ACCELERATED PUBLICATION

The life-extending gene *Indy* encodes an exchanger for Krebs-cycle intermediates

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A longevity gene called *Indy* (for ‘I’m not dead yet’), with similarity to mammalian genes encoding sodium–dicarboxylate co-transporters, was identified in *Drosophila melanogaster*. Functional studies in *Xenopus* oocytes showed that INDY mediates the flux of dicarboxylates and citrate across the plasma membrane, but the specific transport mechanism mediated by INDY was not identified. To test whether INDY functions as an anion exchanger, we examined whether substrate efflux is stimulated by transportable substrates added to the external medium. Efflux of [¹⁴C]citrate from INDY-expressing oocytes was greatly accelerated by the addition of succinate to the external medium, indicating citrate–succinate exchange. The succinate-stimulated [¹⁴C]citrate efflux was sensitive to inhibition by DIDS (4,4′-di-isothiocyano-2,2′-disulphonic stilbene), as demonstrated previously for INDY-mediated succinate uptake. INDY-mediated efflux of [¹⁴C]citrate was also stimulated by external citrate and oxaloacetate, indicating citrate–citrate and citrate–oxaloacetate exchange. Similarly, ef-

flux of [¹⁴C]succinate from INDY-expressing oocytes was stimulated by external citrate, α -oxoglutarate and fumarate, indicating succinate–citrate, succinate– α -oxoglutarate and succinate–fumarate exchange respectively. Conversely, when INDY-expressing *Xenopus* oocytes were loaded with succinate and citrate, [¹⁴C]succinate uptake was markedly stimulated, confirming succinate–succinate and succinate–citrate exchange. Exchange of internal anion for external citrate was markedly pH_o-dependent, consistent with the concept that citrate is co-transported with a proton. Anion exchange was sodium-independent. We conclude that INDY functions as an exchanger of dicarboxylate and tricarboxylate Krebs-cycle intermediates. The effect of decreasing INDY activity, as in the long-lived *Indy* mutants, may be to alter energy metabolism in a manner that favours lifespan extension.

Key words: aging, anion exchange, citrate, dicarboxylate, *Indy*, succinate.

INTRODUCTION

Aging is a complex biological process and involves both genetic and environmental factors. Single gene mutations affecting longevity can be a useful tool for gaining insight into the mechanisms underlying aging [1–3]. Mutations in the *Indy* gene (for ‘I’m not dead yet’) result in an 80–100 % increase in the average lifespan of both adult male and female fruitflies (*Drosophila melanogaster*) without sacrificing fertility or physical activity [1]. The dramatically extended lifespan in *Indy* mutant flies is associated with a great reduction in the levels of *Indy* mRNA and INDY protein that are likely to result in decreased *Indy* activity. The life-extending *Indy* mutation is associated with a decrease in the slope of the mortality curve, implying that the *Indy* mutation causes a lowering of the demographic rate of aging and may directly affect the normal aging process [4]. Studies in *Caenorhabditis elegans* revealed that the disruption of transporters with similar transport properties to INDY also extends the animal’s lifespan [5], demonstrating that the longevity effect of decreasing INDY activity may be conserved across distant evolutionary species [1].

Sequence analysis of *Indy* demonstrated closest similarity to mammalian sodium–dicarboxylate co-transporters. Functional studies in *Xenopus* oocytes showed that INDY indeed functions as a dicarboxylate and tricarboxylate transporter [6,7]. Kinetic measurements indicated that INDY is a relatively high-affinity

transporter as the K_m for succinate was approx. 40 μ M [6,7], a value similar to that of high-affinity mammalian sodium–dicarboxylate co-transporters [8]. In addition, immunocytochemical studies demonstrated that INDY is most prominently expressed on the basolateral membrane of cells in the midgut of *Drosophila melanogaster* and the plasma membrane of fat body and oenocytes [6], confirming that INDY is a plasma-membrane transporter in native tissues. The polarized expression of INDY on the basolateral membrane of the midgut is similar to the membrane site of expression of high-affinity mammalian sodium–dicarboxylate co-transporters [8,9].

Despite its many similarities to mammalian sodium–dicarboxylate co-transporters, several functional properties of INDY were novel for this gene family. Importantly, organic anion transport mediated by INDY is sodium-independent [6,7], in marked contrast with sodium–dicarboxylate co-transporters. In addition, electrophysiological studies detected no electrical current associated with INDY-mediated succinate transport [7], suggesting an electroneutral transport mechanism in contrast with the electrogenic transport mediated by sodium–dicarboxylate co-transporters. Finally, INDY-mediated transport is highly sensitive to inhibition by DIDS (4,4′-di-isothiocyano-2,2′-disulphonic stilbene) [6], which does not strongly inhibit mammalian sodium–dicarboxylate co-transporters. However, the specific transport mechanism mediated by INDY was not identified. The purpose of the present study was to test whether INDY functions as a

Abbreviation used: DIDS, 4,4′-di-isothiocyano-2,2′-disulphonic stilbene.

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DIDS-sensitive anion exchanger, and to test the effect of pH and sodium on a possible exchange process.

MATERIALS AND METHODS

INDY expression in *Xenopus* oocytes

Mature female *Xenopus laevis* frogs (Kaeher, Hamburg, Germany) were subjected to partial ovariectomy under tricaine (3-aminobenzoic acid ethyl ester) anaesthesia (0.2 % for 5–10 min) as described previously [6,10]. In brief, a small incision was made in the abdomen and a lobe of ovary was removed. Subsequently, the oocytes were pre-washed for 20 min in Ca^{2+} -free hypotonic medium (85 mM NaCl, 2 mM KCl, 1 mM MgCl_2 and 5 mM Hepes titrated with Tris base to pH 7.5) to remove blood and damaged tissue. Oocytes then were defolliculated by treatment with 2 mg/ml collagenase (Sigma type I) in Ca^{2+} -free hypotonic solution for 45–90 min with gentle agitation at room temperature (20 °C). After this treatment, oocytes were washed three times in Ca^{2+} -free hypotonic medium, and then washed three times in isotonic Ca^{2+} -containing frog Ringer solution (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl_2 , 1.0 mM MgCl_2 and 5 mM Hepes, pH 7.5).

Indy cDNA was subcloned into the pGH19 expression plasmid as described previously [6]. Plasmid DNA was linearized by *Xho*I digestion, and cRNA was transcribed by using T7 RNA polymerase (mMESSAGE mMACHINE; Ambion). Precipitated cRNA was dissolved in sterile water, and yield and quality were assessed by spectroscopy and agarose gel electrophoresis. On the day of their isolation, oocytes were injected with 50 nl of sterile water or 50 nl of a cRNA solution containing 25 ng of *Indy* cRNA by use of a Drummond microinjector. The injected oocytes were incubated in Ca^{2+} -containing frog Ringer solution at 18 °C for approx. 48 h to allow expression of INDY protein.

Measurements of radiolabelled solute fluxes

For efflux experiments, the respective isotope was microinjected into the oocytes as originally described by Stewart et al. [11]. In brief, individual oocytes were injected with 50 nl of 3.4 mM [^{14}C]citrate or 3.5 mM [^{14}C]succinate (Amersham Biosciences/MP Biomedicals). A total of 10–15 oocytes were lysed after the injection was completed and counted before the experiment (representing 4000–7000 total c.p.m. per oocyte). After approx. 5 min of recovery and two washes in NaCl buffer (100 mM NaCl and 5 mM Hepes, pH 7.5), the efflux assay was initiated by transfer of individual oocytes to a 48-well plate (Beckman Dickinson), with each well containing 1 ml of efflux solution. After 3 min, the efflux solution was removed for scintillation counting. Radioisotope content of the efflux solution was measured by scintillation spectroscopy after addition of 5 ml of scintillation fluid (Opti-Fluor; Packard).

For uptake experiments, oocytes were microinjected with 50 nl of either water or a 1 M solution of sodium lactate, sodium succinate or sodium citrate. After approx. 5 min recovery, oocytes were washed twice in NaCl buffer and then resuspended in the same medium containing [^{14}C]succinate (18 μM). After a 3 min incubation, external isotope was removed by washing the oocytes three times with ice-cold buffer. Radioisotope content of each individual oocyte was measured by scintillation spectroscopy after solubilization in 0.5 ml of 10 % (v/v) SDS and addition of 5 ml of scintillation fluid.

Statistical analysis

Results shown in the histograms are means $\pm$ S.E.M. for 8–16 oocytes. The results were analysed using non-paired Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Figure 1 Effects of external substrates on [^{14}C]citrate efflux from INDY-expressing *Xenopus* oocytes

INDY-expressing oocytes and water-injected oocytes not expressing INDY were microinjected with [^{14}C]citrate and then washed and transferred to various external solutions: NaCl buffer with 1 mM of the indicated substrate, or NaCl buffer with 1 mM of the indicated substrate and 100 μM DIDS. The appearance of radioactivity in the external solution during a 3 min incubation was determined by scintillation spectroscopy. Results are means $\pm$ S.E.M. * $P < 0.05$. N.S., not statistically significant.

RESULTS

Effects of Krebs-cycle intermediates on [^{14}C]citrate efflux

As an initial approach to examine whether INDY can mediate the exchange of dicarboxylate and tricarboxylate Krebs-cycle intermediates, we microinjected [^{14}C]citrate into INDY-expressing oocytes, and tested whether organic anions previously found to be transported by INDY [6,7] can stimulate [^{14}C]citrate efflux. As illustrated in Figure 1, the presence of 1 mM succinate added to the external medium greatly stimulated the efflux of [^{14}C]citrate compared with the control in which 1 mM lactate was added to the external solution. Lactate has been shown previously to have no detectable affinity for INDY [6]. The INDY-mediated citrate efflux was almost completely abolished by 0.1 mM DIDS, which has been shown previously to inhibit INDY-mediated dicarboxylate and tricarboxylate uptake [6]. Importantly, external succinate failed to stimulate [^{14}C]citrate efflux from water-injected oocytes not expressing INDY, confirming that the DIDS-sensitive citrate–succinate exchange is mediated by INDY and not by an endogenous transporter in *Xenopus* oocytes.

Previous studies have shown that INDY-mediated succinate and citrate uptake is not cation-dependent [6,7]. In order to evaluate whether INDY-mediated citrate–succinate exchange is also sodium-independent, we measured succinate-stimulated [^{14}C]citrate efflux in the presence and absence of sodium in the external buffer. The stimulation of efflux of [^{14}C]citrate by external succinate was not altered by the replacement of sodium with potassium (results not shown), confirming that INDY-mediated citrate–succinate exchange is sodium-independent.

Having demonstrated that INDY functions as an anion exchanger, we next examined whether additional INDY substrates added to the external medium can also stimulate [^{14}C]citrate efflux (Figure 1). Similar to succinate, the presence of 1 mM unlabelled citrate in the external medium greatly stimulated DIDS-sensitive

Figure 2 Effects of external substrates on [^{14}C]succinate efflux from INDY-expressing *Xenopus* oocytes

INDY-expressing oocytes and water-injected oocytes not expressing INDY were microinjected with [^{14}C]succinate and then washed and transferred to various external solutions: NaCl buffer with 1 mM of the indicated substrate, or NaCl buffer with 1 mM of the indicated substrate and 100 μM DIDS. The appearance of radioactivity in the external solution during a 3 min incubation was determined by scintillation spectroscopy. Results are means $\pm$ S.E.M. * $P < 0.05$. N.S., not statistically significant. α -ketoglutarate, α -oxoglutarate.

[^{14}C]citrate efflux, consistent with INDY-mediated citrate–citrate exchange. Similarly, the addition of oxaloacetate to the external medium also greatly stimulated the DIDS-sensitive efflux of [^{14}C]citrate, indicating INDY-mediated citrate–oxaloacetate exchange.

Effects of Krebs-cycle intermediates on [^{14}C]succinate efflux

To evaluate the reversibility and anion specificity of INDY-mediated anion exchange in more detail, we tested the ability of INDY to mediate efflux of [^{14}C]succinate in exchange for external dicarboxylates and tricarboxylates. As illustrated in Figure 2, the presence of 1 mM external citrate greatly stimulated the DIDS-sensitive efflux of [^{14}C]succinate from INDY-expressing oocytes, consistent with succinate–citrate exchange. External citrate failed to stimulate [^{14}C]succinate efflux from water-injected oocytes not expressing INDY, confirming that the DIDS-sensitive citrate–succinate exchange is mediated by INDY and not by an endogenous transporter in *Xenopus* oocytes. Taken together, the results in Figures 1 and 2 indicate that INDY-mediated tricarboxylate–dicarboxylate exchange is reversible, as exchange of both internal citrate for external succinate and internal succinate for external citrate were demonstrated.

We next tested the ability of INDY to exchange dicarboxylates of unequal chain lengths. As also shown in Figure 2, the presence of 1 mM external α -oxoglutarate strongly stimulated the DIDS-sensitive efflux of [^{14}C]succinate, indicating that INDY can mediate succinate– α -oxoglutarate exchange. These findings confirmed that INDY is capable of exchanging dicarboxylates of unequal chain lengths across the plasma membrane.

In addition, we tested the ability of INDY to mediate exchange of dicarboxylates with different redox states. The addition of 1 mM fumarate to the external solution greatly stimulated

Figure 3 Effects of internal substrates on [^{14}C]succinate uptake into INDY-expressing *Xenopus* oocytes

INDY-expressing oocytes were microinjected with 50 nl of water or a 1 M solution of the indicated substrate. Oocytes were then washed and transferred to NaCl buffer containing [^{14}C]succinate with or without 100 μM DIDS. After 3 min of incubation, external isotope was removed by washing the oocytes with ice-cold buffer, and radioisotope content was measured by scintillation spectroscopy. Results are means $\pm$ S.E.M. * $P < 0.05$. N.S., not statistically significant.

DIDS-sensitive [^{14}C]succinate efflux, consistent with succinate–fumarate exchange (Figure 2). These findings indicate that INDY can exchange dicarboxylates of different redox states.

Effects of Krebs-cycle intermediates on [^{14}C]succinate influx

To provide additional confirmation for the presence of anion exchange, we investigated whether pre-loading of INDY-expressing oocytes with citrate and succinate facilitates the influx of [^{14}C]succinate. As illustrated in Figure 3, injection of lactate into INDY-expressing oocytes failed to stimulate [^{14}C]succinate uptake compared with INDY-expressing oocytes injected with an equal volume of water. Pre-loading of INDY-expressing oocytes with succinate and citrate greatly stimulated [^{14}C]succinate uptake, and this stimulation by internal anions was completely abolished by DIDS. These findings confirm the presence of DIDS-sensitive succinate–succinate and succinate–citrate exchange.

Effects of pH on succinate- and citrate-mediated [^{14}C]citrate efflux

We had observed previously that INDY-mediated citrate uptake is stimulated at pH 6.0, but that INDY-mediated succinate uptake is pH-independent [6]. It was therefore of interest to test the effect of pH on the ability of external citrate and succinate to stimulate anion efflux by anion exchange. As shown in Figure 4, the effect of external citrate to stimulate [^{14}C]citrate efflux from INDY-expressing oocytes was markedly enhanced at pH 6.0 when compared with pH 7.5. In contrast, the effect of external succinate to stimulate [^{14}C]citrate efflux was pH-independent. At pH 7.5, citrate (pK_a values of 3.1, 4.8 and 6.4) is predominantly in the tricarboxylate form, whereas succinate (pK_a values of 4.2 and 5.6) is predominantly in the dicarboxylate form. Thus these results indicate either that external citrate is preferentially exchanged in the dicarboxylate form and/or that the tricarboxylate form is exchanged together with a proton.

DISCUSSION

We have demonstrated that the transporter encoded by the life-extending gene *Indy* is a disulphonic stilbene-sensitive, sodium-independent anion exchanger that is capable of exchanging pairs

Figure 4 Effects of external pH on citrate- and succinate-stimulated [^{14}C]citrate efflux from INDY-expressing *Xenopus* oocytes

INDY-expressing oocytes were microinjected with [^{14}C]citrate and then washed and transferred to various external solutions: NaCl buffer with 1 mM succinate or citrate at pH 6.0, pH 7.5 and pH 9.0. The appearance of radioactivity in the external solution during a 3 min incubation was determined by scintillation spectroscopy. Results are means $\pm$ S.E.M. * $P < 0.05$. N.S., not statistically significant.

of dicarboxylates and citrate across the plasma membrane. Our findings on the effects of pH suggest that citrate is preferentially exchanged in the dicarboxylate form and/or that the tricarboxylate form is transported together with a proton, whereas transport of dicarboxylates is proton-independent. The function of INDY as an obligate anion exchanger can explain the previously observed electroneutrality of INDY-mediated anion transport [7]. Dicarboxylate–dicarboxylate exchange, citrate–citrate exchange and exchange of a dicarboxylate for citrate with a proton are all predicted to be electroneutral processes. The function of INDY as an obligate anion exchanger is in contrast with that of the mammalian sodium–dicarboxylate co-transporters, which utilize the Na^+ gradient to drive net uphill anion transport across the plasma membrane [12–14]. Thus INDY defines a different class of transporter, exchanging Krebs-cycle intermediates across the plasma membrane independently of sodium.

Interestingly, the function of INDY as an electroneutral anion exchanger of dicarboxylates for citrate plus a proton is very similar to that of the citrate transport protein of the mitochondrial inner membrane [15,16]. Although INDY shows no significant sequence similarity to the mitochondrial citrate transport protein [17], INDY is capable of mediating exchange reactions described for the citrate transport protein, including citrate–succinate exchange (Figures 1, 2 and 3), citrate–oxaloacetate exchange (Figure 1) or citrate–citrate exchange (Figure 1). In addition to tricarboxylate–dicarboxylate exchange, INDY is also capable of exchanging dicarboxylates of unequal chain lengths such as succinate for α -oxoglutarate (Figure 2) and dicarboxylates of different redox states such as succinate for fumarate (Figure 2).

Fatty acid synthesis predominantly occurs in the cytoplasm of the cell and requires acetyl-CoA as the main source. The mitochondrial citrate transport protein plays a major role in this process [17,18]. INDY, which is primarily expressed on the plasma membrane of cells of the midgut and fat body of the fruitfly [6], might have an equally important role in importing citrate from haemolymph into the cells. Citrate can then be cleaved by citrate lyase to acetyl-CoA and used for fatty acid synthesis [18]. The cytosolic pool of citrate is in common for both mitochondrial and plasma membrane transport systems. Thus INDY might

fulfil a physiological function similar to the mitochondrial citrate transport protein in overall cellular metabolism by regulating the amount of citrate in the cytosol for lipid synthesis. A potential role for INDY in lipid metabolism is supported by the recent finding in *C. elegans* that decreased expression of an INDY homologue, the sodium–citrate co-transporter ceNaC-2, results in a reduction in fat content and an increase in lifespan [19].

Given that INDY is a reversible anion exchanger, the net transfer of citrate and caloric or redox equivalents across the plasma membrane mediated by INDY will depend on the relative concentrations of transportable substrates both within the cell and in the extracellular fluid. In the absence of such information, we cannot predict with certainty the effect that a defect in INDY activity would have on cellular metabolism. Nevertheless, the ability of INDY to mediate net transfer of citrate and caloric or redox equivalents across the plasma membrane is consistent with the universal role of energy balance in aging as documented in multiple model systems (e.g. nematode worms, fruitflies and rodents) [20]. This concept is supported by the aforementioned observation that knockdown of expression of the homologous citrate transporter ceNaC-2 increases the lifespan of nematodes [5,19]. Thus it is likely that a reduction in INDY activity in mutant *Indy* long-lived fruitflies alters energy metabolism in a manner similarly conducive to lifespan extension.

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