera, C., Casado, V., Ciruela, F., Schofield, P., Mallol, J., Lluís, C. and Franco, R. (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol. Pharmacol.* **59**, 127–134
- 10 Weihofen, W. A., Liu, J., Reutter, W., Saenger, W. and Fan, H. (2005) Crystal structures of HIV-1 Tat derived nonapeptides Tat(1–9) and Trp2–Tat(1–9) bound to the active site of dipeptidyl peptidase IV (CD26). *J. Biol. Chem.* **280**, 14911–14917
- 11 Rokosu, A. A. (1983) The characterization of an adenosine deaminase from chicken serum. *Comp. Biochem. Physiol. B* **74**, 441–444
- 12 Daddona, P. E. and Kelley, W. N. (1981) Characteristics of an aminohydrolase distinct from adenosine deaminase in cultured human lymphoblasts. *Biochim. Biophys. Acta* **658**, 280–290
- 13 Conlon, B. A. and Law, W. R. (2004) Macrophages are a source of extracellular adenosine deaminase-2 during inflammatory responses. *Clin. Exp. Immunol.* **138**, 14–20
- 14 Tsuboi, I., Sagawa, K., Shichijo, S., Yokoyama, M. M., Ou, D. W. and Wiederhold, M. D. (1995) Adenosine deaminase isoenzyme levels in patients with human T-cell lymphotropic virus type 1 and human immunodeficiency virus type 1 infections. *Clin. Diagn. Lab. Immunol.* **2**, 626–630
- 15 Perez-Rodriguez, E. and Jimenez Castro, D. (2000) The use of adenosine deaminase and adenosine deaminase isoenzymes in the diagnosis of tuberculous pleuritis. *Curr. Opin. Pulm. Med.* **6**, 259–266
- 16 Niedzwicki, J. G., Mayer, K. H., Abushanab, E. and Abernethy, D. R. (1991) Plasma adenosine deaminase2 is a marker for human immunodeficiency virus-1 seroconversion. *Am. J. Hematol.* **37**, 152–155
- 17 Hitoglou, S., Hatzistilianou, M., Gougoustamou, D., Athanassiadou, F., Kotsis, A. and Catriu, D. (2001) Adenosine deaminase activity and its isoenzyme pattern in patients with juvenile rheumatoid arthritis and systemic lupus erythematosus. *Clin. Rheumatol.* **20**, 411–416
- 18 German, D. C. and Kredich, N. M. (1984) A radioenzymatic assay for plasma adenosine. *Anal. Biochem.* **142**, 536–541
- 19 Niedzwicki, J. G. and Abernethy, D. R. (1991) Structure-activity relationship of ligands of human plasma adenosine deaminase2. *Biochem. Pharmacol.* **41**, 1615–1624
- 20 Iwaki-Egawa, S., Namiki, C. and Watanabe, Y. (2004) Adenosine deaminase 2 from chicken liver: purification, characterization, and N-terminal amino acid sequence. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **137**, 247–254
- 21 Andreasyan, N. A., Hairapetyan, H. L., Sargisova, Y. G. and Mardanyan, S. S. (2005) ADA2 isoform of adenosine deaminase from pleural fluid. *FEBS Lett.* **579**, 643–647
- 22 Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. and Taylor, H. L. (1946) Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* **68**, 459–475
- 23 Zavialov, A. V., Buckingham, R. H. and Ehrenberg, M. (2001) A posttermination ribosomal complex is the guanine nucleotide exchange factor for peptide release factor RF3. *Cell (Cambridge, Mass.)* **107**, 115–124
- 24 Aoyagi, S., Hirayanagi, K., Yoshimura, T. and Ishikawa, T. (1982) Preparative separation of nucleosides and nucleotides on a non-ionic gel column. *J. Chromatogr. A* **253**, 133–137
- 25 Zavialov, A. V. and Ehrenberg, M. (2003) Peptidyl-tRNA regulates the GTPase activity of translation factors. *Cell (Cambridge, Mass.)* **114**, 113–122
- 26 Riaz, M. A., Brinkman-Mills, P., Nguyen, T., Pan, H., Phan, S., Ying, F., Roe, B. A., Tochigi, J., Shimizu, Y., Minoshima, S. et al. (2000) The human homolog of insect-derived growth factor, CECR1, is a candidate gene for features of cat eye syndrome. *Genomics* **64**, 277–285
- 27 Perkins, D. N., Pappin, D. J., Creasy, D. M. and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551–3567
- 28 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
- 29 Akalal, D. B., Schein, C. H. and Nagle, G. T. (2004) Mollusk-derived growth factor and the new subfamily of adenosine deaminase-related growth factors. *Curr. Pharm. Des.* **10**, 3893–3900
- 30 Farrugia, A. and Poulis, P. (2001) Intravenous immunoglobulin: regulatory perspectives on use and supply. *Transfus. Med.* **11**, 63–74
- 31 Lin, X. (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. *Development* **131**, 6009–6021
- 32 Meins, M., Burfeind, P., Motsch, S., Trappe, R., Bartmus, D., Langer, S., Speicher, M. R., Muhlenyck, H., Bartels, I. and Zoll, B. (2003) Partial trisomy of chromosome 22 resulting from an interstitial duplication of 22q11.2 in a child with typical cat eye syndrome. *J. Med. Genet.* **40**, e62
- 33 Zurovec, M., Dolezal, T., Gazi, M., Pavlova, E. and Bryant, P. J. (2002) Adenosine deaminase-related growth factors stimulate cell proliferation in *Drosophila* by depleting extracellular adenosine. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 4403–4408
- 34 Dolezal, T., Dolezelova, E., Zurovec, M. and Bryant, P. J. (2005) A role for adenosine deaminase in *Drosophila* larval development. *PLoS Biol.* **3**, e201
- 35 Hoffmann, J. A. and Reichhart, J. M. (2002) *Drosophila* innate immunity: an evolutionary perspective. *Nat. Immunol.* **3**, 121–126
- 36 Sitkovsky, M. V., Lukashev, D., Apasov, S., Kojima, H., Koshiba, M., Caldwell, C., Ohta, A. and Thiel, M. (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu. Rev. Immunol.* **22**, 657–682
- 37 Stubbs, M., McSheehy, P. M., Griffiths, J. R. and Bashford, C. L. (2000) Causes and consequences of tumour acidity and implications for treatment. *Mol. Med. Today* **6**, 15–19
- 38 Pollard, J. W. (2004) Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* **4**, 71–78
- 39 Jin, X., Shepherd, R. K., Duling, B. R. and Linden, J. (1997) Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. *J. Clin. Invest.* **100**, 2849–2857
- 40 Martin, M., Huguet, J., Centelles, J. J. and Franco, R. (1995) Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex. Possible role of adenosine deaminase as costimulatory molecule. *J. Immunol.* **155**, 4630–4643
- 41 Myers, E. and Miller, W. (1988) Optimal alignments in linear space. *CABIOS* **4**, 11–17
- 42 Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **340**, 783–795

Received 26 April 2005/27 May 2005; accepted 1 June 2005

Published as BJ Immediate Publication 1 June 2005, doi:10.1042/BJ20050683