A RECENT ARTICLE addresses an issue which has perplexed several researchers since first raised in 1995: does attractin have an intrinsic dipeptidyl peptidase 4 (DP4) activity? In a new report, a well-established and respected group of enzymologists takes a rigourous scientific approach to the question, and in the process draws the conclusion that attractin does not have an intrinsic DP4 activity, but that there is a co-purifying entity with properties of a soluble CD26 [1]. Since 1995, results from several research groups have been published supporting opposite views and this current report appears to resolve the issue. Nevertheless, the report also raises some intriguing questions, and given that the debate has ranged from the "collegial" to almost "acrimonious" in some spheres, the essay below is an attempt to move objectively through the historical record of the last 12 years.

As first author on the initial publication in 1995 concerning attractin's DP4 activity, it is important for me to note that within the research group doubts and scientific anomalies were raised continuously from 1996 through 2000, at which point the first published results appeared suggesting that circulating soluble DP4 activity was CD26 and not attractin [2]. At that time, the author decided that enzymology should be left to enzymologists, he would focus on the many other apparent properties of attractin, and he would be happy to distribute reagents to whoever requested them (as did many researchers) and that eventually a consensus would arise.

1989-1994: The lead-up to our studies on circulating DP4

Under the guidance of Prof. Stuart Schlossman and Dr Chikao Morimoto, tremendous advances had been made in our understanding of human CD26 [3], initially characterised by the Reinherz group and Schlossman as a human T cell activation antigen through reactivity with the Ta1 monoclonal antibody [4]. Several years later, other researchers established the identity between CD26 and the long-established cell surface DP4 activity of many cells [5], subsequently confirmed by the cloning of human CD26 [6]. Given the high representation of CD26/DP4 in kidney, liver and endothelial cells, it would be difficult to find a critical systemic role for activated T cell CD26 were it not for the impressive stimulation-related upregulation – in the other cell types mentioned above, CD26/DP4 is constitutively expressed and is not subject to event-driven activation. This suggested a specific role for CD26 in T cell activation and crosslinking of CD26 appeared to upregulate CD3/TcR-mediated signalling [5, 7, 8]. The upregulation of signalling was intriguing – in large part because the cytoplasmic tail of CD26 is very short with no signalling capacity. A possible clue to mechanism was provided by the effort to identify a low molecular weight band which was continuously co-precipitated with cell-surface iodinated CD26 following incubation with 1F7 mAb. Schlossman and Morimoto made identification of this moiety a priority and after a Herculean effort, Dr Junichi Kameoka in the laboratory identified the band as adenosine deaminase (ADA). CD26 had now been identified as the long-sought-after main human adenosine deaminase binding protein [9]. The ability to bind ADA is not common to CD26 of all species and may reflect differing roles for DP4. Nevertheless, the proximal binding of ADA to the activated T cell surface would protect T cells from the toxic effects of adenosine accumulation and provide a means for recycling inosine for nucleotide synthesis, both of which would aid human T cell survival and promote proliferation [10].

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Accordingly, CD26 now played the proud role of being the canonical representative of DP4 activity, of being the ADA-binding protein, and of being a *bona fide* T cell activation antigen. Within the laboratory, Dr Toshiaki Tanaka produced membrane CD26-transfected Jurkat T cells and showed that crosslinking of CD26 upregulated CD3/TcR-mediated activation of IL2 secretion, and that DP4-inactive CD26 was less efficient in this role, suggesting a direct role for the DP4 enzymatic activity in this process [11]. The mechanism for costimulatory activity remains unclear, nevertheless the DP4 activity and the ADA-binding activity were clearly both properties of the extracellular domain. The obvious next step was to determine whether a recombinant ectodomain of CD26 could recapitulate functionality apparently mediated by membrane CD26 and whether such properties were simply a measure of DP4 activity, ADA-binding activity or whether there was a separate signal-transducing receptor upon the activated T cells with which CD26 ectodomain could interact.

Dr Tanaka produced a recombinant CD26 (sCD26) secreted by transfected CHO cells where the secreted protein corresponded to the entire ectodomain, was glycosylated, had DP4 activity, and retained 1F7 antigenicity [12]. The intent then was to determine whether the presence of sCD26 affected the responses of human T cells to various stimuli. Examination of the issue of a serum DP4 activity and its relationship to membrane CD26/DP4 was already well-established. At that time, there were already numerous reports of DP4 activity in both human and animal sera correlating with various pathologies. Further reports demonstrated that the immune cell-bound DP4 activity was restricted to T lymphocytes, and that in conditions where circulating T cells were reduced (i.e. by thoracic duct drainage), this was accompanied by a subsequent reduction in serum DP4 activity [13]. This would seem to lead to the remarkable conclusion that not only was T cell CD26/DP4 expression regulated, but also that T cells were uniquely poised to somehow produce a secreted form despite the total lymphoid DP4 activity being dwarfed by that of liver, kidney and endothelial cells in the normal state. In certain hepatic cancers or disease, however, serum DP4 activity did increase significantly [14-16]. If we wanted to test the responses of human T cells to sCD26, we would first have to determine relative levels of DP4 activity in serum used in media for stimulation (serum-free lymphocyte stimulation media was not routinely used at that time).

An assay was developed to measure CD26-reactive material in human plasma, using the recombinant sCD26 as a standard. Two in-house anti-CD26 mAb, 5F8 capture and 1F7-biotin for development, were used in an ELISA assay while 5F8 capture was used to measure immobilised DPPIV activity [12]. As expected, DP4 activity more-or-less correlated with antigen levels ($r = 0.7$), but the detected levels in plasma seemed surprisingly high: 5-15 μg/ml. In another anomalous result, levels of sCD26 shown to enhance responses to tetanus toxoid (100ng/ml to 5μg/ml) were significantly lower than the level already apparently circulating. It thus became critical to establish the relationship of the CD26-like DP4 material circulating to our “standard” recombinant sCD26. Given these high levels in plasma, and the likely T cell origin, we set about purifying the material. Since the material to be purified would be compared with recombinant sCD26, we had to be certain that the standard sCD26 was a good

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representative. We were concerned with the apparently high levels of sCD26 measured in donor plasma/serum, forcing us to consider the possibility that the ELISA assay may be systematically overestimating the amount of sCD26 in serum. This could occur if a significant fraction of our recombinant sCD26 standard was denatured during the elution from immunoaffinity columns, resulting in reduced antigenicity. To specifically overcome this, we used a new non-denaturing procedure to purify the sCD26, as described in our next report, "*Although we had a large panel of antibodies available, we did not use these for affinity purification, since we found that in every case the harsh elution conditions required (glycine-HCl, pH1-2, or 3M KSCN) resulted in a reduction in the DPPIV specific activity of at least one order of magnitude*".

1995-1998: gp175 to DPPT-L to attractin

Since there did not seem to be a strong difference in DP4 activity between serum and plasma, we used serum due to easier availability of bulk amounts with the added bonus that the fibrinogen/fibrin would already have been "removed". In order to preserve the DP4 activity, we used a multi-step purification procedure with no exposure to strong denaturing reagents [17]. The only criterion for picking particular fractions during the preparation was the presence of DP4 activity. Two main steps appeared to be critical: ion exchange and concanavalin A lectin-binding. Ten per cent of the DP4 reactivity was recovered with a 5000-fold increase in specific activity which seemed to be specifically associated with a 175kDa glycoprotein (gp175) quite distinct from 105-110kDa CD26. As we noted at the time, "*In two preparations (out of seven total), we were also able to copurify some lower molecular mass DPPIV activity corresponding to the 105-kDa form of CD26, but quantitation by laser densitometry confirmed that this was always less than 6% of the total preparation, and separated away at the preparitive native electrophoresis stage*" [17]. The purified serum DP4 appeared by silver staining to be homogenous with no 105kDa contamination (Fig.1A), and labelled at the expected molecular weight with ^{3}H-DFP. Nevertheless, it was perplexing that there seemed to be some epitope reactivity with a panel of anti-CD26 mAb. In a separate experiment not published, identical amounts of gp175 and sCD26 were radioiodinated, separated by gel electophoresesis and the dried gels developed as autoradiographs. Doubling dilutions of sCD26 were run out to determine the approximate % level contamination of gp175 by sCD26 if present (Fig.1B). No sCD26 contamination of the iodinated gp175 was detected and the gp175 did not bind ADA. In fact, the only other labelled protein in some preparations apart from gp175 was subsequently identified as angiotensinogen by mass spectroscopic analysis of tryptic peptides (Duke-Cohan; unpublished results). If the gp175 was contaminated with 105kDa sCD26, the levels appeared to be less than 2-3% while the specific DP4 activity of the gp175 was around 40% that of sCD26. Similarly, a panel of anti-CD26 antibodies immobilized around 40% the activity of a similar amount of sCD26, from which the conclusion we drew at the time was that gp175 was a separate gene product with some structural similarity to CD26 ectodomain. Also implied was the conclusion that although sCD26 could clearly be found in serum/plasma, the dominant DP4 activity was associated with the gp175 moiety.

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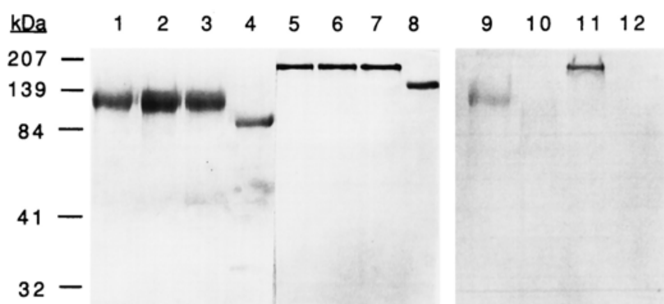

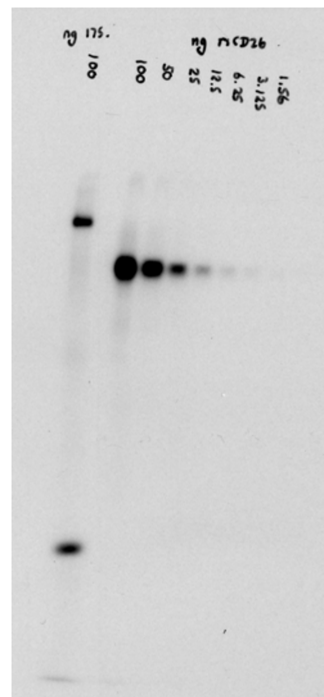

Figure 1. A. (above) Lanes 1-4, 9, 10: purified recombinant CD26. Lanes 5-8, 11, 12: purified serum attractin. Lanes 1-8: silver stain. Lanes 9-12: periodic acid-Schiff staining. Compare intact sCD26 (lane 1) with attractin (lane 5), and deglycosylated sCD26 (lane 4) with deglycosylated attractin (lane 8) [*from Ref. 17*]. B. (right) iodinated gp175 (100ng; lane 1) shows no apparent contamination with sCD26 (doubling dilutions of iodinated sCD26 from 100ng to 1.56ng; lanes 2-8). The lower band in the attractin lane was later identified as angiotensinogen [*Duke-Cohan, unpublished results*]

Working within a Department renowned for its definition of human leukocyte antigens through monoclonal antibody development [18], we believed that definitive evidence one way or the other could be provided by developing specific antibodies. Monoclonal antibody development was set in motion but this takes time so for immediate results we initiated production of polyclonal antibody. Although we could not identify sCD26 in preparations of gp175, the antibody developed against the injected attractin clearly had a limited reactivity against sCD26, which could be removed by passage over a sCD26 affinity column. Meanwhile 1F7 anti-CD26 mAb showed absolutely no reactivity against serum-purified gp175 or material similarly isolated from serum-free media conditioned by activated human T cells. We took this to indicate some aspect of the shared epitopes as previously observed, but which were maintained in the native state but not the denatured condition. Despite the enigma of apparent epitope similarity, the specific antibody clearly demonstrated that gp175 was distinct from sCD26, had different expression kinetics on activated T cells and was released from activated T cells [19]. This last parameter was reinforced by the observation that renal-derived ACHN cells proliferating at a high rate have extremely high levels of surface membrane CD26/DP4 but did not release this activity into culture supernatant during growth. In contrast, activated T cells could release a DP4 activity which clearly escalated with proliferation, and purification of the DP4 activity from activated T cell conditioned serum-free media yielded a 175 kDa moiety indistinguishable from serum gp175 with no indication of sCD26. We thus felt that the serum gp175 DP4 activity originated from activated T cells. Further, we felt justified in renaming gp175, using the rather unwieldy acronym DPPT-L meaning **D**i**p**eptidyl **p**eptidase IV from **T** cells and **L**arge [19].

As an aside, it is nevertheless clear that there is sCD26 in serum/plasma which had been purified by immunoaffinity chromatography [20]. Peptide sequencing indicated

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the likely cleavage point but the basis for its generation (i.e. the specific protease responsible) remains unclear.

DPPT-L was remarkably resistant to proteolysis, but eventually we were able to sequence and identify some peptides. It was immediately apparent that none of the peptides bore any resemblance to CD26 sequence. By back-translating the peptides, some potential expressed sequence tags (EST) derived from mRNA were identified, and full-sequencing of the original clones harbouring the EST led to isolation of a full-length mRNA. The encoded protein did not contain a transmembrane domain, was replete with motifs characteristic of extracellular proteins involved in adhesion, and was clearly an orthologue of a *C. elegans* transmembrane protein of unknown function. Most importantly, every single peptide we had found by tryptic digestion was identified within the open reading frame, reinforcing the apparent purity of our initial preparation. The 5' coding region was unusual in that DPPT-L was clearly a secreted protein but no classical signal peptide was identified. The amino terminal did however contain a region very similar to a catalytic serine domain although other residues of a catalytic triad were not identified. The lack of a signal peptide in our construct was practically overcome by inserting the cDNA into a vector encoding a bovine IgG signal sequence in the context of an optimum start codon. Nevertheless, even after stable transfection into CHO cells, the DPPT-L was not secreted, and from other studies appeared to have a microsomal location which remained its fate until a secretory event was activated. This complicated downstream purification, necessitating lysis prior to purification through the introduced C-terminal $His_{(6)}$ tag. The purified material, despite no exposure to denaturing techniques, had only 25% the enzymatic activity of purified serum DPPT-L, and only 10% the activity of recombinant sCD26. This was a concern, nevertheless the DP4 activity was there and similarly to serum DPPT-L, no 105kDa sCD26 activity could be detected by silver staining or Western blotting with 1F7 anti-CD26 antibody. Several of the anti-CD26 antibodies could still immunoprecipitate a band at 175kDa detectable by anti-DPPT-L polyclonal antibody. Nevertheless, we felt it necessary to add the statement: "*Because we purify attractin with nondenaturing techniques, there exists the possibility that soluble CD26 binds to the purified attractin and is carried through the purification process*".

Given the number of extracellular adhesion-associated motifs, we tested and confirmed that secreted DPPT-L could mediate by some mechanism T cell migration to adherent monocytes. Since it did not cause aggregation of either cell alone, and since this effect was time dependent occurring maximally at 48h, a role in regulation of migratory events was suggested. We postulated that this may reflect a DP4-mediated regulation of chemokine gradients. Given the low enzymatic activity of the recombinant form on comparison with recombinant sCD26, we made a conscious decision to refocus the name upon its apparent dominant activity and changed the name to attractin [21]. Nevertheless, the DP4 activity did not appear to be due to contamination with CD26, we had found no evidence by any technique that CD26 could interact with attractin, and we began to consider several possibilities:

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a) Attractin (DPPT-L) is an enzyme but DP4 activity is a weaker "collateral" activity of a second as yet undiscovered main enzymatic activity.

b) Attractin is a co-factor for CD26 activity, allowing significant increases in apparent specific activity for a very low level of contaminating CD26.

c) There is heterogeneity in the sCD26 pool where a very small fraction has very high enzymatic activity and this happens to copurify with attractin during our non-denaturing purification protocols.

d) There is heterogeneity in the attractin pool – some has DP4 activity, some does not, and our CHO recombinant form was depleted of the active form while the serum was enriched in this form. This led to the question of significant post-translational processing, leading from a pre-protein to a mature protein with enzymatic activity, as occurs for many proteinases to prevent inappropriate digestion.

In our subsequent articles we began to introduce these concepts into the Discussion sections as an acknowledgement of the inconsistencies and the ongoing discussion in the research group, and our uncertainty concerning the DP4 activity of attractin. Our uncertainty fluctuated in degree according to results and publications of others where sometimes we felt quite confident that the DP4 activity was intrinsic, at other times we were quite perplexed. It should also be stressed that numerous individuals wrote to us concerning the DP4 activity of attractin and we always quite openly voiced the concerns that were part of our internal debate.

To address this uncertainty, and given the variability of human sera, samples, and immune reactions (which was our primary interest), we set about identifying a mouse equivalent of attractin. Given the orthologue already identified in *C. elegans*, we were confident that this would be a formality, if not particularly easy. We were fortunate enough to identify some mouse EST clones which matched up very well with the 5' end of human attractin, and we set about sequencing the full inserts and generating probes to screen mouse libraries. We made significant progress identifying the apparent 5' third of the mouse attractin mRNA. What was immediately apparent was a difference downstream of the start codon: the mouse form encoded a signal peptide.

1999: Mahogany – many doors open but where does DP4 fit in?

Between late 1998 and early 1999, our search for a mouse model benefited immeasurably from contact with Dr. Greg Barsh at Stanford. His interest stemmed from his work on the *Mahogany* and *Mahoganoid* strains of mice, long used as models for study of pigmentation regulation, but also of interest since these mice when crossed with mutant mice bearing the agouti A^y allele were not only resistant to the induction of yellow phaeomelanin pigment but also did not become morbidly obese. Substantiating this latter effect, the mice consistently weighed less than normal littermates when allowed to eat *ad libitum* [22]. Although the *Mahogany* (*mg*) and *Mahoganoid* (*md*) mutations had been mapped, the mutant genes had not been characterised, which had become one of the main aims of the Barsh laboratory. The chromosomal locations of *mg* and *md* were different

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but the phenotypic effects were genetically epistatic to each other meaning that they seemed to operate at the same biochemical level; specifically, downstream of agouti protein and upstream of the melanocortin receptor Mc1R. Following publication of our sequence for secreted human attractin, Dr Barsh came to visit us and told us that they were sequencing a *Mahogany* candidate gene (mgca) from the 3' end. Our two sequences were aligned generating a full-length mRNA with an ectodomain with greater than 90% homology to secreted human attractin, a transmembrane domain and a significant cytoplasmic domain. Further studies confirmed that this was the transcript altered in various *mg* alleles, and was the normal product of the wild-type gene. Several matters were immediately resolved. First, this transmembrane form of attractin had no detectable DP4 activity, and did not harbour the putative catalytic serine, the consequence of the major sequence diversity between mouse and human being localised to the 5' end [23].

With the help of Greg Barsh and Teresa Gunn, we used the mouse sequence to design primers for walking along human chromosome 20p13 region, sequencing large overlapping BAC clones. The purpose here was to unequivocally identify the 5' end of attractin and to determine whether there was a genomic basis for a membrane as well as a secreted form of attractin, and whether any difference in sequence might account for DP4 activity. We identified the coding exons and could show unequivocally that the human attractin gene could generate both a secreted and a membrane form by alternative splicing, that both mRNA are produced and that they may be differentially expressed in cells. We were also able to show that the 5' region of the genomically-identified secreted and membrane human attractin differed from that we had previously published. As we wrote: "*In the initial attractin cDNA clone contig we described (AF034957), the 5' region contains an internal deletion of 222bp in comparison both with a cDNA isolated subsequently (AF106861) and with the sequence predicted here from the genomic structure. Two independent clones containing this deletion were identified in our initial screen, and it remains to be determined whether this is a true transcript or an artifact arising during library construction….*" [24]. This was important since the putative catalytic serine was lost in the new amended sequences. No DP4 identity could be associated with the membrane expression of the new transmembrane construct, and the secreted material had an activity barely but consistently above background. Since our apparently homogenous serum attractin still had strong activity, we were realistically stuck between two camps: either there was a very high activity sCD26 isoform that co-purifies in essentially undetectable amounts with serum attractin, or the recombinant attractin forms expressed in CHO cells were being secreted without some appropriate post-translational processing generating active enzyme.

2000-2003: Doubt and support?

In 2000, a seminal article was published by the De Meester group in Belgium [2]. Using ADA-binding, they were able to remove more than 90% of the serum DP4 activity and show that by size and reactivity, it was CD26. Attractin does not bind ADA. Back-calculating concentration from the published results, the serum concentration was probably in the range 0.3-0.5 μg/ml sCD26 with a specific activity of ~40U/mg. On comparison with our original ELISA/activity results published in 1994, we were finding monoclonal antibody-based reactivity in serum of 5-15μg/ml based on our standard

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recombinant CD26 which only had an activity of ~4U/mg. This means that our exclusion of CD26 activity was based on a standard an order of magnitude less enzymatically active and, based on antibody binding, probably significantly denatured. Thus, the earlier statement based on Fig. 1B that if contamination was present, it was less than 2-3% could now be modified to less than 0.2-0.3% which is probably a level we would not pick up by Western blotting or silver staining. Given this result, we felt that the DP4 activity of attractin was really questionable, and it became less of a focus in our research.

In an article in 2002 [25], we explicitly outlined some of the doubts stating: "*It is possible that enzyme activity is either the result of an undefined catalytic fold or of an association with a cofactor or enzyme leading to DPPIV activity or is the result of posttranslational modifications from a proenzyme*". The aspect of post-translational modifications and mature protein structure led us to undertake amino terminal sequencing of purified attractin where we were able to identify an unusually long signal peptide of 83 amino acids and confirm that the mature protein sequence began at position 84 [25]. None of our studies to date have identified a structural basis for the DP4 activity of attractin. At this point, we felt justified in leaving the DP4 question open.

Nevertheless, the possibility that DP4 may be a "gatekeeper" to physiological degradation of bioactive peptides, particularly the incretins helping regulate levels of endogenously-secreted insulin, meant that the relative activities of molecules with DP4 activity became of paramount importance in determining the prospects for therapeutic DP4 inhibitors. One company, *probiodrug AG* in Germany, whose research line is based completely upon development of physiologically-relevant inhibition of protease activity, undertook further investigation of attractin's DP4 activity and improved our confidence in our earlier results when they published an article outlining the purification of serum attractin with an associated DP4 activity that did not seem to be due to CD26 contamination [26]. They further confirmed the unusually long signal peptide.

2004-2006: Big developments, big pharma, basic research:

Research on using DP4 inhibitors to therapeutically regulate metabolic processes was gaining steam, and the possibility that non-CD26 entities may retain DP4 activity even in the presence of inhibitors was troubling and required elucidation. In 2006, another article appeared apparently confirming a DP4 activity associated with attractin and independent of CD26 [27]. This activity, on the surface of monocytes, occurred in the absence of CD26 expression, confirmed both by immunofluorescence and quantitative rt-PCR. Further, anti-attractin antibody could precipitate the activity – the problem of cross-reactivity rearing its ugly head again? In any event, here there seemed to be further evidence of a non-CD26-associated DP4 activity with attractin as a main candidate.

2007: New results, new questions:

Meanwhile, the research team at *probiodrug* was continuing with its examination of serum-purified attractin. Large amounts of serum were processed and attractin was purified. Inhibitor profiles suggested that the DP4 activity of attractin and recombinant sCD26 were very similar. In contrast, however, DP4 activity measured *in situ* following

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isoelectric focussing clearly shows that attractin and sCD26 have different pI and that the DP4 activity of sCD26 associates with material at the pI of CD26, and the DP4 activity of attractin associates with material at the pI of attractin [1]. The physical properties are not the same. This was further confirmed by measuring the *in situ* proteolytic activity following non-denaturing native gel electrophoresis; once again the DP4 activity of CD26 ran with CD26, while the DP4 activity of attractin ran with attractin. This would seem to confirm that attractin really does have DP4 activity but then a crucial experiment was performed. Although the *probiodrug* attractin preparation does not seem as clean as that used in our early studies (see Fig. 1A above), the DP4 activity could be completely removed by binding to immobilised ADA [1]. This really supports the proposal that the DP4 activity of serum-purified attractin is an ADA-binding high activity CD26-like molecule. The only contentious aspect of their report concerns an apparent duplication of a previously reported experiment where CD26 cleaved sequential peptides from RANTES while the attractin DP4 activity cleaved only one dipeptide and stopped [28]. In fact, it is well-known that apparent DP4 substrate motifs may often only be cleaved in the context of a short peptide used in their report [1, 29] while in our earlier report we were using full-length (68 aa) RANTES [28]. Nevertheless, this would simply indicate now that the DP4 activity of serum attractin represents a CD26 with cleaner activity than that represented by recombinant or porcine kidney CD26. There are clearly some tissue-specific post-translational modifications of serum sCD26 that lend it different biophysical and functional properties to that of recombinant CD26 or purified kidney CD26 and give it physical separation properties identical to those of attractin. Given the low physical presence and high activity, the *probiodrug* report suggests in a practical but somewhat rueful aside that DP4 activity may actually be used as a marker for attractin purification, which is exactly what we and they had done in previous reports.

In the recent words of a good colleague, the history of attractin is "an excellent example of a life paradox: you have discovered the molecule on the basis of its nonexistent attribute" (!).

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