Stucture and function of the dicarboxylate transporter *INDY*

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The mechanisms underlying aging are complex. Rogina et al. discovered a new class of longevity gene called *Indy (I'm not dead yet)*[4], which had significant homology to several Na-dicarboxylate transporters in the kidney proximal tubule. Two Indy genes have been cloned to date, Indy-1 and Indy-2[4]. In studies of *Drosophila* enhancer-trap lines, mutations in the Indy-1 gene resulted in a doubling of mean life-span[4]. Functional studies in *Xenopus* oocytes showed that the Indy-1 protein (*INDY*-1) represents a novel transporter of dicarboxylates and citrate across the plasma membrane by a mechanism not involving cotransport with Na^+ or H^+. Our recent efflux studies in *Xenopus* oocytes showed that *INDY*-1 can function as an organic anion exchanger of dicarboxylic and tricarboxylic Krebs cycle intermediates[2]. Despite its close homology to *INDY*-1, the cellular localization and functional properties of *INDY*-2 are unknown. We tested the hypothesis that *INDY*-2 can function as dicarboxylate transporter in the plasma membrane. To test this hypothesis we fused green fluorescence protein (GFP) to the NH_2-terminal domains of *INDY*-1 and *INDY*-2 and examined their subcellular localization and function in the *Xenopus* oocyte expression system.

Oocytes were isolated from Xenopus laevis frogs (Nasco, Fort Atkinson, WI) as previously described [1,3]. Indy 1 and Indy 2 plasmids were obtained from Drs. Blanka Rogina and Stephen Helfand (Department of Genetics and Developmental Biology, School of Medicine, University of Connecticut Health Center, Farmington CT). *Indy-1 and Indy-2* cDNA were subcloned into the pGH19 expression plasmid as previously described[1]. The eGFP was cloned in frame into pgh Indy-1 and Indy-2 using Sma/Eco47 III -BglII/BamHI sites resulting in N-terminal eGFP-Indy-1 and eGFP-Indy-2 fusion constructs. The cloning was confirmed by sequencing. Plasmid DNA was linearized by *Xho*I digestion, and cRNA was transcribed by using T7 RNA polymerase (mMESSAGE mMACHINE, Ambion, Austin, TX). Precipitated cRNA was dissolved in sterile H_2O, 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 H_2O or 50 nl of a cRNA solution containing 25 ng of Indy-1 or Indy-2 cRNA by use of a Drummond microinjector. The injected oocytes were incubated in Ca^{2+}-containing frog Ringer solution at 18°C for ~48 h to allow for expression of INDY protein. Uptake and efflux studies of [14C]succinate or [14C]citrate were performed in individual oocytes by scintillation spectroscopy. Fluorescence microscopy was conducted in the Imaging Core using an Olympus Fluoview laser scanning miscroscope. *INDY*-GFP fluorescence was measured using an argon/krypton laser at 488 nm, emission was measured at 515 nm. Images were analysed using NIH Image J 1.30 and CorelDraw 9.0. Data are expressed as means ± SEM of n oocytes investigated. Student's t-test or ANOVA were used ($p<0.05$).

We first studied the cellular localization of INDY. We injected *Xenopus* oocytes with cRNA of Indy-1, Indy-1-GFP, Indy-2, Indy-2-GFP or H_2O. Fig. 1 shows green fluorescence at the surface membrane of *Xenopus* oocytes injected cRNA of Indy-1-GFP (n= 21). In contrast, virtually no green fluorescence was detected at the surface membrane of *Xenopus* oocytes injected with cRNA of Indy-2-GFP (n=20). There was no fluorescence in oocytes injected with non-tagged Indy-1 (n=11), non-tagged Indy-2 (n=9) or H_2O (n=21).

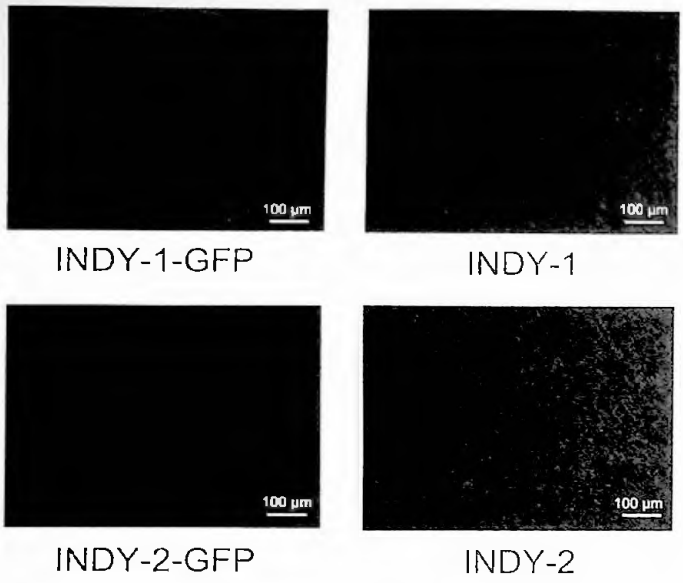

Fig. 1: Representative images of GFP-tagged *INDY* expressed in Xenopus oocytes. The cRNA of Indy-1, Indy-1-GFP, Indy-2, Indy-2-GFP or H_2O was injected into *Xenopus* oocytes. The GFP fluorescence was measured 2 days after injection by confocal microscopy with an objective lens (x10). The bars indicate 100 µm.

We next performed uptake studies with ^{14}C-labeled citrate at the 1 and 5 positions. Oocytes were washed twice at room temperature in 1 ml transport buffer (100 mM NaCl/5 mM Hepes, pH 7.5) before incubation in 500 μl of the same solution containing [^{14}C]citrate (9 μM). After a 30-min incubation, external isotope was removed by washing the oocytes three times with 1 ml of ice-cold buffer. Radioisotope content of each individual oocyte was measured by scintillation spectroscopy after solubilization in 0.2 ml of 10% (vol/vol) SDS and addition of 3 ml of scintillation fluid (Opti-Fluor, Packard). Radioisotope uptake of oocytes injected with cRNA of Indy-1, Indy-2, Indy-1-GFP and Indy-2-GFP was compared to radioisotope uptake of oocytes injected with H_2O. Fig. 2 shows that radioisotope uptake rates of oocytes injected with cRNA of Indy-1 were ~350 cpm/oocyte. Despite fluorescence in the surface membrane (Fig. 1), radioisotope uptake rates of oocytes injected with cRNA of Indy-1-GFP were only ~50 cpm/oocyte. Presumably, the low uptake rates result from insufficient expression of *INDY-1*. However, another reason could be an inhibitory effect of GFP on *INDY-1* function. Radioisotope uptake rates of oocytes injected with Indy-2 or Indy-2-GFP were ~50 cpm/oocyte. Radioisotope uptake rates of oocytes injected with H_2O was ~50 cpm/oocyte (not shown in Fig. 2).

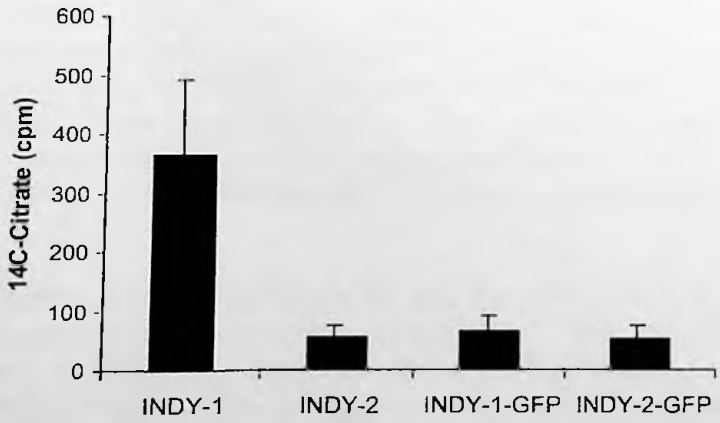

Fig. 2: Summary of data on uptake of [^{14}C]-citrate in *Xenopus* oocytes injected with cRNA of Indy-1, Indy-2, Indy-1-GFP and Indy-2-GFP. Radioisotope uptake is given in counts per minute. 10 oocytes each from the same batch of cells.

In conclusion, we were able to show that *INDY-1* is expressed in the plasma membrane using GFP-tagged *INDY-1* as expected from our previous flux studies[1,2]. We were not able to detect GFP-tagged *INDY-2* in the surface membrane of *Xenopus* oocytes. It is possible that *INDY-2*-GFP interferes with intracellular trafficking. Alternatively, translation into the protein might have been compromised by cRNA contamination or *INDY-2* might be expressed at locations other than the plasma membrane (e.g., Golgi apparatus, mitochondria, etc.). In agreement with the absence of *INDY-2*-GFP in the plasma membrane, our uptake studies did not show transport activity of *INDY-2*. Further studies are needed to examine whether *INDY-2* is expressed in the plasma membrane, including Western blots using antibodies against *INDY-2*. Once INDY-2 can be localized in the plasma membrane of *Xenopus* oocytes, the transport properties of *INDY*-2 can be studied using flux studies.

We recently showed that *INDY*-1 functions as an anion exchanger of dicarboxylic and tricarboxylic Krebs cycle intermediates. We found that substrate efflux through *INDY*-1 is stimulated by transportable substrates added to the external medium[2]. We performed similar efflux experiments with oocytes injected with cRNA of Indy-2. We found no significant efflux of [^{14}C]-citrate or [^{14}C]-succinate in oocytes injected with cRNA of Indy-2. Because of the low uptake rates of *INDY-1* in this study the meaning of the later result is questionable. Further studies are required to examine whether *INDY-2* may provide efflux of dicarboxylic and tricarboxylic Krebs cycle intermediates across the plasma membrane.

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1. Knauf F, Rogina B, Jiang Z, Aronson PS, Helfand SL. Functional characterization and immunolocalization of the transporter encoded by the life-extending gene Indy. *Proc Natl Acad Sci USA* 99(22):14315-9, 2002

2. Knauf , F., Teichert, C., Mohebbi, N., Herold , D., Rogina , B., Helfand, S., Aronson 3 , P.S., Gollasch, M., Luft, F.C., Identification of the transport mechanism mediated by life-extending gene Indy. *Nephrol. Dial. Transplant.* 18 (Suppl 4): 8, M20, 2003.

3. Löhn M, Muzzulini U, Essin K, Tsang SY, Kirsch T, Litteral J, Waldron P, Conrad H, Klugbauer N, Hofmann F, Haller H, Luft FC, Huang Y & Gollasch M. Cilnidipine is a novel slow-acting blocker of vascular L-type calcium channels that does not target protein kinase C. *J Hypertens.* 20(5):885-93, 2002.

4. Rogina, B., Reenan, R. A., Nilsen, S. P. & Helfand, S. L. Extended life-span conferred by cotransporter gene mutations in Drosophila. *Science* **290,** 2137-40, 2000.