The concentrative nucleoside transporter family (SLC28): new roles beyond salvage?

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Abstract

The concentrative nucleoside transporter (CNT) family (SLC28) has three members: SLC28A1 (CNT1), SLC28A2 (CNT2) and SLC28A3 (CNT3). The CNT1 and CNT2 transporters are co-expressed in liver parenchymal cells and macrophages, two suitable models in which to study cell cycle progression. Despite initial observations suggesting that these transporter proteins might contribute to nucleoside salvage during proliferation, their subcellular localization and regulatory properties suggest alternative roles in cell physiology. In particular, CNT2 is a suitable candidate for modulation of purinergic responses, since it is under the control of the adenosine 1 receptor. Increasing evidence also suggests a role for CNT2 in energy metabolism, since its activation relies on the opening of ATP-sensitive K+ channels. Animal and cell models genetically modified to alter nucleoside transporter expression levels may help to elucidate the particular roles of CNT proteins in cell physiology.

Nucleosides, nucleotides and the need for their salvage

Nucleosides and nucleotides play multiple roles in cell physiology. These molecules can be considered both as nutrients and as modulators of cell homeostasis. This dual role is explained by the fact that nucleosides and nucleotides are not only nucleic acid precursors, but also key determinants of energy metabolism, acting as metabolic activators in biosynthetic pathways (UDP), as energy transfer units (ATP and GTP), as well as coenzymes (NAD and FAD). Moreover, some nucleosides (adenosine) and nucleotides (ATP) are also ligands for purinergic receptors (P1 and P2), and transducers of endocrine signals (cAMP), thus contributing to the modulation of a broad range of cellular events. Although it is plausible to consider that diet might be a significant source of nucleotides/nucleosides, de novo biosynthesis, principally located in liver parenchymal cells, is crucial for whole-body nucleotide/nucleoside homeostasis. However, de novo synthesis is energetically costly. For instance, inosine monophosphate synthesis from α-D-ribose-5-phosphate requires five molecules of ATP, glycine, aspartate, two molecules of glutamine and two of N10-formyl-tetrahydrofolate, as a further source of carbon atoms. Moreover, cell types showing high turnover, such as enterocytes and cells of the immune system, do not appear to show significant endogenous nucleotide synthesis, and mostly rely on extracellular nucleoside supply. Recycling of the nucleoside units required for replenishment of the cellular nucleotide pool is known as salvage, and salvage pathways rely on the ability of the cell to take up these molecules from the extracellular milieu. Since nucleosides are hydrophilic molecules, plasma membrane transporters are required for this process to occur.

Nucleoside transporters

Nucleoside transport across the plasma membrane is mediated by transporter proteins belonging to the SLC28 and SLC29 families. SLC29 proteins are designated equilibrative nucleoside transporters (ENTs), a name which is probably not appropriate, because orthologues in protozoa are proton linked (for an excellent recent review on SLC29 protein members, see [1]). ENT transporters show low affinity for substrates, albeit with broad selectivity. Although four members (ENT1, ENT2, ENT3 and ENT4) have been identified to date, ENT1 and ENT2 are the only ones for which extensive molecular and functional characterization has been undertaken [1]. Interestingly, ENT2 is a somewhat unusual nucleoside transporter, given that it has also been shown to translocate nucleobases, although with apparent low affinity, but high turnover number for transport [2].

Low-affinity, broad-selectivity, equilibrative transport processes have long been known to be ubiquitous in mammalian cells (for a review, see [3]), whereas the occurrence of high-affinity, concentrative transport was initially believed to be restricted to selected epithelia, such as those of the small intestine [4,5], kidney [6] and choroid plexus [7,8]. Evidence for the co-expression of more than one concentrative Na+-dependent transport system, differing in substrate selectivity, was first provided in intestinal epithelial cells in the late 1980s [9], thus anticipating the occurrence of at least one purine- and one pyrimidine-prefering nucleoside transporter. Subsequently, a putative purine and pyrimidine broad-selectivity transport system was identified [10]. These three activities were named N1/cif, N2/cit and
N3/cib respectively (for a comprehensive review, see [3]). The proteins responsible for these transport activities belong to the SLC28 family, which is now known to have three members: SLC28A1, SLC28A2 and SLC28A3, also designated as CNT1 (concentrative nucleoside transporter 1), CNT2 and CNT3 (for an excellent recent review on SLC28 proteins, see [11]). Detailed functional characterization of Na\(^{+}\)-dependent nucleoside transporter processes in renal and B cells revealed the occurrence of additional N-type transport systems with unique substrate and pharmacological properties [12–14]. However, it remains to be seen whether these are in fact variants of the known CNT transporter proteins or are associated with members of other protein families.

Both CNT and ENT transporters also recognize most nucleoside-derived drugs used in anti-cancer treatment, and thus, they are pharmacological targets that might influence drug bioavailability, and consequently, response to treatment. This review will not focus on these particular aspects of nucleoside transporter selectivity, which have recently been reviewed elsewhere by us and others [15,16].

CNT transporters in a somewhat unusual epithelial model: the hepatocyte

Despite the initial view that CNT-type transporter expression was restricted to selected epithelia (i.e. enterocytes, and renal and choroid plexus epithelial cells), we and others demonstrated the occurrence of these transport systems in basolateral plasma membrane vesicles from rat liver [17,18], whilst in isolated hepatocytes nucleoside uptake processes were consistent with the co-expression of N1/citf and N2/cit transport systems [19]. The first CNT2 orthologue was indeed cloned from rat liver by Arias and colleagues [20] (at that time called SPNT for sodium-purine nucleoside transporter), whereas we later cloned the pyrimidine-prefering hepatic nucleoside transporter [21], which turned out to be identical to the intestinal CNT1 cloned previously by Young and colleagues [22].

Analysis of enriched plasma membrane fractions using isofrom-specific antibodies revealed a different subcellular localization for the two transporter proteins [21]. By combining immunogold electron microscopy, immunofluorescence and highly resolutive fractionation methods, leading to the isolation of canalicular and sinusoidal membranes, endosomes and caveolae-enriched fractions from rat liver, we proposed a model for CNT1 and CNT2 trafficking and subcellular localization in hepatocytes [23]. This model suggested that CNT1 and CNT2 are initially inserted into the sinusoidal plasma membrane, albeit in different microdomains, since CNT2 was not identified in caveolin-enriched fractions. This event might determine their functional status, since most of the transport activity found in basolateral membranes is CNT2 related. CNT1 may then be internalized into the early endosome compartment, trafficking into the apical canalicular membrane, in which CNT1 functional activity can be detected [23]. This study also revealed a significant intracellular presence of the two isoforms, although CNT1 was much more abundant, opening the possibility that trafficking processes also contribute to the modulation of the functional activity of CNT1 and CNT2 at the plasma membrane; this hypothesis is currently under investigation in our laboratory.

The initial observation that Na\(^{+}\)-dependent nucleoside transport activity is increased in plasma membrane vesicles from regenerating rat livers [24], later demonstrated to implicate both CNT isoforms [21], could be interpreted as a merely adaptive response to cell proliferation, thus promoting nucleoside salvage. Nevertheless, CNT1 and CNT2 expression in hepatocytes is associated with differentiation and maintenance of the hepatic phenotype [25–27], whereas poorly differentiated, highly proliferating liver parenchymal cells, such as some hepatoma cell lines and fetal hepatocytes, show low or negligible expression of CNT-type transporters [25–27]. Indeed, we now know that CNT1 and CNT2 expression is much broader than initially expected [28], and recently, using a tissue array approach to study nearly 300 independent epithelial gynaecological tumours, we demonstrated that whereas ENT1 expression is highly retained, CNT1 protein is lost in a significant number of tumours [29]. This would argue against CNT proteins being required for salvage during cell proliferation. Although CNT2 mRNA and CNT1 protein levels appear to be cell cycle regulated in FAO hepatoma cells [25,30], it has been demonstrated, at least for CNT1, that although expression oscillates during cell cycle progression, it is not in fact required for proliferation [30]. CNT1 protein is dramatically up-regulated when cells are treated with drugs that are able to interfere with intracellular nucleotide metabolism, such as hydroxyurea, a known inhibitor of ribonucleotide reductase [30]. This is more consistent with a role for CNTs as fine sensors of intracellular nucleotide metabolism. Moreover, we have recently shown that multifunctional cytokines implicated in the process of ‘hepatocyte priming’ prior to cell cycle progression, such as TNF-α (tumour necrosis factor α) and IL-6 (interleukin 6), up regulate CNT1 protein without causing any significant change in CNT2 expression [31]. This effect is completely dependent on the differentiated status of the cell, and its physiological significance is further supported by the evidence that the livers of TNF-α receptor I knockout mice show a dramatic reduction in CNT1 protein levels that can be increased by administration of IL-6 [31]. Interestingly, CNT2 protein levels increase in both FAO hepatoma cells and primary cultures of rat hepatocytes after treatment with TGF-β1, a known anti-proliferative, proapoptotic agent (S. Fernández-Veledo, F. Casado and M. Pastor-Anglada, unpublished work). Thus, although the rapid increase in levels of CNT1 and CNT2 protein during the early proliferative regeneration response of the liver following partial hepatectomy initially favoured the view that CNTs are responsible for salvage, we now believe that their different subcellular localization, at least in hepatocytes, along with their differential regulation, supports additional roles for CNTs in cell physiology. To determine whether this applies to other epithelia will require...
Further research. Recently, the laboratories of Giacomini and Unadkat described the subcellular localization of transfected tagged CNT1 and CNT2 proteins in epithelial cell models [32,33]. Although their data support the view that in absorptive epithelia CNT1 and CNT2 are located on the apical side (in accordance with earlier literature reporting functional assays using brush border membrane vesicles), significant CNT2 insertion has also been reported to occur at the basolateral side, as is the case in hepatocytes [32].

Different transporters, different functions?
Evidence that CNT up-regulation is not involved in cell proliferation, but rather in cell activation and/or apoptosis, comes from studies using murine bone marrow macrophages, a primary culture model suitable for discriminating these cell responses. MCSF (macrophage colony-stimulating factor)-induced proliferation is completely blocked when macrophages are treated with either interferon-γ or LPS (lipopoly saccharide): the first treatment leads to cell activation, and the second leads eventually to apoptotic death. MCSF-induced proliferation is associated with a selective up-regulation of ENT1, without significant changes in CNT1 and CNT2 expression and activity [34]. Pharmacological blockade of ENT1 function by NBT1 (nitrobenzylthioinosine) results in the inhibition of thymidine incorporation into DNA, and impairment of the proliferative response [34]. Interestingly, under these conditions, uridine incorporation into RNA is not modified [34], thus suggesting that substrate channelling is dependent on the uptake pathway, and also anticipating different functions for each nucleoside transporter protein. Although ENT1 seems to be implicated in MCSF-dependent proliferation, and its content also appears to be co-ordinated with the cell cycle in human cancer cells [35], ENT1 mRNA remains unaltered during cell cycle progression in rat FAO hepatoma cells, although basal levels of expression are initially much higher than those found in rat hepatocytes [25]. Nevertheless, a putative role for ENT1 in cell proliferation and growth is challenged by the recent observation that ENT1 function by NBTI (nitrobenzylthioinosine) results in a decrease in ENT1 mRNA content and related activity, whereas the expression of both concentrative nucleoside transporters, CNT1 and CNT2, is markedly up-regulated [34]. Interestingly, the latter response is STAT-1 independent, since it is also present in macrophages isolated from STAT-1 knockout mice. Nevertheless, the decrease in ENT1-related transport activity appears to be mediated by STAT1-dependent post-transcriptional events [37]. LPS treatment of murine bone marrow macrophages similarly up-regulates CNT1 and CNT2 expression, via both TNF-α-dependent and -independent mechanisms [38]. These observations support the view that opposite cell responses are characterized by the selective up-regulation of particular nucleoside transporter proteins, thus raising the question of what specific functions CNT transporters might have, beyond the mere salvage of extracellular nucleosides.

New roles for CNT2 beyond salvage?
High-affinity concentrative nucleoside transporters might be good candidates for mediating efficient extracellular adenosine retrieval, probably contributing to the modulation of purinergic responses. Although ENT1 has traditionally been proposed as a major player in the regulation of adenosine levels (for a recent review, see [1]), this transporter protein is not concentrative and shows much lower apparent affinity for purine nucleosides than do CNTs. Moreover, up-regulation of CNT1 and CNT2 in murine bone marrow macrophages after either LPS or TNF-α treatment [38] is consistent with CNTs playing a compensatory role in purinergic signalling by down-regulating the adenosine receptor activation that is known to impair LPS-induced production of TNF-α and nitric oxide in macrophage cell lines [39]. Nevertheless, it is also unlikely that all CNT isoforms play similar roles in adenosine retrieval from the extracellular medium. Human CNT1 is inhibited by adenosine, although adenosine itself is either not a substrate [40] or is very poorly transported [41]. Whether or not this apparent discrepancy is the result of the increasing number of polymorphic variants initially identified by Giacomini and colleagues for human CNT1 [42], it is likely that adenosine is poorly transported by this isoform, particularly compared with the purine-preferring nucleoside transporter CNT2 and the most recently cloned member of the SLC28 family, CNT3.

The first evidence of purinergic regulation of the CNT2 transporter has recently been obtained in our laboratory, by demonstrating that in liver cells, adenosine 1 receptor (A1R) activation leads to a transient, glucose-dependent increase in CNT2 transport activity that is completely dependent on the opening of ATP-sensitive K+ channels [43]. Moreover, A1R, CNT2 and several subunits of these channels co-localize in FAO hepatoma cells [43]. Although this does not prove that CNT2 activation modulates adenosine receptor function, it strongly suggests that CNT2 has other physiological roles beyond nucleoside salvage. The recent finding that CNT2 transcripts are widespread in the brain, with prevalent expression in amygdala, hippocampus, cerebellum, and specific neocortical regions, along with the evidence that CNT2 mRNA levels are significantly depleted in the cerebral cortex of sleep-deprived rats (a situation in which extracellular adenosine increases for neuroprotection) [44], further supports this view.

Concluding remarks
Analysis of the subcellular localization and regulatory properties of CNT (SLC28) proteins in epithelia, and particularly in liver parenchymal cells and macrophages, indicates that members of the SLC28 protein family might play physiological roles beyond nucleoside salvage. CNT2 is a suitable candidate to modulate purinergic responses, although it may also contribute to energy metabolism, since
adenosine uptake activated by adenosine receptor agonists depends on ATP-sensitive K⁺ channel function and glucose metabolism. Animal and cell models genetically modified to alter nucleoside transporter expression levels will help to further elucidate the particular roles CNT and ENT proteins play in cell physiology.

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References


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