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Characterisation of intestinal peptide transporter of the Antarctic haemoglobinless teleost *Chionodraco hamatus*

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Summary

H^+/peptide cotransport was studied in brush-border membrane vesicles (BBMV) from the intestine of the haemoglobinless Antarctic teleost *Chionodraco hamatus* by monitoring peptide-dependent intravesicular acidification with the pH-sensitive dye Acridine Orange. Diethylpyrocarbonate-inhibited intravesicular acidification was specifically achieved in the presence of extravesicular glycyl-L-proline (Gly-L-Pro) as well as of glycyl-L-alanine (Gly-L-Ala) and D-phenylalanyl-L-alanine (D-Phe-L-Ala). H^+/Gly-L-Pro cotransport displayed saturable kinetics, involving a single carrier system with an apparent substrate affinity ($K_{m,app}$) of 0.806±0.161 mmol l^{-1}. Using degenerated primers from eel and human (PepT1) transporter sequence, a reverse transcription-polymerase chain reaction (RT-PCR) signal was detected in *C. hamatus* intestine. RT-PCR paralleled kinetic analysis, confirming the hypothesis of the existence of a PepT1-type transport system in the brush-border membranes of icefish intestine.

Functional expression of H^+/peptide cotransport was successfully performed in *Xenopus laevis* oocytes after injection of poly(A)$^+$ RNA (mRNA) isolated from icefish intestinal mucosa. Injection of mRNA stimulated D-Phe-L-Ala uptake in a dose-dependent manner and an excess of glycyl-L-glutamine inhibited this transport. H^+/peptide cotransport in the Antarctic teleost BBMV exhibited a marked difference in temperature optimum with respect to the temperate teleost *Anguilla anguilla*, the maximal activity rate occurring at approximately 0°C for the former and 25°C for the latter. Temperature dependence of icefish and eel intestinal mRNA-stimulated uptake in the heterologous system (oocytes) was comparable.

Key words: brush-border membrane vesicle, *Xenopus laevis* oocyte, PepT1, H^+/peptide cotransport, fish, intestine, Antarctic fish.

Introduction

In mammals intestinal absorption of di- and tripeptides provided by dietary protein digestion is mediated by electrogenic brush-border membrane (BBM) transport processes. Peptide influx, coupled to an inwardly directed H^+ gradient, is additionally driven by the inside-negative transmembrane electrical potential (Ganapathy et al., 1994). Peptide transporters are unique among the solute transporters in presenting H^+ as the cotransported species and the capability to bind and translocate a high number of different substrates (Ganapathy et al., 1994; Meredith and Boyd, 2000). Besides peptides, this transporter is able to carry pharmacologically active molecules such as β-lactam antibiotics (Kramer et al., 1992; Okano et al., 1986; Tsuji, 1995; Wenzel et al., 1995), angiotensin converting enzyme (ACE) inhibitors such as captopril (Hu and Amidon, 1988) and renin inhibitors (Kramer et al., 1990). The molecular nature of the peptide transporters has been recently elucidated by the cloning of highly homologous cDNAs, encoding transmembrane proteins that exhibit membrane-potential-dependent transport of peptides and peptide-like drugs, coupled to proton translocation (Boll et al., 1995; Fei et al., 1994; Liang et al., 1995; Saito et al., 1995; Daniel, 1996).

Peptide transport has also been well demonstrated in teleosts where, as in higher vertebrates, peptide uptake occurs by carrier-mediated mechanisms and is highly stimulated by an inside-negative trans-membrane electric potential (Thamotharan et al., 1996; Maffia et al., 1997). Furthermore, an inwardly directed trans-membrane H^+ gradient has resulted coupled to peptide transport, either alone (Thamotharan et al., 1996) or in cooperation with the membrane potential (Maffia et al., 1997; Thamotharan et al., 1996). Proton/glycyl-sarcosine (Gly-Sar) cotransport has been described in BBM of absorbing cells of tilapia *Oreochromis mossambicus* intestine and

rockfish *Sebastes caurinus* intestine and pyloric ceca (Thamotharan et al., 1996). Moreover, H^+/glycyl-glycine, H^+/glycyl-L-proline and H^+/D-phenyl-L-alanine cotransport has been described in eel *Anguilla anguilla* intestinal BBM (Verri et al., 1992, 2000; Maffia et al., 1997).

To extend information on peptide transporters in lower vertebrates, we have investigated the presence of such a carrier in a very particular family of teleost, Channichthyidae (icefish). The members of this family have been ecologically confined for the last 13 million years to the low-temperature Antarctic waters, developing an extremely high degree of stenothermy and endemism (Eastman, 1993). In particular we investigated the presence of a H^+/peptide cotransport, characterising several of its functional properties in brush border membrane vesicles (BBMV) isolated from *C. hamatus* intestine and making a functional comparison with the peptide transporter of eel intestine. Icefish and eel dipeptide transporters were functionally expressed in *Xenopus laevis* oocytes, and part of the icefish dipeptide transporter nucleotide sequence was compared to the mammalian intestinal PepT1-type transporter by RT-PCR approach. Finally, the effects of temperature on transporter functionality were investigated by measuring the temperature-dependent substrate uptake.

Materials and methods

Materials

Specimens of *Chionodraco hamatus* Lönnberg, each weighing 200–250 g, were collected in the Ross sea (Antarctica) off the Italian Antarctic base Terranova Bay and maintained without feeding in seawater aquaria at 0°C. Eels *Anguilla anguilla* at the yellow stage were purchased from the Acquatina Lagoon (Frigole, Italy). Female *Xenopus leavis* (NASCO, Fort Atkinson, WI, USA) were maintained at 18°C in a 12 h:12 h light:dark cycle and kept in pairs in 10 liter plastic tanks containing 5 cm of chlorine-free tapwater. Frogs were fed 'frog brittle' twice a week, followed by a change of water 1 h after feeding. All chemicals were reagent grade and purchased from Merck (Darmstadt, Germany). Valinomycin was obtained from Sigma (St Louis, MO, USA). Custom-synthesised D-[^{3}H]phenylalanyl-L-alanine (D-[^{3}H]Phe-L-Ala; specific activity 9 Ci mmol l^{-1}) was obtained from Zeneca (Billingham, UK), while the fluorescent dye Acridine Orange (AO) was from Eastman Kodak (Rochester, NY, USA).

Isolation of poly(A)$^+$ RNA

Total RNA was isolated from the scraped mucosa of the whole icefish and eel intestine, using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) (Chomzynski and Saachi, 1987). Poly(A)$^+$ RNA (mRNA) was purified by chromatography using oligo(dT)-cellulose affinity column (Sambrook et al., 1989).

Reverse transcription-polymerase chain reaction (RT-PCR)

1 μg poly(A)$^+$ RNA samples from icefish and eel intestinal mucosa were subjected to RT-PCR using the GeneAmp RNA-PCR kit (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Briefly, reverse transcription was performed for 12 min at 42°C in the presence of oligo(dT)$_{16}$ primer and the resulting cDNA was subjected to PCR using degenerated primers based on the human (PepT1, complete cds; Liang et al., 1995) and eel (AY167576 GenBank) intestinal peptide transporter sequence (forward primer: 5′-GACTCGTGGCTSGGRARGTTC-3′, starting at nucleotide 279, and reverse primer: 5′-CCAGTCCAKCCAG-TGCKCCCTCT-3′, starting at nucleotide 848).

PCR amplification was performed for 35 cycles with 95°C denaturation for 1 min, 48°C annealing for 1 min and 72°C extension for 1 min, followed by a final synthesis at 72°C for 7 min. RT-PCR products were separated by 1% agarose gel electrophoresis, stained by ethidium bromide (1 mg l^{-1}) and visualised under UV light using the Gel-Doc System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR product was subcloned into TOPO TA cloning vector (Invitrogen, CA, USA), and subjected to sequencing. Sequence alignment was done using the Clustal W program (EMBL-EBI).

Transport activity in intestinal brush border membrane vesicles (BBMV)

Preparation of BBMV

BBMV were prepared from the whole intestine of the fishes as previously described (Maffia et al., 1996; Verri et al., 2000). Protein concentration was measured with the Bio-Rad protein assay kit, using lyophilised bovine serum albumin as a standard. Intra- and extravesicular buffers had the same ionic strength, anion concentration and osmolarity.

Measurements of fluorescence quenching

BBM intravesicular acidification was assessed by monitoring the fluorescence quenching of the pH-sensitive dye Acridine Orange as previously detailed (Verri et al., 2000). BBMVs were prepared in a buffer containing (in mmol l^{-1}): 100 KCl, 100 mannitol, 2 Hepes, adjusted to pH 7.4 with Tris. To start the experiment, 20 μl of BBMVs (250 μg of protein) were injected into 1980 μl of cuvette buffer containing 3 μmol l^{-1} Acridine Orange, 5 μmol l^{-1} valinomycin, 0.5% ethanol, 100 mmol l^{-1} mannitol, 2 mmol l^{-1} Hepes, adjusted to pH 7.4 with Tris, and either KCl, choline chloride or dipeptide. When present, peptides replaced mannitol iso-osmotically. To obtain faster re-equilibration of the transient transmembrane H^+ asymmetry, 20 μl of 3 mol l^{-1} KCl solution were added into the cuvette at the time indicated. Fluorescence signals were recorded by a Perkin-Elmer LS-50B spectrofluorometer equipped with an electronic stirring system and a thermostatically controlled cuvette holder and managed by the Perkin-Elmer Fluorescence Data Manager software for PC (Perkin-Elmer Ltd, Buckinghamshire, UK). Excitation and emission wavelengths were 498 and 530 nm, respectively, and both slit widths were set to 5 nm. Each experiment was repeated at least three times using membranes prepared from different animals. Within a single experiment, each data point represents 3–5 replicate measurements.

For diethylpyrocarbonate (DEP) inhibition of proton accumulation BBMVs were incubated for 1 h at 0°C in buffer containing (in mmol l^{-1}) 280 mannitol, 20 K_2HPO_4/KH_2PO_4, pH 6.4, either in the presence (100 mmol l^{-1} ethanol stock solution) or in the absence (ethanol only) of 2 mmol l^{-1} diethylpyrocarbonate (DEP). In both cases, the final ethanol concentration did not exceed 1%. Then, to eliminate excess DEP, which affects the Acridine Orange fluorescence signal, DEP-treated and untreated BBMVs were diluted in 35 ml of buffer containing (in mmol l^{-1}) 100 mannitol, 100 KCl, 2 Hepes, adjusted to pH 7.4 with Tris, and centrifuged at 50000 ***g*** for 30 min. This washing procedure was repeated twice. To start the experiment, BBMVs (20 µl, 250 µg of protein) loaded with (in mmol l^{-1}) 100 mannitol, 100 KCl and 2 Hepes, adjusted to pH 7.4 with Tris, were injected into 1980 µl of cuvette buffer containing 3 µmol l^{-1} Acridine Orange, 5 µmol l^{-1} valinomycin, 0.5% ethanol and either (in mmol l^{-1}) 100 choline chloride, 80 mannitol and 2 Hepes, adjusted to pH 7.4 with Tris, plus 20 Gly-L-Pro. The net peptide-dependent H^+ fluxes were obtained by subtracting H^+ flux in the absence of peptide (control) from the total H^+ flux in the presence of peptide (Gly-L-Pro), expressed as $\Delta F\%\ mg^{-1}$ protein min^{-1}, where $\Delta F\%$ is the fluorescence quenching.

For the temperature-dependence experiments, icefish and eel BBMVs (20 µl, 250 µg of protein), prepared in the same buffer as above, were injected into 1980 µl of cuvette buffer containing 3 µmol l^{-1} Acridine Orange, 5 µmol l^{-1} valinomycin, 0.5% ethanol, 100 mmol l^{-1} mannitol, 2 mmol l^{-1} Hepes, adjusted to pH 7.4 with Tris, and 100 mmol l^{-1} choline chloride or 100 mmol l^{-1} choline chloride and 20 mmol l^{-1} Gly-L-Pro. The Gly-L-Pro dependent proton uptake and passive diffusion (choline chloride) were measured at increasing temperatures: –2, 0, 2, 4, 8, 12, 18, 24 and 30°C for *C. hamatus* and 1, 5, 9, 12, 18, 25, 30 and 40°C for *A. anguilla*.

Kinetic analysis

BBMV (20 µl, 250 µg of protein), prepared in a buffer containing (in mmol l^{-1}): 100 mannitol, 100 KCl, 2 Hepes, adjusted to pH 7.4 with Tris, were injected into 1980 µl of an incubation buffer containing 3 µmol l^{-1} Acridine Orange, 5 µmol l^{-1} valinomycin, 0.5% ethanol, 100 mmol l^{-1} choline chloride, 100 mmol l^{-1} mannitol, 2 mmol l^{-1} Hepes, adjusted to pH 7.4 with Tris and increasing Gly-L-Pro concentrations from 0.5 to 20 mmol l^{-1}, iso-osmotically compensated by decreasing mannitol concentrations. Kinetic parameters were determined by non-linear regression analysis, based on the Marquardt algorithm (Marquardt, 1963) by using the software package Statgraphics (STSC, Rockville, MD, USA). Carrier-mediated Gly-L-Pro-dependent H^+ influx kinetics were determined by a curve-fitting procedure using the iterative non-linear regression method based on the following Michaelis–Menten type equation: $\Delta F\%=(\Delta F\%\times[S])/(K_{m,app}+[S])$, where $\Delta F\%$ was peptide-dependent H^+ influx, $[S]$ extravesicular peptide concentration and $K_{m,app}$ the concentration that yielded one half $\Delta F\%_{max}$.

Transport activity in Xenopus laevis *oocytes*

Oocytes and injections

Mature females *X. leavis* frogs were anaesthetised by partial immersion in a solution of ethyl-3-aminobenzoate (MS-222; 1.5 g l^{-1}). Oocytes at stages V and VI were removed through a small incision in the abdomen and separated from the ovarian lobes using fine forceps. Following collagenase treatment for 1 h, defolliculated oocytes were allowed to recover in Barth's medium (Colman, 1984) overnight at 18°C. Oocytes were injected into the vegetal pole with 40 nl of either mRNA solution (1 ng nl^{-1}) or RNAse-free water using a World Precision Instrument nanoliter injector (Sarasota, FL, USA). Oocytes were then maintained at 4 or 18°C in daily changes of Barth's medium for up to 3 days.

Uptake measurements in oocytes

X. laevis oocytes, injected with 40 nl of either water or poly(A)$^+$ mRNA (1 ng nl^{-1}) prepared from icefish and eel intestinal mucosa cells, were incubated either at 4°C or 18°C for 3 days. Then dipeptide (D-Phe-L-Ala) uptake was measured, at 0°C and 18°C, in a pH 6.5 buffer. 5–10 oocytes were used for each experimental point. Uptake measurement was initiated by placing the oocytes in 200 µl of the uptake medium (100 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$ and 1 mmol l^{-1} $CaCl_2$, buffered with 5 mmol l^{-1} MES/Tris, pH 6.5) containing [^{3}H]-D-Phe-L-Ala (10 µCi ml^{-1}). After incubation the uptake solution was removed and the oocytes were washed three times with 3 ml of ice-cold buffer (100 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, and 1 mmol l^{-1} $CaCl_2$ and was buffered with 5 mmol l^{-1} MES/Tris, pH 6.5). Each single oocyte was then placed into a scintillation vial, dissolved in 250 µl of 1% SDS, followed by the addition of 1 ml of Ready-Safe scintillation fluid (Beckman, Fullerton, CA, USA) and counted (1 min per oocyte) using liquid scintillation spectrometry.

In the dose-dependence study of the expression of D-Phe-L-Ala uptake, oocytes were injected with either 40 nl of water or increasing amounts of icefish intestinal mucosal cells mRNA (13, 19, 23 and 40 ng) and then incubated at 18°C for 3 days. 5 oocytes were used for each uptake determination. D-Phe-L-Ala uptake in the oocytes was determined at 18°C for 1 h after 3 days of incubation at 18°C. The composition of the uptake medium and the concentration of radiolabel were the same as for the uptake experiments.

In the competition experiments oocytes were injected with 40 nl of either water or mRNA (1 ng nl^{-1}) isolated from icefish and eel intestinal mucosa cells. 5 oocytes were used for each uptake determination. Uptake of peptide was measured at 18°C for 1 h after 3 days of incubation at 18°C. The composition of the uptake medium was the same as for the uptake experiments. The concentrations of [^{3}H]-D-Phe-L-Ala and glycyl-L-glutamine (Gly-Gln) were 2 mmol l^{-1} (10 µCi ml^{-1}) and 10 mmol l^{-1}, respectively.

In the temperature dependence experiments oocytes were injected with 40 nl of poly(A)$^+$ mRNA (1 ng nl^{-1}) isolated from eel and icefish intestine and then incubated at 18°C for 3 days.

5 oocytes were used for each uptake determination. D-Phe-L-Ala uptake was evaluated by pre-incubating the oocytes for 30 min at different temperatures (0, 12, 18, 25°C) and then measuring the peptide uptake at 18°C for 1 h. The composition of the uptake medium was the same as described above. The concentration of D-Phe-L-Ala was 2 mmol l^{-1} (10 μCi ml^{-1}).

Statistics

Each experiment was repeated at least three times using membranes prepared from different animals. Within a single experiment, each data point represents 3–5 replicate measurements. Data points reported in the figures are given as means ± standard error (S.E.M.). Error bars are shown wherever they exceed the size of the symbols.

Results

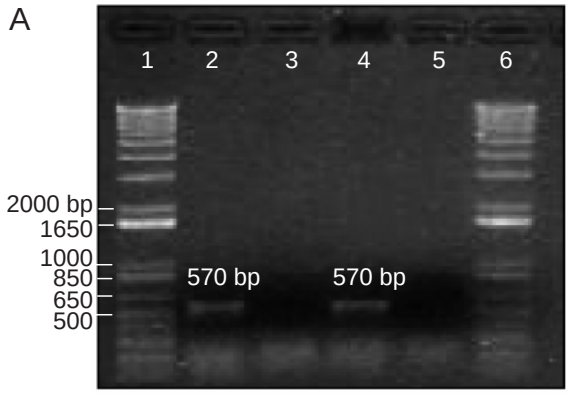
Detection of a RT-PCR product in icefish intestine by specific primers

Using degenerate primers designed on the basis of the human and eel intestinal peptide transporter sequence, PepT1-related RT-PCR products of approx. 570 base pairs (bp) were amplified from mRNA isolated from whole icefish and eel intestine (Fig. 1A). The partial nucleotide sequence of the resulting cDNA of icefish showed 73% similarity to hPepT1 and presumably encoded an amino acid sequence with 74% similarity to hPepT1 (Fig. 1B).

Peptide-dependent H^+ uptake in BBMV

The functional expression of a peptide transporter in the icefish intestine, working at low temperatures, was confirmed by the observation of a peptide-dependent proton influx in isolated BBMV, followed by monitoring Acridine Orange fluorescence quenching at 0°C (Fig. 2A). As shown in this figure, the addition of vesicles to the cuvette, in short-

A



B

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PepT-ice        ------------------------------------------------------------
hPepT1          MGMSKSHSFFGYPLSIFFIVVNEFCERFSYYGMRAILILYFTNFISWDDNLSTAIYHTFV 60

PepT-ice        --------------DSWLGRFKTIVYLSIVYMLGQVVMAISAIHDLTDANMDGTPDNMTL 46
hPepT1          ALCYLTPILGALIADSWLGKFKTIVSLSIVYTIGQAVTSVSSINDLTDHNHDGTPDSLPV 120
                              *****:***** ***** :**.* ::*:*:**** * *****.:.:

PepT-ice        HVALSMVGLILIALGTGGIKPCVAAFGGDQFLDHQEKQRSTFFSIFYLSINAGSLLSTVI 106
hPepT1          HVVLSLIGLALIALGTGGIKPCVSAFGGDQFEEGQEKQRNRFFSIFYLAINAGSLLSTII 180
                **.**:** *************:******* : *****. *******:*********:*

PepT-ice        TPILRAQECGINTKQSCYPLAFGVPAALMVVALVVFILGSRMYIKTPPQGNIMVKVCTCI 166
hPepT1          TPMLRVQQCGIHSKQACYPLAFGVPAALMAVALIVFVLGSGMYKKFKPQGNIMGKVAKCI 240
                **:**.*:***::**:*************.***:**:*** ** *  ****** **..**

PepT-ice        GFAVKNRFRHRSSQYPKRAHWLDW---------------------------------- 190
hPepT1          GFAIKNRFRHRSKAFPKREHWLDWAKEKYDERLISQIKMVTRVMFLYIPLPMFWALFDQQ 300
                ***:********. :*** *****

PepT-ice        ------------------------------------------------------------
hPepT1          GSRWTLQATTMSGKIGALEIQPDQMQTVNAILIVIMVPIFDAVLYPLIAKCGFNFTSLKK 360

PepT-ice        ------------------------------------------------------------
hPepT1          MAVGMVLASMAFVVAAIVQVEIDKTLPVFPKGNEVQIKVLNIGNNTMNISLPGEMVTLGP 420

PepT-ice        ------------------------------------------------------------
hPepT1          MSQTNAFMTFDVNKLTRINISSPGSPVTAVTDDFKQGQRHTLLVWAPNHYQVVKDGLNQK 480

PepT-ice        ------------------------------------------------------------
hPepT1          PEKGENGIRFVNTFNELITITMSGKVYANISSYNASTYQFFPSGIKGFTISSTEIPPQCQ 540

PepT-ice        ------------------------------------------------------------
hPepT1          PNFNTFYLEFGSAYTYIVQRKNDSCPEVKVFEDISANTVNMALQIPQYFLLTCGEVVFSV 600

PepT-ice        ------------------------------------------------------------
hPepT1          TGLEFSYSQAPSNMKSVLQAGWLLTVAVGNIIVLIVAGAGQFSKQWAEYILFAALLLVVC 660

PepT-ice        -------------------------------------------------
hPepT1          VIFAIMARFYTYINPAEIEAQFDEDEKKNRLEKSNPYFMSGANSQKQM 708
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Fig. 1. (A) Expression of PepT1-related mRNA in icefish and eel intestine by RT-PCR. The PepT1 isoform was detected in the intestine of icefish (lane 2) and eel (lane 4; positive control). The identity of each isoform was confirmed by PCR product size (570 bp) and sequencing (data not shown). Lanes 1 and 6: markers; lanes 3 and 5: RT-PCR control (RNA without reverse transcriptase). (B) Comparison of primary amino acid sequences of icefish and human PepT1 H^+/peptide cotransporters. Asterisks indicate indentical amino acids; colons, conserved substitution (small, acidic, basal or hydroxyl+ Amine+Basic, etc.); stops, semi-conserved substitution.

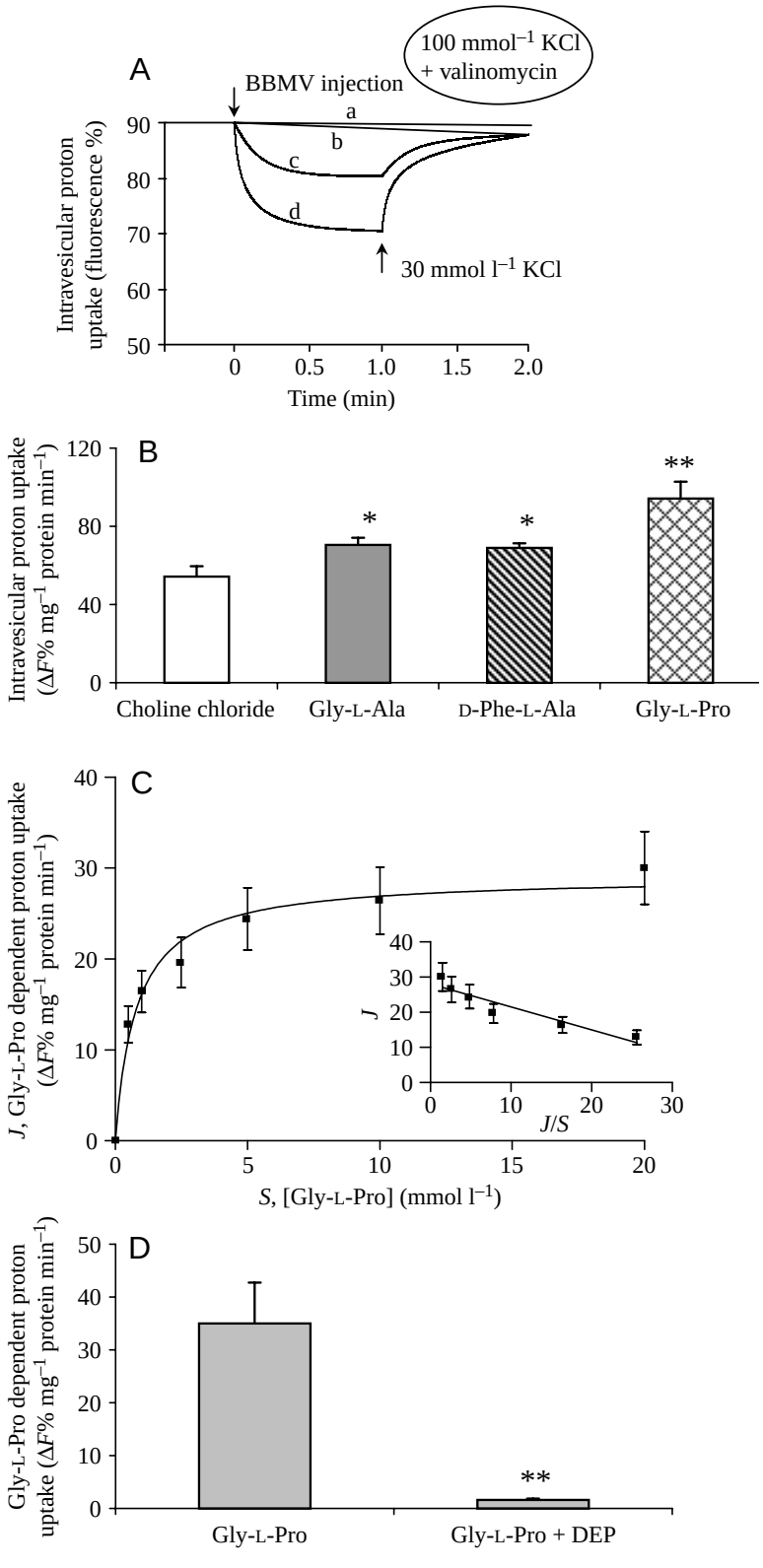

Fig. 2. (A) Peptide-dependent Acridine Orange fluorescence quenching in icefish intestine brush-border membrane vesicles (BBMVs). In addition to valinomycin, the cuvettes contained 100 mmol l^{-1} KCl (trace a); 100 mmol l^{-1} KCl + 20 mmol l^{-1} Gly-L-Pro (trace b); 100 mmol l^{-1} choline chloride (trace c); 100 mmol l^{-1} choline chloride + 20 mmol l^{-1} Gly-L-Pro (trace d). (B) Effects of exogenous dipeptides on H^+ influx rate (trace d – trace c, N=5). (C) Dependence of the initial rate of H^+ influx on increasing extravesicular Gly-L-Pro concentrations. Inset, the Woolf–Augustinsson–Hofstee plot of the experimental data (N=3). (D) Diethylpyrocarbonate (DEP) inhibition of proton accumulation in icefish intestinal BBMVs (N=4). Values were significant at *P<0.05; **P<0.01.

circuited conditions ($[K^+]_i$=$[K^+]$=100 mmol l^{-1}, plus valinomycin), did not produce any significant change in AO fluorescence quenching, either in the absence (trace a) or in the presence (trace b) of 20 mmol l^{-1} glycyl-L-proline (Gly-L-Pro) in the extravesicular medium. However, when a transmembrane electrical potential was imposed ($[K^+]_i$=100 mmol l^{-1}, $[K^+]$= 1 mmol l^{-1} plus valinomycin), a transient fluorescence quenching was observed due to intravesicular acidification (trace c), which was further enhanced in the presence of 20 mmol l^{-1} Gly-L-Pro (trace d). Specific peptide-dependent H^+ influx was determined by subtracting from this value the fluorescence quenching in the absence of peptide (trace d – trace c). As shown in Fig. 2B, the icefish transporter is able to recognise different dipeptides. When specific peptide-dependent H^+ influx was measured at saturating concentrations of three different dipeptides, Gly-L-Pro exhibited higher maximal velocities with respect to glycyl-L-alanine (Gly-L-Ala) and D-phenylalanyl-L-alanine (D-Phe-L-Ala). Several kinetic features of the icefish peptide transporter were determined by monitoring Gly-L-Pro-dependent H^+ influx, at 0°C, in the presence of increasing peptide concentrations (Fig. 3C). Non-linear regression analysis of the experimental data yielded calculated values at 0°C for V_{max}=29.06±1.27 ΔF% mg^{-1} protein min^{-1} and $K_{m,app}$=0.806±0.161 mmol l^{-1}. The linear relationship observed in a Woolf–Augustinsson–Hofstee plot (Segel, 1975) (inset to Fig. 3C) strengthened the hypothesis for the presence of a single carrier system for Gly-L-Pro transporter. The hypothesis of the presence of a peptide transporter in intestinal icefish BBMV is further strengthened by the significant inhibition of Gly-L-Pro dependent proton influx that occurs in the presence of DEP, a reactive agent specific for hystidyl-residues that inhibits mammalian peptide transporters (Fei et al., 1997; Miyamoto et al., 1986; Terada et al., 1996) (Fig. 2D).

Peptide uptake in mRNA-injected oocytes

Measurement of peptide uptake in mRNA-injected oocytes (Fig. 3A) revealed no expression of the intestinal peptide transporter in oocytes pre-incubated for 3 days at 4°C,

compared with a marked expression in those pre-incubated at 18°C (i.e. the physiological temperature of *X. laevis*). Injection of increasing concentrations of poly(A)$^+$ mRNA prepared from *C. hamatus* enterocytes into *X. laevis* oocytes led to a dose-dependent stimulation of peptide transport (Fig. 3B), as shown by the [^{3}H]-D-Phe-L-Ala oocyte uptake activity after 3 days of incubation at 18°C. Intestinal peptide transporters such as hPepT1 are characterised by their ability to transport a variety of di-/tripeptides. Unlabeled peptide (glycyl-glutamine, Gly-Gln) competes with radiolabeled D-Phe-L-Ala for the uptake process in mRNA-injected oocytes (Fig. 3C), demonstrating the competition between different dipeptides for the icefish intestinal peptide transporter heterologously expressed in *X. laevis* oocytes.

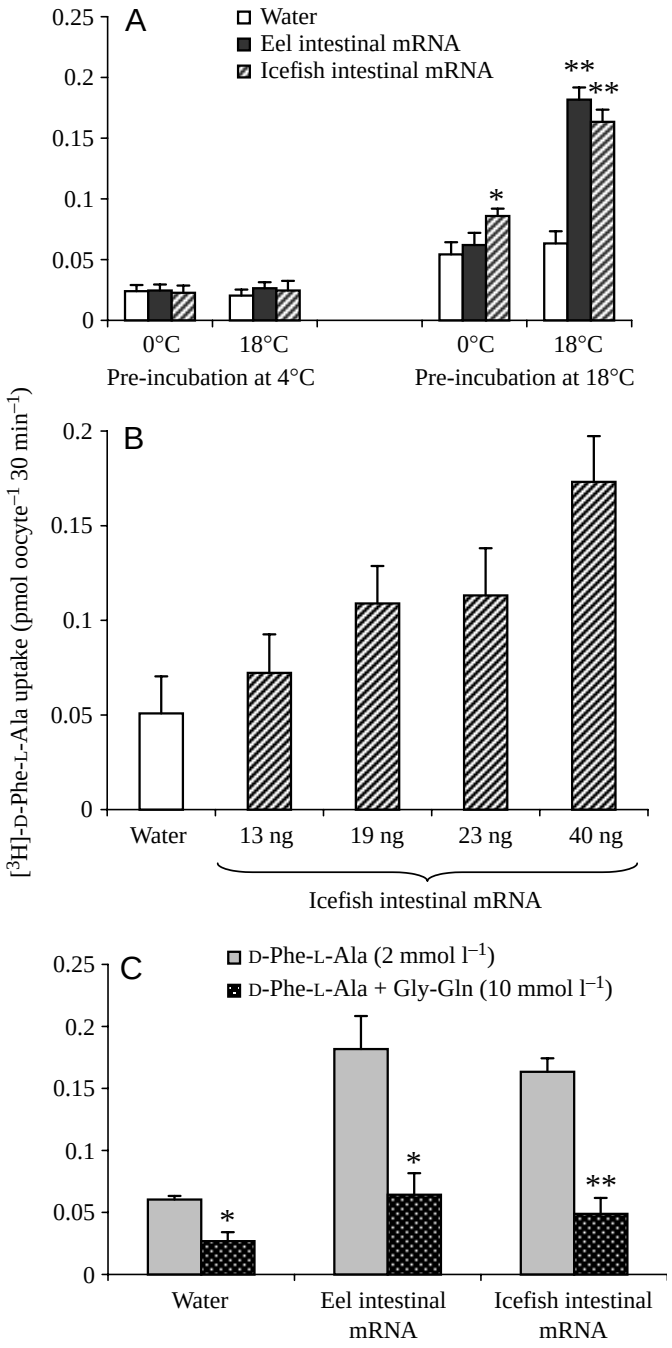

Fig. 3. (A) [^{3}H]-D-Phe-L-Ala uptake in oocytes injected with either water or poly(A)$^+$ mRNA. Values are means ± S.E.M. for 7–10 oocytes per condition, and are representative of two similar experiments; see Materials and methods for details. (B) Dose-dependent expression of D-Phe-L-Ala uptake (N=4). (C) Competition for peptide uptake in oocytes by the simultaneous presence of pairs of peptides (N=4).

Temperature dependence of the peptide transport system in the homologous (BBMV) and heterologous (oocyte) systems

Total dipeptide uptake in icefish and eel BBMVs is shown in Fig. 4 (upper traces) together with passive diffusion (lower traces). Passive diffusion was almost constant at temperatures up to 10°C for the icefish and 25°C for the eel, then started to decrease toward zero. The net, carrier-mediated dipeptide uptake measured in BBMV and oocytes is shown in Fig. 5, where differences in temperature dependence, relative to the expression system under consideration, can be seen.

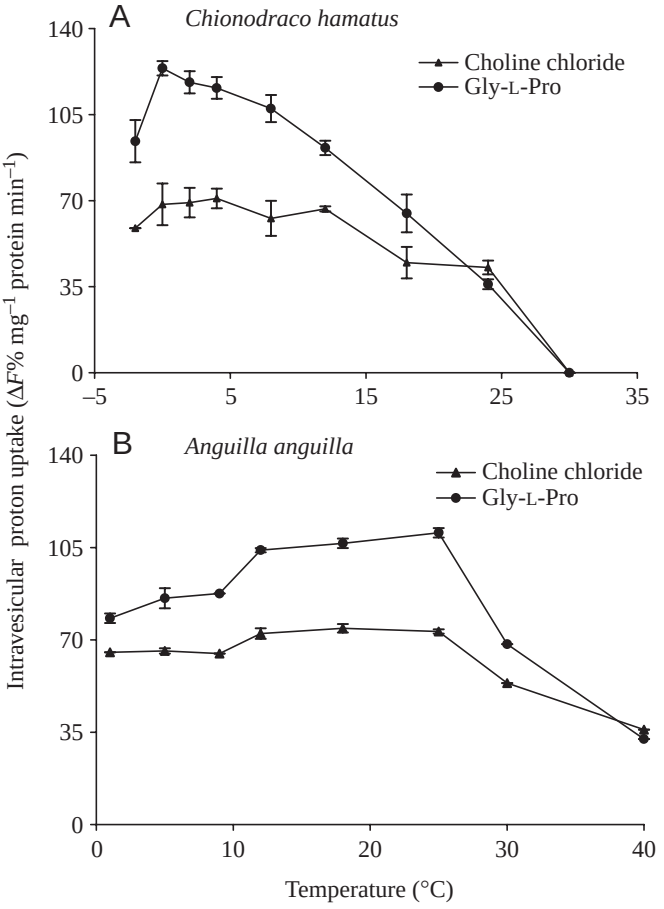

Fig. 4. Temperature-dependence of the peptide transporter in BBMV in *C. hamatus* (A) and *A. anguilla* (B). The incubation mixture contained 100 mmol l^{-1} choline chloride (circles) or 100 mmol l^{-1} choline chloride + 20 mmol l^{-1} Gly-L-Pro (triangles). For details, see Materials and methods.

Discussion

In mammals, peptide transporters of the brush border membrane of intestinal and renal epithelial cells are responsible for the absorption or re-absorption of di- and tripeptides. These transporters are driven by electric potential gradients and couple the transport of oligopeptides with H^+ (Ganapathy et al., 1994). In addition to their natural substrates, peptide transporters are also capable of binding and translocating many pharmacologically active peptidomimetics (Amidon et al., 1994; Tsuji, 1995; Meredith and Boyd, 2000). Two H^+/peptide cotransporters, a lower affinity PepT1 and a higher affinity PepT2, have been cloned and functionally characterised in higher vertebrates (Fei et al., 1994; Liu et al., 1995). Although the molecular structures of peptide transporters are almost unknown in teleost fish, the presence of a 'low affinity type' peptide transporter has been described in the intestinal brush border membranes of herbivorous (*Oreochromis mossambicus*; Thamotharan et al., 1996) and carnivorous teleosts (*Sebastes caurinus* and *Anguilla anguilla*; Verri et al., 1992; Thamotharan et al., 1996; Maffia et al., 1997). As in higher vertebrates, the peptide transporter in teleosts operates electrogenically by coupling proton translocation to substrate transport. Some of the basic characteristics of peptide transport in fish have been determined; the kinetics of transport of different substrates and the possible involvement of more than one peptide transporter phenotype have been recently analysed in eel (Verri et al., 2000) where, within the 0.1–10 mmol l^{-1} substrate range, only one active transport system seems to operate, although the presence of other peptide transporters that might operate at different substrate concentrations cannot be excluded.

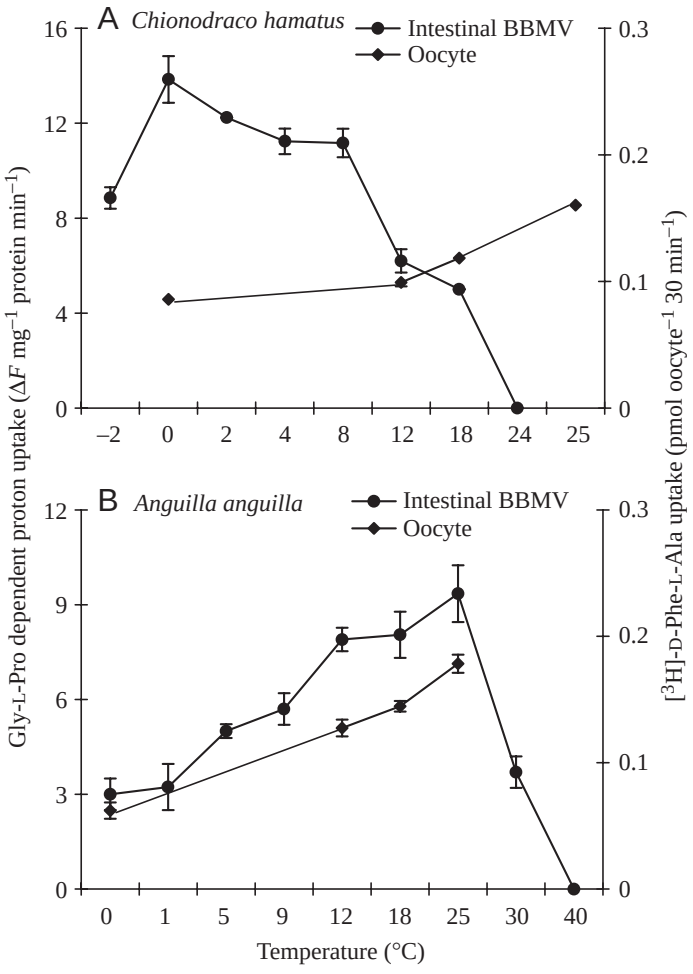

Fig. 5. Temperature-dependence of the expression of the peptide transporter in oocytes (diamonds; *N*=4). Icefish (A) and eel (B) carrier-mediated dipeptide uptake measured in brush-border membrane vesicles (BBMVs; filled circles) is also shown for comparison.

In the present work we have investigated the presence and characteristics of a peptide transport system in a member of the Channichthyidae (teleosts, sub-order Notothenioids), a family whose members possess unique eco-physiological characteristics deriving from their 13 million-year-long confinement in the cold waters of the Antarctic seas. The most dramatic physiological peculiarities of these animals include sub-zero temperature survival while avoiding freezing, the absence of red blood cells, aglomerular kidneys, etc., but whether they have any 'metabolic' adaptation to the low temperatures is still under debate (Eastman, 1993; Hardewig et al., 1998; Somero, 1995; Giardina et al., 1998; Guderley, 1998), and their exact feeding and predation behaviours, as well as mechanisms of food digestion, absorption and assimilation, are still quite unknown. Adaptation of membrane transport processes is a common component of the wide range of physiological and adaptive reactions to cold shown by ectothermic organisms. As well as the generality of poikilotherm enzymatic processes, substance transport through the cell membrane is impaired by the low environmental temperatures, which can directly affect protein stability and functionality and the physical state of the lipid microenvironment.

As for cold-adapted transporters, the functional and structural characterisation of some carrier proteins belonging to Antarctic fish (Maffia et al., 1996, 2000, 2001; Maffia and Pellegrino, 1999; Storelli et al., 1998) and other vertebrates (Tibbits et al., 1992; Xue et al., 1999; Elias et al., 2001; Dode et al., 2001) has been recently undertaken. Although many functional characteristics of these transporters are similar to those of their warm-adapted homologues, in some cases they display distinctive adaptive features to cold, such as low-temperature adapted kinetic parameters (K_{cat}, E_a, K_m), a narrow range of temperature optimum, etc. (Maffia et al., 1996; Storelli et al., 1998; Tibbits et al., 1992), the reasons for which are still unknown at the molecular level. In addition to the sodium-glucose cotransporter already described in Antarctic Notothenioids (Maffia et al., 1996), we have now extended our research on cold-adapted carrier proteins to a proton oligopeptide transporter (POT) (Paulsen and Skurray, 1994) in the intestine of the Antarctic haemoglobinless teleost *Chionodraco hamatus*. A functional comparison was performed on the same transporter of the temperate teleost *Anguilla anguilla*. Reverse transcription of

mRNA extracted from icefish and eel intestinal mucosa cells and subsequent amplification by PCR using degenerate primers derived from eel and human (PepT1) H^+/peptide transporters led to an identification of 570 bp cDNA in both fishes (Fig. 1A). Partial nucleotide sequence of the resulting cDNA of icefish revealed 73% similarity to hPepT1, and it presumably encodes an amino acid sequence with 74% similarity to hPepT1. These data constitute a first evidence for the presence of a PepT1-related mRNA product in the mRNA pool isolated from *C. hamatus* intestinal mucosa, thus suggesting the possible functional expression of a PepT1-related protein in the absorbing epithelium of the icefish. The hypothesis was confirmed by the observation of a dipeptide uptake activity in intestinal BBMV, whose main characteristics resemble those of the well-known human low-affinity, high-capacity transporter and eel and tilapia transporters (Fig. 2). Also dipeptide uptake by the icefish transporter was voltage-dependent and proton-coupled (Fig. 2A), with a relatively low substrate specificity, as it has generally been found for the PepT1 isoforms studied to date (Fig. 2B; Ganapathy et al., 1994; Liang et al., 1995; Thamotharan et al., 1996; Maffia et al., 1997; Verri et al., 2000; Meredith and Boyd, 2000). Proton influx shows a hyperbolic function with substrate concentration typical of a carrier-mediated process (Fig. 2C), and the corresponding Woolf–Augustinsson–Hofstee plot (Segel, 1975) (inset to Fig. 2C) confirmed that Gly-L-Pro-dependent H^+ influx in the icefish intestinal BBMV occurs by a single carrier, whose kinetic parameters at 0°C are V_{max}=29.06±1.27 ΔF% mg^{-1} protein min^{-1} and $K_{m,app}$=0.806± 0.161 mmol l^{-1}. The finding that treatment of BBMVs with 2 mmol l^{-1} DEP, a known inhibitor of proton-peptide cotransport (Fei et al., 1997; Miyamoto et al., 1986; Terada et al., 1996), inhibits approximately 95% of peptide-dependent H^+ influx in icefish intestine (Fig. 2D), further confirms the identification of the transporter as a hPepT1-related isoform. DEP inhibition suggests that histidyl residues in *C. hamatus* peptide transporter are located at, or near, critical catalytic site(s). In higher vertebrates, specific DEP-reactive residues, i.e. histidyl residues 57 and 121 (Fei et al., 1997; Terada et al., 1996), are crucial for the transporter function. These histidyl residues are suggested to be involved in H^+ binding and translocation. Whereas mutation of histidine 57 in both rat and human PepT1 transporters completely blocked transport, mutation of histidine 121 appeared to abolish transport activity only in the human PepT1, and not in the rat PepT1 (Fei et al., 1997; Terada et al., 1996). Although we possess only preliminary data on the secondary and tertiary structure of the transporter protein, our studies reveal that active side histidyl residues in the icefish transporter are essential for function, as judged by loss of proton transport capability on DEP treatment (Verri et al., 2000).

The expression of the POT in oocytes of *Xenopus laevis* gave us the possibility of analysing the relationship between transport activity and the lipid milieu in which the transporter is embedded (i.e. the cell membrane). The expression of the proton-peptide cotransporter in the heterologous system was carried out at two different incubation temperatures by injecting enterocyte mRNA in the oocytes and maintaining them at 4°C or 18°C for 3 days. Then the transport activity was measured in oocytes from the two pools at the environmental temperatures of the two species: 0 and 18°C. A first result arising from this trial, was the absence of any measurable transport activity in the oocytes incubated at 4°C. On the other hand, in the oocytes incubated at 18°C, it was possible to detect at 0°C a low but significant transport activity for the icefish transporter only, whereas noticeable transport activity was detected at 18°C for both the icefish and eel transporters (Fig. 3A). These results suggest an 'intrinsic' capacity of the transport protein of the Antarctic species to actively perform substrate transport at 0°C, with respect to the eel. Furthermore, the absence of detectable transport activity in oocytes incubated at 4°C suggests that frog oocytes face physiological difficulties in expressing the heterologous transporter in a functionally active form at this low temperature. An explanation of these phenomena could be the necessity of the cell membrane to possess an optimal fluid state for a perfect positioning and functioning of its transporters, receptors, channels, etc. But the cell biochemical machinery of the frog is also markedly impaired at 4°C, while at 18°C (i.e. its environmental temperature) processes such as transcription, translation, post-translational modifications, membrane positioning can occur normally and efficiently. Injection of increasing concentrations of poly(A^+) mRNA prepared from *C. hamatus* enterocytes into *X. laevis* oocytes produced a dose-dependent peptide transport activity (Fig. 3B), as shown by the [^{3}H]-D-Phe-L-Ala oocyte uptake activity after 3 days of incubation at 18°C. The [^{3}H]-D-Phe-L-Ala uptake in water-injected oocytes can be explained by a simple diffusion mechanism and/or the presence of an endogenous transport activity in the frog oocyte membrane. Icefish peptide transporter appears to share the same low substrate specificity as the mammalian protein PepT1 (Meredith and Boyd, 2000), characterised by its ability to transport a variety of di-/tripeptides. The capacity of unlabeled peptide (glycyl-glutamine, Gly-Gln) to compete with radiolabeled D-Phe-L-Ala for the uptake process in mRNA-injected oocytes (Fig. 3C) demonstrates the competition between different dipeptides on the icefish intestinal peptide transporter heterologously expressed in *X. laevis* oocytes. Substrate competition in water-injected oocytes demonstrates that at least part of the uptake that normally occurs in the frog oocyte depends on active transport.

More detailed characterisation of the temperature effects on transport activity of the peptide transport system in homologous (BBMV) and heterologous (oocyte) systems was carried out by measuring passive diffusion and active transport at different temperatures (Figs 4, 5). Passive diffusion was constant up to 10°C in icefish BBMV and 25°C in eel BBMV (Fig. 4), then decreased toward zero with further temperature increments. Membrane passive diffusion is a process that, in the absence of any particular specificity of the diffusion systems (i.e. highly specific channels; Maffia and Acierno, in press), is quite

independent from the temperature within a range that permits the cell membrane to retain a sufficient degree of integrity. The differences shown by our data are in good agreement with the different physicochemical profiles of the membranes of the two fishes, adapted to Antarctic and temperate waters, respectively (Acierno et al., 1996; Maffia and Acierno, in press). This temperature specificity appears more evident when passive diffusion is subtracted from total transport (Fig. 5). In this case, as expected, the temperature range of physiological activity of the transporter is narrower, in agreement with the complex temperature requirements of a trans-membrane carrier protein and the specificity of proteo-lipid interactions (Maffia and Acierno, in press; Sharpe et al., 2002). The structure of a protein that spans the entire cell membrane is markedly affected by the physicochemical state of the lipid bilayer, which provides the required degree of plasticity to permit the conformational protein changes necessary for substrate translocation. Interestingly, within this narrow temperature range, the optimum temperature for transport corresponds with the respective temperatures to which the two fish are adapted (icefish) or acclimated (eel). Indirect confirmation of this behaviour is provided by the active transport activity measured for the same transporters expressed in *X. laevis* oocytes. The increased activity up to 25°C (i.e. the acclimation temperature of the frog) suggests that the narrow optimal temperature range is basically derived from the general efficiency of the membrane, rather than being a specific thermodynamic effect on the carrier protein itself. In any event, both the intrinsic structural characteristics (i.e. amino acid sequence) and specific proteo-lipid membrane interactions can contribute, alternatively or in parallel, to the adaptive features displayed by temperature-adapted enzymes and transporters.

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