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Nucleobase Transporters as a Novel Tool in Molecular Pharmacology

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S u m m a r y: Nucleobases and their analogues are used as powerful anticancer, antiviral and antimicrobial agents. The majority of pathogenic microorganisms and several mammalian cell types possess highly specific nucleobase transport systems which are promising gateways for drug delivery into target cells. In addition, parasitic protozoa due to their inability to synthesize purines or pyrimidines, are absolutely dependent on nucleobase uptake from the host, and thus inhibition of their transport systems will interrupt their life cycle. Understanding the molecular mechanisms underlying the expression and function of different nucleobase transporters promises to be a critical first step in the systematic development of novel pharmacological therapies. However, up to date no gene encoding a specific nucleobase transporter has been cloned from neither mammalian cells nor pathogenic microorganisms. Our laboratories have developed a model microbial system to clone and study nucleobase transporters. We will discuss studies concerning structure-function relationships in the most significant family of nucleobase transporters We will also present our current efforts to perform functional analysis of putative nucleobase transporters identified in databases and to clone the nucleobase transporters of Candida albigans, Leishmania major and Trypanosoma brucei.

INTRODUCTION

There are several reasons underlying the medical importance of nucleobase (purine & pyrimidine) transporters. First, purine metabolism and transport is an essential part of most living cells for the production of ATP and GTP and the synthesis of nucleic acids. This is reflected in the fact that several mammalian cells have been shown to possess specific nucleobase transporters (erythrocytes, T cells, endothelial and glial tissues, jejunum, kidney, choroid plexus, placenta and cardiac myocytes). Furthermore, proper urate (an oxidized purine) renal handling is of particular importance in humans where, apart from glomerular filtration, and tubular reabsorption and secretion, post-secretory reabsorption, results in elevated urate plasma levels which are thought to play an important role in antioxidant defensive mechanisms. Second, many nucleobase analogues have been and continue to be synthesized and evaluated as antiviral, antimicrobial and anticancer agents, e.g. acyclovir, dideoxyinosine, 5-deoxycitidine and 5-flurouracil. To exert their therapeutic effect, these analogues must first cross the cell membrane thorough the action of nucleobase or nucleoside transporters. Third, most parasitic protozoa which cause severe and widespread diseases of man (malaria, leishmaniasis, toxoplasmosis, trypanosomiasis) totally depend on purine uptake from the host as they lack the ability for nucleobase biosynthesis. Thus, a better understanding of the molecular mechanisms of nucleobase transport in these parasites is of primary significance for therapeutical treatments and the development of new drugs, emphasized by the fact that present day treatments are very inefficient. To date no gene encoding a nucleobase transporter has been cloned from neither mammalian cells nor pathogenic microorganisms. This markedly contrasts research on other families of solute transporters including those specific for nucleoside transporters. The only known nucleobase transporters

come from bacteria, yeast, filamentous fungi, and very recently from plants. These proteins constitute four families. The first, called the Nucleobase-Ascorbate Transporter (NAT) family, includes fungal (*Aspergillus nidulans*), bacterial (Bacillus) and plant (maize) uric acid-xanthine transporters, the uracil transporters of E. coli and Bacillus species and the mammalian ascorbate transporters. All other sequences belonging to this family have been identified in silico and their functions remain unknown. The second, called for the time being the AzgA-Like Transporter family (ALT; not published) is represented by only a single known fungal (*A.nidulans*) adenine-guanine-hypoxanthine transporter and includes homologues from other fungi, bacteria, plants and metazoans. The third, called the Purine-Related Transporter family (PRT), includes only microbial members (yeast and bacterial) and is represented by the yeast adenine-guanine-hypoxanthine-cytosine transporter. The fourth, called the Purine Permease transporter family (PUP), is only found in plants and is represented by a single known transporter specific for purine-related solutes and several homologues of unknown function. Based on the information outlined above our laboratories have developed a system to clone and study nucleobase transporters from human cells and pathogenic microorganisms using the fungus *A. nidulans*.

RESULTS AND DISCUSSION

Rationale of our approach.

We are following a double approach to study nucleobase transporters using *A. nidulans* a model system. First, we use a molecular genetic approach to understand the determinants controlling structure-function relationships in the uric acid-xanthine transporter of the fungus *A. nidulans*, the best studied member of the NAT family. Second, we introduce nucleobase transporter homologues of unknown function, identified in data bases and cloned by PCR or screening techniques, into an *A. nidulans* mutant strain lacking all endogenous purine-pyrimidine transporters. This strain allows the direct identification of any novel gene encoding a functional nucleobase transporter. Knowledge combined from the two approaches allows the identification of amino acid residues critical for the specificity and kinetics of different transporters. Finally, we have initiated the construction of genomic and cDNA libraries from parasitic protozoa, which are known to possess very active nucleobase transporters. These libraries are constructed in a novel high-efficiency replicative vector and will be used to complement *A. nidulans* purine transporter mutants. Complementing clones should correspond to plasmids harboring genes expressing homologous (NAT) or analogous purine-pyrimidine transporters from protozoa.

Structure-function relationships in the NAT family

Chimeric transporters.- A. nidulans has two NAT homologous of similar but not identical function. These are known as UapA and UapC. Both are hydrophobic proteins of 12-14 transmembrane segments (TMS) which transport uric acid, xanthine and their analogues with different kinetics. Using an in vitro recombination system we have constructed UapA/UapC chimeric molecules and answered to the question how do they function, if they do so. Chimeric genes were expressed into an *A. nidulans* mutant strain lacking all endogenous purine transporter genes. Our results showed that the region including L8-TMS9-L9-TMS10-L10 is critical for both the function and specificity of these transporters. If its origin was UapA, chimeric transporters functioned as a UapA protein.

Targeted mutations.- To further investigate the role of the L8-TMS9-L9-TMS10-L10 region, we designed and studied the effect of a number of conservative mutations on UapA function and specificity. Our results showed that N450 is absolutely essential for function, while residues E412, R414 and Q449, while not absolutely essential for function, they are critical for determining the specificity of the transporter towards different purines. Most interesting results came from the analysis of a mutation in residue Q449. In particular, mutation Q449E resulted in a UapA molecule with "relaxed" specificity. UapA-Q449E transports its physiological substrates xanthine and uric acid with reduced capacity, but has also acquired the novel property to bind very efficiently, and transport with reduced capacity, non-physiological substrates such as guanine, 6-thioguanine, 8-azaguanine or hypoxanthine. We proposed that residue 449 might in fact participate in molecular contacts with position C-2 of the purine ring. Interestingly, substitution Q449P,

which positions a P residue found in the homologous mammalian ascorbate transporters, results in a mutant strain with hypersensitivity to ascorbic acid.

Second-site suppressors.- To further investigate the functional role of amino acid residues in UapA we isolated second-site suppressors that restore or alter the function of selected mutant strains. Mutation Q449E was selected as the original mutation to be suppressed. Among revertants we isolated a uapA mutant with the novel capacity to transport all purines and uracil. This mutant results from substitution F569S located in TMS14 of UapA. Further kinetic analysis showed that UapA-Q449E/F569S binds all purines, uracil, analogues of these bases which have substitutions at C-5, C-6 or C-8, but not at C-2, adenosine, inosine and ascorbic acid. In the absence of the original mutation Q449E, UapA-F569S binds similar substrates, but with different kinetics. It seems that the simultaneous presence of substitutions Q449E and F569S has an additive effect in the "process" of converting UapA into a high-affinity hypoxanthine transporter. On the other hand, substitutions Q449E and F569S have an opposing effect on UapA affinity for xanthine. Our work shows that single amino acid substitutions involving polar residues in L9-TMS10-L10 or TMS14 alter the specificity of UapA. Such mutations are located at the two edges, or within, two amphipathic α-helix transmembrane segments. We believe that amphipathic α-helices TMS10 and TMS14 are likely to form part of the UapA hydrophilic pathway through which nucleobases are transported. This work also shows that the specificity of a NAT protein can be easily shifted towards novel substrates, which are transported with high affinity and capacity. It should be emphasized that in other cases it has proved difficult to engineer complete specificity changes in enzymes or transporters while at the same time maintaining wild-type levels of activity.

Cloning and Expression of putative nucleobase transporter homologues in A. nidulans

Several homologues of *A. nidulans* nucleobase transporters (NAT and ALT families) exist in archea, bacteria, fungi, plants, protozoans and metazoans. We have chosen to investigate the biochemical function of several of them by expression in an *A. nidulans* strain lacking its own endogenous nucleobase transporters. We selected four homologues for the following reasons. 1. A maize NAT homologue because plant transporters seem to have a similar mechanism of transport (H^+ symport) with fungal transporter, and thus serve as a promising starting experiment to establish the determinants for heterologous expression. 2. A *Candida albigans* NAT homologue not only because of the medical importance this pathogenic fungus but also because first line drugs against it are nucleobase analogues. 3. Human NAT and ALT homologues and Leishmania NAT and PUR homologues for obvious medical reasons. We have already succeeded the functional expression of the maize homologue and showed that it functions as a uric acid-xanthine transporter. The human NAT homologue, SVCT1, has recently been shown to function as a vitamin C transporter. Cellular expression of SVCT1 has proven problematic in our standard *A. nidulans* strain lacking its endogenous nucleobase transporters. However, by making use of a novel mutant strain in which targeting of transporter proteins into the plasma membrane is increased, we showed that SVCT1 expression causes hypersensitivity to L-ascorbic acid. We are currently investigating by mutational analysis whether SVCT1 can be converted into a nucleobase transporter. The expression of a second human ALT homologue, which is expressed in T cell lymphomas, and of the *C. aligans* NAT homologue will be discussed. Finally, Leishmania full-length NAT and PUR homologous genes are in progress and will also be discussed.

CONCLUSIONS

We have developed a model system to express and study putative nucleobase transporters from organisms of medical importance. This system will also be extended for cloning by direct genetic complementation nucleobase transporters not present in databases. In parallel we investigate the molecular determinants controlling structure-function relationships in an *A.nidulans* member of NAT, the largest and most conserved family of nucleobase transporters. Conclusions from these studies are complementary not only to our efforts to understand the function and specificity of nucleobase transporters from other organisms but also to ongoing efforts for the functional expression of these proteins into *A. nidulans.*