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Short communication

Aplysia mollusk-derived growth factor is a mitogen with adenosine deaminase activity and is expressed in the developing central nervous system

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Abstract

Mollusk-derived growth factor (MDGF), the first growth factor to be characterized in *Aplysia*, was purified and characterized and has both adenosine deaminase activity and stimulates cell proliferation in vitro. MDGF is structurally related to a new subfamily of adenosine deaminase-related growth factors that require enzymatic activity to stimulate cell proliferation, a unique property of known growth factors. We examined the expression of MDGF protein in the CNS since *MDGF* mRNA increased in the developing CNS, and recent data suggest that inosine is involved in neuronal reorganization and restoration of essential circuitry after CNS injury. MDGF levels transiently increased during embryonic and post-metamorphic development and in the developing CNS, but was undetectable in adult CNS. No effects on morphology or neurite extension of adult *Aplysia* neurons were observed.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Transmitters in invertebrates

Keywords: ADA; Adenosine deaminase-related growth factor; *Aplysia*; Developing central nervous system; Insect-derived growth factor; MDGF; CECR1

Growth factors involved in cell proliferation and differentiation have a wide distribution in vertebrates and invertebrates, and studies with invertebrate models have provided insights into complex mechanisms of growth factor signaling [12,21,24,49]. However, attempts to purify and characterize endogenous growth factors in *Aplysia* have been largely unsuccessful. To date, only two molluscan neurotrophic factors have been purified and characterized: *Lymnaea* cysteine-rich neurotrophic factor [22] and *Lymnaea* epidermal growth factor (L-EGF) [25,26,56].

An insect-derived growth factor (IDGF) purified from conditioned medium of an insect cell line stimulates the proliferation of embryonic NIH-Sape-4 cells from the fly *Sarcophaga* in an autocrine manner [29]. IDGF is present in unfertilized mature eggs and is maintained throughout embryonic development, suggesting that it might be a maternal protein important for early embryonic development. IDGF showed no significant homology to other proteins in the database except for *Aplysia* atrial gland granule-specific antigen (AGSA), a glycoprotein that was initially thought to play a role in the structure and function of the cortex of secretory granules [54].

We recently characterized an atrial gland cDNA for AGSA that led to a correction in the sequence, and renamed the predicted protein mollusk-derived growth factor (MDGF) to emphasize its increased homology to IDGF [6]. *MDGF* mRNA increased markedly in the

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developing CNS during a period when significant proliferation of *Aplysia* neurons occurs [6].

In this study, we have characterized MDGF by examining the developmental expression of MDGF protein, immunoaffinity purifying MDGF, and demonstrating that MDGF has adenosine deaminase activity and mitogenic activity. MDGF stimulates cell proliferation of embryonic NIH-Sape-4 cells at picomolar concentrations. In addition to expression in the atrial gland in the reproductive tract [54], MDGF protein expression is transiently elevated during CNS development. We hypothesize that MDGF may play a growth factor role during periods of cell proliferation in the CNS by modulating extracellular adenosine levels. In this regard, recent data suggest that inosine is involved in neuronal reorganization and restoration of essential circuitry after CNS injury [17].

All developmental stages of *Aplysia californica* were obtained from the University of Miami-NIH *Aplysia* Resource Facility, including freshly laid egg cordons, premetamorphic stage 6 animals, post-metamorphic stages 7–12 animals, juveniles (1–40 g), late juveniles (41–125 g), and adults (>125 g). Large adult *A. californica* (150–500 g) were obtained from Marine Research and Educational Products (Escondido, CA, USA). Animals and egg cordons were housed in aquaria containing artificial seawater at 14±2 °C. For egg cordons, sections (~8–12 cm segments) were removed each day from an egg mass, snap frozen on dry ice, and stored at −70 °C. For stages 6–12 animals, the entire animal or small groups of animals were snap frozen. For larger animals (>10 g), *Aplysia* CNS were removed and snap frozen.

Peptide synthesis and antiserum production were performed by Sigma–Genosys (The Woodlands, TX, USA). Two peptides were synthesized corresponding to MDGF precursor residues 26–40 (APLTSKAAYLLKRNSC; the N-terminus of MDGF after signal sequence cleavage) and residues 510–525 (CVKTSVEGLKPHINDRSamide; the C-terminus of MDGF). A cysteine residue was included at the C-terminus of the N-terminal peptide and the N-terminus of the C-terminal peptide, and both were used for conjugation to bovine serum albumin. Two New Zealand white rabbits were each immunized with protein conjugates of both peptides. The two peptides were linked to the same affinity column and MDGF antiserum was affinity purified.

Total protein was purified from egg cordons, entire *A. californica* (stages 6–12), and adult CNS that were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) [18] for *MDGF* mRNA expression studies [6]. Protein was quantified (BCA Protein Assay; Pierce, Rockford, IL, USA), and samples of each developmental stage and tissue (30 μg) were fractionated on denaturing 10% polyacrylamide gels (Bio-Rad, Hercules, CA, USA) [35] and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with MDGF antiserum (1:3000 dilution), washed, incubated with peroxidase-conjugated anti-rabbit IgG (1:3000 dilution), treated with ECL Plus blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and exposed to X-ray film.

Using Gateway Cloning and Bac-to-Bac Systems (Invitrogen), an MDGF PCR product was transferred into pDest10 vector for expression of N-terminal His-tagged MDGF. Sf9 cells transfected with MDGF bacmid DNA were grown in Sf-900 II SFM, and recombinant MDGF (~61–kDa) was purified using His·Bind Quick 900 cartridges (Novagen, Madison, WI, USA). Recombinant MDGF contained significant chitinase contamination of the same molecular weight, and was therefore purified by immunoaffinity purification.

Recombinant MDGF was inactive in adenosine deaminase (ADA) and cell proliferation assays; recombinant MDGF expressed in *E. coli* was insoluble. In contrast, recombinant adenosine deaminase-related growth factor-A (ADGF-A) and ADGF-D, which contained a C-terminal His-tag, exhibited activity [59], suggesting that the N-terminus may be sensitive to inhibition by a tag. Therefore, MDGF was purified from atrial gland extracts using Seize X Protein G Immunoprecipitation kits (Pierce). Atrial glands from adult *A. californica* were homogenized in 10 mM EDTA/10 mM EGTA/20 mM Tris–HCl/1 mM PMSF (pH 7.4), sonicated, centrifuged (20,000×*g*; 20 min), the supernatant purified on Spin X Cups (Pierce), the protein eluted, exchanged with 10 mM Na_2PO_4 buffer (pH 7.4; Centricon YM-30, Millipore, Bedford, MA, USA), and stored at 4 °C or −20 °C and −70 °C for long-term storage.

ADA activity was determined by measuring the rate of ADA-dependent change at A_{265} resulting from the conversion of adenosine to inosine [1,31]. Assays were performed by adding 100 μmol adenosine to 50 mM KH_2PO_4 buffer, followed by addition of purified MDGF. One unit of enzyme activity was defined as the amount of enzyme catalyzing the deamination of 1 μmol of adenosine/min ($-\Delta A = 8.6\ \text{min}^{-1}\ \text{ml}^{-1}$) at 265 nm [3].

Growth factor activity was tested in an insect cell proliferation assay that detects low concentrations of IDGF [29]. After optimizing the assay, NIH-Sape-4 cells (100 μl; RIKEN Cell Bank, Tsukuba Science City, Japan) were plated at a density of 1×10^5 cells/ml in M-M medium [43] in 96-well microtiter plates containing penicillin/streptomycin/amphotericin B. Cells were incubated 2–3 days (28±1 °C) in the presence or absence of MDGF, and cell number was determined using the alamarBlue assay [4,29,30], which correlates increased cell number and enhanced cellular metabolism with generation of reducing activity; cultures were incubated with alamarBlue (15 μl) for 1.5 h.

Since the alamarBlue assay measures changes in cell number and not cell proliferation directly, we used BrdU incorporation to confirm that increases in cell number were due to cell proliferation. NIH-Sape-4 cells (100 μl) were

plated at a density of 1×10^5 cells/well and allowed to attach for 4 h before adding MDGF (1 ng/ml; the concentration giving maximal growth response using alamarBlue assay). Eight hours prior to fixation, BrdU was added (5 ng/ml). Cells were aspirated and centrifuged (400 rpm) onto poly-L-lysine-coated coverslips for 5 min. To detect BrdU incorporation into DNA, cells were washed twice with HG solution (Hank's balanced salt solution with calcium and magnesium, 0.1% glucose, and 15 mM HEPES) containing 2% normal goat serum (NGS) and fixed for 30 min in methanol at −20 °C. Cells were washed once with water and then DNA was denatured by incubating in 2 N HCl for 10 min at 37 °C followed by washing once with 2 ml/2 cm^2 of 0.1 M borate buffer (pH 8.4). After two more washes with HG washing solution/2% NGS, non-specific binding was blocked by adding 2% NGS and 0.1% Triton X-100 in HG for 15 min at 37 °C. Cells were incubated with blocking buffer containing a monoclonal antibody to BrdU (anti-BrdU, diluted 1:1000; Alexa Fluor-594 conjugate, Molecular Probes, Eugene, OR, USA) and DAPI (5 μg/ml; Sigma) for 1 h at 37 °C. Cells were washed twice with HG/0.1% Triton X-100 and once with water. Coverslips were mounted on glass microscope slides using 20 μl Fluoromount G/coverslip, sealed with nail polish, and stored at 20 °C.

Approximately 300 cells per treatment were counted on a Zeiss AxioPlan II microscope (equipped for epifluorescence) with a Black and White Axiocam. Using Axiovision 3.0 digital microscopy software (Zeiss), images were captured and the total cells and proliferating cells were counted. The DNA-intercalating dye DAPI was used to stain cell nuclei and to obtain the total number of viable cells. Cells that stained BrdU positive were counted as proliferating and the percentage of proliferating cells out of the total number of viable cells for the different treatments was calculated. Statistical analysis was done using an unpaired *t* test.

Sensory neurons from *Aplysia kurodai* (70–120 g) were removed from ventral-caudal clusters of pleural ganglia with a segment (100–500 μm) of major axon intact and plated on poly-L-lysine-coated tissue culture dishes; culture dishes and medium were prepared as described previously [38,50]. For sensory-motor coculture, sensory neurons isolated from the pleural ganglia were cocultured with the abdominal ganglia motor neuron LFS [36]. Sensory neurons from the same ganglia were divided and placed in dissociated cell culture randomly in four groups and treated with: (1) MDGF (100 ng/ml) plus phosphate buffer (0.5 mM); (2) hemolymph (50%); (3) hemolymph (50%) plus phosphate buffer; or (4) phosphate buffer dissolved in 50% isotonic L15 medium. Culture dishes contained five to 15 sensory neurons that were grown for 2 days at 18 °C in each medium condition, and the percentage of neurons showing de novo neurite outgrowth from the primary growth cone was calculated.

Excitatory postsynaptic potentials (EPSPs) were measured [36] and the cultures subsequently received: (1) five pulses of 5-HT (10 μM) for 5 min at 15 min intervals; (2) MDGF (100 ng/ml) in phosphate buffer; or (3) phosphate buffer alone for 1 day before the second EPSP measurement.

Cultured *A. kurodai* sensory neurons were impaled with a single microelectrode (10–20 MΩ resistance) and held at initial membrane potentials. After stabilization for 5–10 min, depolarizing current pulse steps ranging from 0.1 to 0.3 nA with a duration of 500 ms were pre-tested in each sensory neuron to determine the threshold of current that produced a single spike before treatment. Immediately after baseline recordings, MDGF (100 ng/ml) in phosphate buffer (0.5 mM), 5-HT (10 μM), or phosphate buffer solution were applied to the bath for 5 min. The 500 ms pulse was delivered 5 min after treatment. The number of spikes during the 500 ms pulse reflected membrane excitability. Only one sensory neuron was recorded from each culture plate.

Aplysia californica develop in four phases [33,34]: embryonic development; larval development, which extends from hatching until metamorphosis (stages 1–6); metamorphic development; and post-metamorphic development (stages 7–12), which extends from metamorphosis until just before reproductive maturity.

Protein was isolated from egg cordons, stages 6–12 juvenile animals, juvenile CNS, and adult CNS, and MDGF was detected by Western blot analysis. MDGF was expressed during embryonic development (Fig. 1A), and the protein size (57 kDa) was consistent with that predicted for MDGF (57.3 kDa) [6]. MDGF was detected in egg cordons, suggesting that the protein may be important during oocyte development. Egg cordons were maintained at a temperature (14 °C) similar to that of California ocean temperatures, therefore development proceeded more slowly than at the temperature used in previous studies (25 °C) [33,34]. As a result, it was not possible to correlate the MDGF levels with a particular stage of embryonic development.

To determine whether MDGF protein was expressed in the CNS of post-embryonic developing animals, a range of stages were examined. When whole animals were examined, MDGF was detectable beginning in pooled stages 6–9 animals and consistently reached a peak in stage 12 animals (Fig. 1B). MDGF levels routinely declined in the CNS of animals in the 10 g size range, increased again in 20 to 60 g developing animals, and then declined in larger ($\geq$80 g) animals. MDGF was undetectable in adult CNS, consistent with MDGF RT-PCR [6] and AGSA northern blot studies [54].

Analysis of the protein families database Pfam [7] clearly suggested that MDGF and related proteins constitute a new subfamily of adenosine deaminase-related growth factor proteins that show sequence and functional similarity to the enzyme ADA (Fig. 2), but are not related to any known growth factors [59]. To examine whether

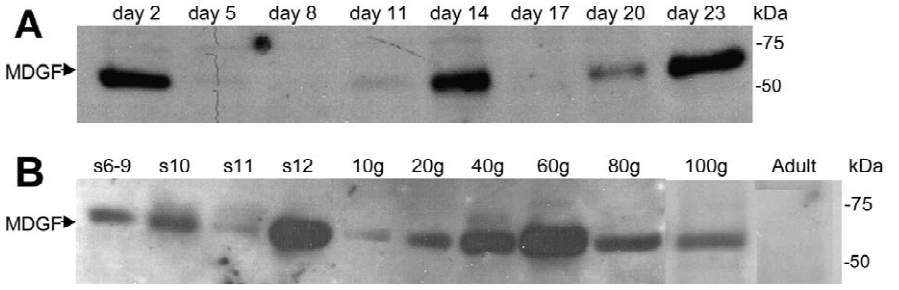

Fig. 1. MDGF protein is expressed in *Aplysia* egg cordons and the developing CNS. (A) Immunoblot analysis showing that MDGF levels in *Aplysia* egg cordons transiently increase during embryonic development ($n=3$). (B) MDGF is present in the developing CNS but not in adult CNS. For stages 6–12 juveniles, protein was isolated from the entire animal; for larger animals ($\geq$10 g), protein was isolated from the CNS ($n=3$–6 for all stages). Rainbow molecular weight markers (Amersham Pharmacia Biotech) are in kilodaltons.

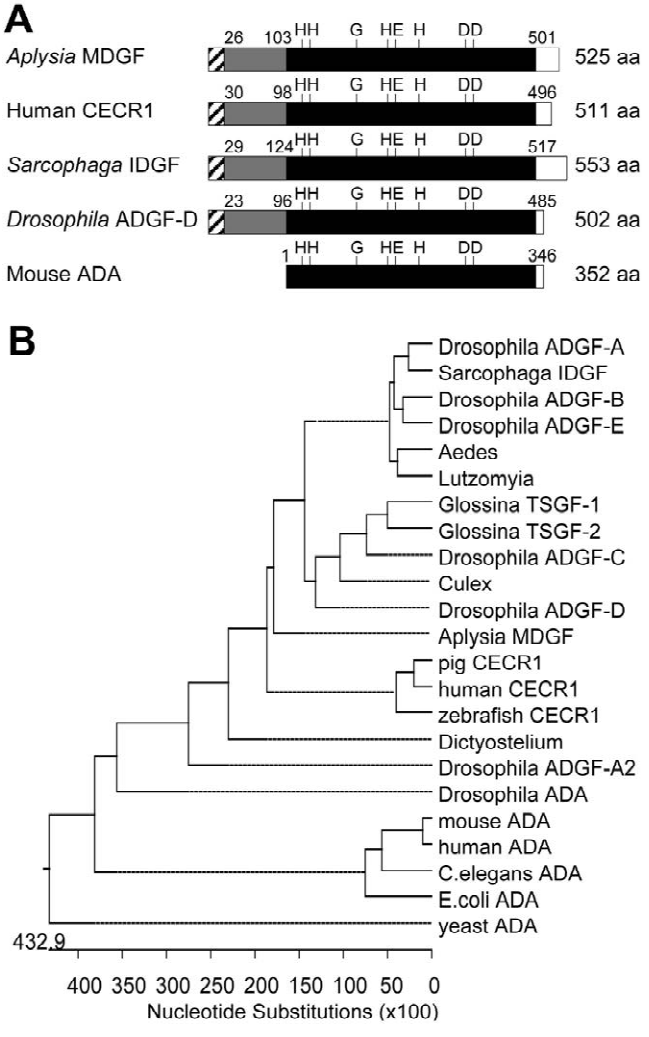

Fig. 2. MDGF-related growth factors. (A) Structural organization of *Aplysia* MDGF (accession No. AAD13112), human cat eye syndrome critical region gene 1 (CECR1), *Sarcophaga* IDGF, *Drosophila* ADGF-D, and mouse ADA. Signal peptides are batched. ADGF-specific N-terminal regions are shaded light grey. ADA domains are indicated by solid bars. Active site residues in the ADA region are indicated. Small gaps introduced to align each sequence optimally are not shown. (B) Phylogenetic analysis of MDGF-related growth factors and classical ADAs was generated based on an alignment of MDGF-related proteins, including classical ADAs; alignments were made using the MegAlign software (DNASTAR, Inc.) based on the ClustalW algorithm [55].

ADA activity was present in extracts of adult atrial glands and other tissues, atrial glands, albumen glands, large hermaphroditic ducts (Fig. 3A), and CNS were assayed for ADA activity. ADA activity was detected only in atrial gland extracts (Fig. 3B), consistent with northern blot analyses, demonstrating that AGSA mRNA was restricted to the atrial gland [54].

A Coomassie Blue-stained gel of atrial gland extract supernatant (Fig. 3C, lane 1) and purified MDGF (Fig. 3C, lane 2) are shown. The antiserum recognizes MDGF by immunoblot analysis (Fig. 3C, lane 3). This MDGF was tested in an ADA activity that measures the conversion of adenosine to inosine, and found to be an active adenosine deaminase (81 units/mg; Fig. 3D).

The mitogenic activities of *Drosophila* ADGF-A and ADGF-D (Table 1) depend on their ADA activity [59]. To obtain the best control condition, we needed to culture cells in a medium that supported their survival but did not cause an appreciable increase or decrease in cell number since this would interfere with analysis of the mitogenic response. NIH-Sape-4 cells were cultured for 72 h in M-M medium containing 0.3% FBS and MDGF (0.1 to 10 ng/ml; Fig. 4A). Addition of 0.3% FBS to the M-M culture medium resulted in a fairly constant cell number when compared to cells cultured in M-M medium alone, where we observed morphological changes consistent with cell death in some cells. MDGF caused an increase in cell number that was maximal at 1 ng/ml. At that concentration, a two-fold increase in cell number was observed relative to controls. NIH-Sape-4 cells cultured in M-M medium alone did not increase in cell number, and no detectable changes in cell morphology were observed following MDGF treatment (Fig. 4B and C).

To confirm that the two-fold increase in cell number is due to an increase in cellular proliferation, BrdU labeling and DAPI staining were used. Proliferating and total cells were identified and counted (Fig. 4D and E). Following terminal pulse-labeling with BrdU for 2.5 h, MDGF-treated cells (1 ng/ml) showed about a five-fold increase ($P<0.0001$) in the number of proliferating cells when compared to controls (41.8%, MDGF-treated cells; 7.8%, controls; Fig. 4F).

MDGF may play a developmental role in embryos and the immature CNS. MDGF protein reaches high levels during two periods of post-embryonic development. The first peak occurs in stage 12 animals (whole animals examined), and the second occurs in the CNS of 40–60 g *Aplysia*; both peaks coincide with the highest *MDGF* mRNA levels observed during development [6]. The greatest increase in cell number is known to occur during stage 12 [13,28]. Accompanying the increased neuronal proliferation during stage 12, a 150-fold increase in neuropilar volume occurs with resulting formation of synaptic interactions [13]. The significance of the second peak in MDGF expression in the CNS of 40–60 g animals is not clear. *MDGF* mRNA [6] and MDGF protein are not detectable in the CNS of adult *Aplysia*. MDGF did not appear to induce neurite outgrowth or cause other changes in the morphology of *A. kurodai* sensory neurons, affect their membrane excitability, or induce long-term synaptic facilitation in sensory-motor neuron co-cultures.

The ADA family was previously classified into two groups: (1) cellular enzymes involved in purine metabolism that are critical for maintenance of a competent immune system; genetic defects in them are correlated with severe combined immunodeficiency diseases [27]; and (2) double-stranded RNA-specific ADAs that contain unique RNA-binding domains; these ADAs are thought to participate in RNA editing of specific mRNA [32]. Since IDGF has ADA activity and no significant sequence identity was found with classical ADAs except for their ADA domains, IDGF and other ADGF proteins comprise a third group [59] that share 30–50% sequence identity and a longer N-terminal region than classical ADA enzymes (Fig. 2, Table 1).

The 3D structure of murine ADA contains a parallel α/β barrel motif with eight central β strands and eight peripheral α helices with a zinc atom in its oblong-shaped

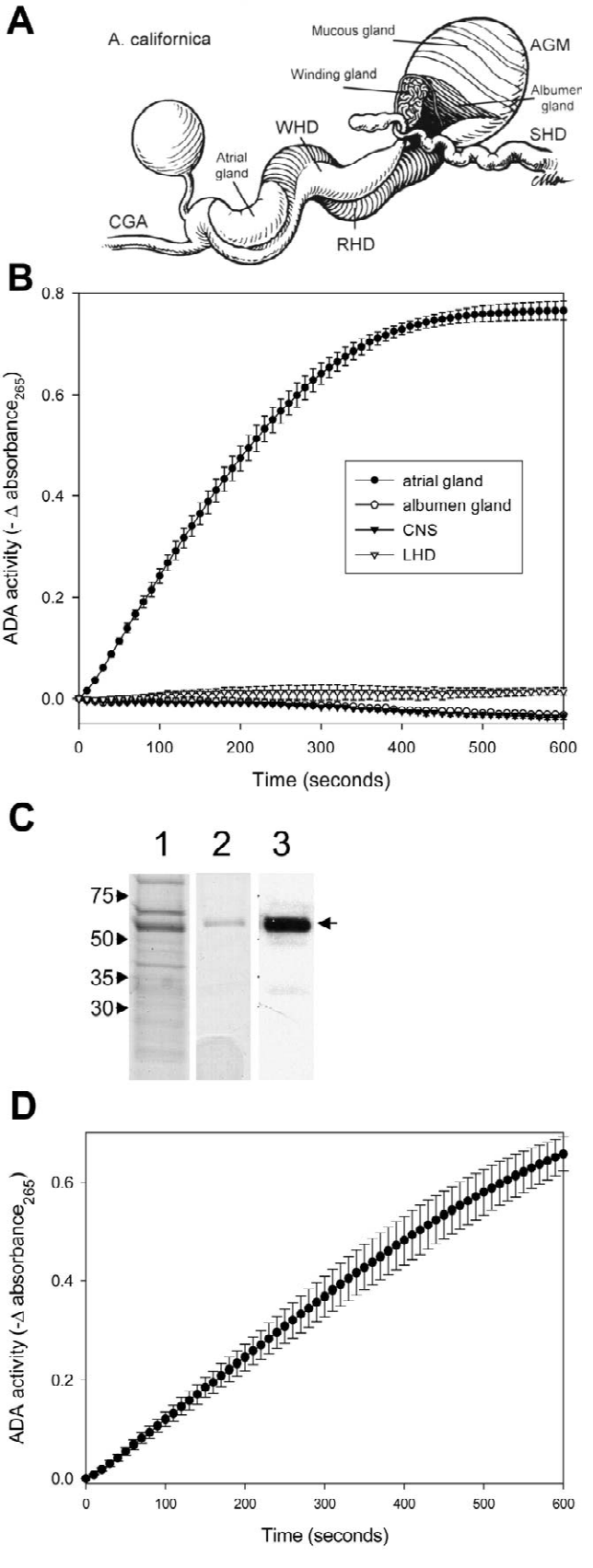

Fig. 3. Adenosine deaminase activity is restricted to the *Aplysia* atrial gland. (A) Reproductive tract of *Aplysia*. Following ovulation, eggs are transported from the ovotestis (not shown) through the small hermaphroditic duct (SHD) to the accessory genital mass (AGM), where they are fertilized and packaged into a cordon. Packaging occurs as the eggs are transported past the exocrine mucous, winding and albumen glands. The AGM is connected to the common genital aperture (CGA) by the large hermaphroditic duct, which is composed of the red hemiduct (RHD; the oviduct) and the white hemiduct (WHD; the copulatory duct). The exocrine atrial gland is associated with the oviduct and secretes into the large hermaphroditic duct. The large sac-like structure to the left is the gametolytic gland. (B) Reproductive organs and CNS of adult *Aplysia* were homogenized, and extracts (100 ng) were incubated with 100 μmol adenosine and the ADA-dependent change in A_{265} was monitored. (C) Purification of MDGF. Lane 1: Coomassie-Blue-stained supernatant of atrial gland extract (10 μg). Lane 2: Immunoaffinity-purified MDGF. Lane 3: Affinity-purified MDGF in lane 2 was transferred to PVDF membrane and probed with MDGF antiserum. The arrow indicates a 57-kDa band recognized by MDGF antiserum. Rainbow molecular weight markers (Amersham Pharmacia Biotech) are in kilodaltons. (D) Affinity-purified MDGF (10 ng) has adenosine deaminase activity. Data represent the mean±S.E. ($n=4$).

Table 1
Catalytic residues involved in ADA activity are conserved in ADA-related growth factors and classical ADAs

Enzyme	Zn^{2+}-binding			Catalytic residues					ADA activity	Mitogenic activity	Ref. and/or Accession number
ADA-related growth factors											
Aplysia MDGF[a]	H117	H119	H361	D446	G329	E364	H389	D447	Yes	Yes	[6]; present study
Human CECR1	H	H	H	D	G	E	H	D	N.D.[b]	N.D.	[45,51]; NP_059120
Pig CECR1	H	H	H	D	G	E	H	D	N.D.	N.D.	[40]; P58780
Zebrafish CECR1	H	H	H	D	G	E	H	D	N.D.	N.D.	[40]; P58781
Sarcophaga IDGF	H	H	H	D	G	E	H	D	Yes	Yes	[29]; BAA 11812
Drosophila ADGF-A	H	H	H	D	T	E	H	Y	Yes	Yes	[40,59]; AAL40912
Drosophila ADGF-A2 (MSI[c])	H	H	H	D	G	E	N	D	N.D.	Yes	[40,41]; NP_525020
Drosophila ADGF-B	H	H	H	D	G	E	H	D	N.D.	N.D.	[40,59]; AAL40920
Drosophila ADGF-C	H	H	H	D	G	E	H	D	N.D.	N.D.	[40,59]; AAL40911
Drosophila ADGF-D	H	H	H	D	G	E	H	D	Yes	Yes	[40,59]; AAL40912
Drosophila ADGF-E	K	H	H	G	G	Q	H	S	No	No	[40,59]; AAL40910
Lutzomyia	H	H	H	D	G	E	H	D	Yes	N.D.	[15,16] AAF78901
Glossina TSGF-1	H	H	H	D	G	E	H	S	+[d]	N.D.	[37]; AAD52850
Glossina TSGF-2	H	H	H	D	G	E	L	A	+[d]	N.D.	[37]; AAD52851
Aedes	H	H	H	D	G	E	H	D	+[d]	N.D.	[46]; AAL76033
Culex	H	H	H	D	G	E	H	D	+[d]	N.D.	[46]; AAK97208
Anopheles	H	H	H	D	G	E	H	D	N.D.	N.D.	
Dictyostelium	H	H	H	D	G	E	H	D	N.D.	N.D.	AAM08455
Classical ADAs											
Mouse ADA[a]	H15	H17	H214	D295	G184	E217	H238	D296	Yes	N.D.	DUMSA
Human ADA[a]	H	H	H	D	G	E	H	D	Yes	N.D.	AAH0767
C. elegans ADA	H	H	H	D	G	E	H	D	N.D.	N.D.	NP_501087
Drosophila ADA	H	H	H	D	G	E	H	D	No	No	[59];AAF5433
Yeast ADA	H	H	H	D	S	E	H	D	Yes	N.D.	NP_014258
E. coli ADA	H	H	H	D	G	E	H	D	Yes	N.D.	[14]; BAA154

[a] ClustalW alignment using Megalign software reveals conservation of amino acids corresponding to identified catalytic residues of mouse and human ADAs [44,52,57,58]. The positions of the catalytic amino acids are indicated for *Aplysia* MDGF and mouse ADA.
[b] N.D., not determined.
[c] ADGF-A2 is also known as male-specific IDGF or MSI.
[d] ADA activity has been observed in tissue extracts, but the protein has not been purified.

catalytic pocket [58]. The side chains of His15, His17, His214, and Asp295 are important for the coordination of the zinc atom [58] and the enzymatic mechanism for the deamination reaction is thought to involve Gly184, Glu217, His238, Asp295, and Asp296 [44,52,57]. With few exceptions, ADA-related growth factors share conserved Zn^{2+}-binding and catalytic residues (Table 1).

The ability of MDGF to stimulate cell proliferation was not unexpected, since *Drosophila* ADGF-A and ADGF–D (Table 1) and calf ADA [30] stimulate NIH-Sape-4 cell proliferation [59]. In adult *Aplysia*, ADA activity is restricted to atrial glands, and MDGF exhibits potent ADA activity in vitro, strongly suggesting that the ADA-like domain of MDGF is responsible for the ADA activity (Fig. 2, Table 1). MDGF stimulates maximal proliferation of NIH-Sape-4 cells at a concentration similar to that of IDGF [29,30]. Growth stimulation by MDGF is unlikely to be due to the production of inosine analogs in the medium, since adenosine analogs and inosine analogs cause essentially no growth stimulation of NIH-Sape-4 cells [30].

Drosophila ADGFs stimulate cell proliferation by depleting extracellular adenosine levels [59]. *Drosophila* $C1.8^{+}$ cells exhibit dramatic increases in survival, polarization, and proliferation in response to minimal medium lacking adenosine, and the addition of ADGF-A has no effect [59]. When adenosine is added to the medium, the cells lose polarity and fail to proliferate, and these effects can be prevented by addition of ADA or ADGF [59]. Although these findings suggest that the mitogenic response of NIH-Sape-4 cells is due solely to adenosine depletion, it is also possible that ADA-related growth factors may exert some of their effects via a receptor-mediated mechanism.

There are specific binding sites for IDGF on the surface of NIH-Sape-4 cells, and IDGF binds to these sites with a K_d of about 2×10^{-10} M [30], however the IDGF receptor has not been isolated or characterized. It has been proposed that the receptors for IDGF are modified with an adenosine moiety, and that when IDGF binds to these receptors, it may deaminate the adenosine moiety, and this process may be a prerequisite for signal transduction. Alternatively, the unique N-terminal region of this subfamily of growth factors may have classic growth factor functions by binding to receptors on the surface of target cells to induce a signaling cascade, and the ADA domain is necessary to modify another protein that is needed for the signal to be transmitted. It is also possible that the cell response is due to a combination of binding of ADA-related growth factor

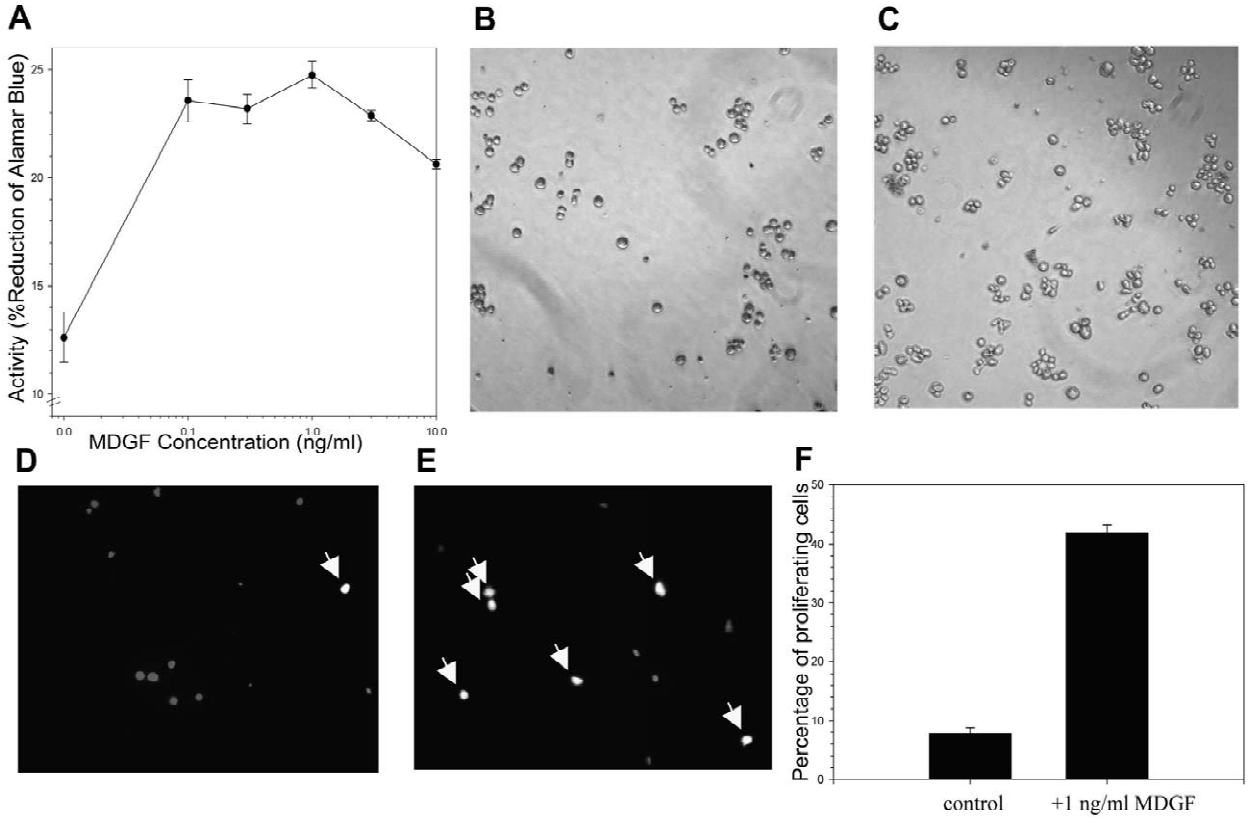

Fig. 4. MDGF increases the number of NIH-Sape-4 cells in vitro. (A) Cells were plated at low density (1×10^5 cells/100 μl/well) in M-M medium containing 0.3% FBS and allowed to attach to the substratum before adding purified MDGF (0.1–10 ng/ml). After 72 h, the percent reduction of alamarBlue was determined. Data represent the mean±S.E. of four experiments. (B) NIH-Sape-4 cells cultured for 72 h in M-M medium lacking MDGF (control). (C) NIH-Sape-4 cells cultured for 72 h in M-M medium containing 1 ng/ml MDGF. (D, E) BrdU/DAPI staining of NIH-Sape-4 cells. Cells were plated in microtiter plates and the growth factor assay was performed as described in the text. To distinguish non-proliferating versus proliferating cells, the cells were incubated with a combination of DAPI and a BrdU monoclonal antibody conjugated to Alexa Fluor-594. The figure shows the difference in (D) control versus (E) MDGF-treated cells. Arrows point to BrdU-positive proliferating cells. (F) The number of BrdU-positive cells was counted and divided by the total number of cells (DAPI-stained) to determine the percentage of proliferating cells. The number of proliferating cells was increased significantly in MDGF-treated cells. Data are expressed as the mean±S.E. ($n=3$) and statistical analysis was performed using an unpaired *t*-test (*$P<0.0001$).

to a receptor and the ADA-mediated modulation of adenosine and/or inosine levels. An ADA-binding protein (CD26) has been described that binds to a cluster of charged residues in human ADA [47,48]. Interestingly, two of these three residues are conserved in *Aplysia* MDGF and the third residue is conservatively substituted.

Adenosine is a peripheral nociceptive agent [11] and ADA-related growth factors may be involved in the regulation of extracellular adenosine levels. For example, *Lutzomyia* ADA is thought to reduce adenosine levels to prevent or attenuate host perception of an insect bite [15]. ADA catalyzes the conversion of adenosine or 2′-deoxyadenosine to inosine or 2′-deoxyinosine, thereby regulating their intra- and extracellular concentrations [23]. ADA has a wide species distribution and its sequence is highly conserved [5,10,14,39,44]. ADA deficiency is associated with severe combined immunodeficiency disease in humans [2,19,20], and the effects are thought to be due largely to the accumulation of ADA substrates and their subsequent conversion to dATP, which inhibits ribonucleotide reductase, a key enzyme in DNA synthesis [20].

Addition of adenosine and its subsequent conversion to inosine is involved in stimulating axon outgrowth, expression of growth associated protein (GAP-43), and reverses inhibition of axon outgrowth due to 6-thioguanine in goldfish and rat retinal ganglion cells, suggesting that axon outgrowth in CNS neurons involves an intracellular purine-sensitive mechanism [9]. In vivo studies of axon growth after unilateral transection of the rat corticospinal tract show that minipump application of inosine to rat sensorimotor cortex stimulated extensive sprouting of pyramidal cells into the denervated spinal cord white matter [8], resulting in the formation of new corticospinal tract synapses in the denervated spinal cord [53]. This axonal reorganization is accompanied by improved performance on sensorimotor tasks for inosine-treated rats with unilateral cortical infarcts [17]. These data suggest that inosine is involved in neuronal reorganization and restoration of

essential circuitry after CNS injury. It is not known whether there is a correlation between inosine levels and the observed increase in MDGF during stage 12 of development.

The high levels of *MDGF* mRNA and protein in ovo and in the developing CNS suggest a potential role for MDGF in the developing *Aplysia* CNS. MDGF may play a role in regulating extracellular adenosine and inosine levels during CNS development to provide a permissive environment for growth, development, and, perhaps, repair of the CNS. Although we see no effect on *A. kurodai* sensory neurons, MDGF may affect the proliferation of other neuron types, neural stem cells, or glial cells.

Adenosine released during rat spinal cord injury can reach an extracellular concentration of 100 μM [42]. This raises the question as to whether MDGF plays a role in reducing the levels of extracellular adenosine after nerve injury in *Aplysia*, and whether a reduction in these levels could facilitate injury repair.

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