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Mutations in the Chinese hamster ovary cell *GART* gene of de novo purine synthesis

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Abstract

Mutations in several steps of *de novo* purine synthesis lead to human inborn errors of metabolism often characterized by mental retardation, hypotonia, sensorineural hearing loss, optic atrophy, and other features. In animals, the *phosphoribosylglycinamide transformylase (GART)* gene encodes a trifunctional protein carrying out 3 steps of *de novo* purine synthesis, phosphoribosylglycinamide synthase (GARS), phosphoribosylglycinamide transformylase (also abbreviated as GART), and phosphoribosylaminoimidazole synthetase (AIRS) and a smaller protein that contains only the GARS domain of GART as a functional protein. The *GART* gene is located on human chromosome 21 and is aberrantly regulated and overexpressed in individuals with Down syndrome (DS), and may be involved in the phenotype of DS. The GART activity of GART requires 10-formyltetrahydrofolate and has been a target for anti-cancer drugs. Thus, a considerable amount of information is available about GART, while less is known about the GARS and AIRS domains. Here we demonstrate that the amino acid residue *glu75* is essential for the activity of the GARS enzyme and that the *gly684* residue is essential for the activity of the AIRS enzyme by analysis of mutations in the Chinese hamster ovary (CHO-K1) cell that require purines for growth. We report the effects of these mutations on mRNA and protein content for GART and GARS. Further, we discuss the likely mechanisms by which mutations inactivating the GART protein might arise in CHO-K1 cells.

Keywords

somatic cell mutations; gene evolution; enzyme activity; metabolism; genetic disorders

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1. Introduction

The *de novo* purine biosynthetic pathway is a target for currently used cancer chemotherapy agents, and new agents continue to be developed. Aberrant purine synthesis leads to significant pathology in humans. For example, purine overproduction due to genetic alterations in PRPP synthase (PRPS1) resulting in increased activity leads to mental retardation, sensorineural deafness, ataxia, and hyperuricemia that can result in kidney damage and gouty arthritis if untreated (Becker et al., 1994). Loss of function mutations in PRPS1 leads to conditions with related phenotypes: Arts syndrome, characterized by mental retardation, early onset hypotonia, ataxia, delayed motor development, hearing impairment, and optic atrophy (de Brouwer et al. 2007), a variant of Charcot-Marie Tooth syndrome (CMTX5), which is characterized by inherited peripheral neuropathy, sensorineural hearing loss, gating disturbance, and visual loss, and Rosenberg-Choutorian syndrome, which has features similar to CMTX5 (Kim et al., 2007). Deficiency of adenylosuccinate lyase (ADSL), step 8 in Figure 1, leads to psychomotor delay often accompanied by autistic features, hypotonia, and seizures (Spiegel et al., 2006; Mouchegh et al., 2007). Phosphoribosylaminoimidazolecarboxamide transformylase/IMP synthase (ATIC) deficiency leads to a syndrome characterized by dysmorphic features, severe neurological defects, and congenital blindness (Marie et al., 2004). Thus, defects in several steps of *de novo* purine synthesis lead to inborn errors of metabolism, often with similar features, and it seems reasonable to hypothesize that defects in other enzymes in the pathway may also lead to inborn errors.

One enzyme in the pathway of special interest is GART. In mammals, GART is part of a trifunctional protein, also known as GART, which carries out two additional steps of *de novo* purine synthesis, GARS and AIRS (Figure 1). GART function, which requires 10-formyltetrahydrofolate as a cofactor, is the best characterized of the three steps because it has been a target for cancer chemotherapy (Dahms et al., 2005). However, even though some inhibitors of GART showed some promise, none have become a mainstay of therapy. The GARS and AIRS domains of GART, which do not require folates or other cofactors, are less well characterized and present additional chemotherapeutic targets.

The GART gene is located on human chromosome 21, and is trisomic in DS (Moore et al., 1977; Patterson, 2007). Individuals with DS have ~1.5-fold elevated levels of purines in their bodily fluids (Pant et al., 1968). Persons with DS, in addition to essentially ubiquitous intellectual disability of varying severity, almost always have hypotonia and have an increased risk of sensorineural deafness, all of which are common features of inborn errors of purine metabolism. Moreover, *GART* is aberrantly regulated in the developing cerebellum of individuals with DS (Brodsky et al., 1997) and is overexpressed in fetal heart of individuals with DS (Li et al., 2006). We and others hypothesize that *de novo* purine synthesis may be elevated in individuals with DS because of increased dosage of the *GART* gene and that this may be related to the intellectual disabilities and other health problems confronted by individuals with DS (Brodsky et al. 1997; Li et al., 2006). If GART activity is related to the phenotype of DS, then methods that reduce the activity of GART to levels seen in euploid individuals might alleviate some of the consequences of DS. Understanding the mechanism of action of the various activities of GART may aid in developing ways to reduce the activity of one or more of the enzymatic steps of GART for this purpose.

In addition to its possible relevance to human health and development, the evolution, structure, and basic genetics of GART are inherently interesting. Mammals, vertebrates, and insects produce two proteins from the *GART* gene, one the full length trifunctional protein, and one containing only the amino-terminal GARS domain (Brodsky et al., 1997; Aimi et al., 1990; Henikoff et al., 1986). The monofunctional GARS protein is produced through cleavage and polyadenylation of the *GART* gene transcript in the intron separating the last *GARS* exon and

the first *AIRS* exon (Kan and Moran, 1995; Brodsky et al., 1997; Kan and Moran, 1997). In yeast, the GARS and AIRS domains are carried on a single protein, while GART is on a separate protein. In plants or bacteria, each of the three activities is carried on a separate protein (Li et al., 1999). Thus, there are important differences in the structure and organization of the genes and proteins in different organisms. How this situation arose evolutionarily is unclear.

Two important approaches to understanding the mechanisms of action of the enzymes of purine synthesis are analysis and comparison of the structures of the proteins and analysis of mutations in the genes encoding the enzymes. Both mutations generated by targeted mutagenesis and naturally occurring mutations associated with human inborn errors of metabolism have been studied. Structural analysis can be informative to guide generation of hypotheses, but these need to be tested experimentally. The GART domain of the trifunctional GART gene has been cloned and expressed, allowing study of the GART monofunctional human protein. Structural and functional comparisons of the human and *E. coli* protein as well as targeted mutagenesis experiments have revealed important differences between these proteins (Manieri et al., 2007; Zhang et al., 2002). Similarly, structural and functional analysis coupled with targeted mutagenesis of the *ADSL* locus reveals important differences between the human, *B. subtilis*, and *E. coli* enzymes (Sivendran et al., 2004; Sivendran and Colman, 2008). Thus, specific serine residues in the *E. coli* enzyme were hypothesized to be important for catalysis. This was then proven by targeted mutagenesis of the relevant residues in the *E. coli*, *B. subtilis*, and human *ADSLs* (Tsai et al., 2007; Sivendran and Colman, 2008). One important point from this work was that the *E. coli* *ADSL* might not be a particularly good model for either the human or *B. subtilis* enzymes (Sivendran and Colman, 2008). These findings demonstrate that comparisons of the bacterial and human structures of enzymes, including GART, while useful, must be interpreted carefully and that hypotheses based on these comparisons need to be validated experimentally, by analysis of normal and mutant proteins.

A further complexity in the case of GART is the multifunctional nature of the mammalian enzyme and the recently demonstrated existence of functional complexes between GART and the other enzymes of de novo purine synthesis under growth conditions requiring purines (An et al., 2008). Determination of the amino acids responsible for this mammalian multiprotein complexing will most likely require study of mutants of the mammalian enzymes.

So far, no examination of mutants affecting the activities or structures of the mammalian GARS and AIRS domains of GART has been reported. Therefore, to elucidate the role of GART in mammalian development, health, and disease, to contribute to the development of additional anti-cancer drugs, and to aid in understanding the evolution and structure of GART, we isolated CHO-K1 mutants that are deficient in the GARS or AIRS enzyme activities (Brodsky et al., 1997; Patterson, 1975). These mutants were isolated solely on the basis of their requirement for purines for growth. This means that these mutations may cause amino acid substitutions or may be due to other changes, for example in the production or stability of GART mRNA or protein. If they result in amino acid substitutions, since there is no bias (except that they affect the function of the enzyme) these mutants may reveal unexpected insights into which amino acids are important for the function and/or stability of GART. These mutants fall into two complementation groups, AdeC and AdeG, as determined by somatic cell hybridization experiments. Here we report the identification of the mutations in CHO-K1 purine auxotrophs of the AdeC and AdeG complementation groups and characterize the purine deficiency phenotype in these CHO-K1 mutant cell lines.

2. Materials and Methods

2.1 Cell lines and cell culture

The AdeC and 55-1 mutant cell lines have been partially characterized previously (Brodsky et al., 1997). 55-1 is a member of the AdeG complementation group. Therefore, in this manuscript we refer to it as AdeG (55-1). Previously, for example in Brodsky et al. (1997), it was referred to simply as 55-1. For standard, nonselective, growth CHO cell lines were grown in Ham's F12 (Ham, 1965) medium supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS), penicillin, streptomycin, and the antifungal-amphotericin B.

2.2 Isolation and Northern blot analysis of GARS and GART mRNA

RNA was isolated using the RNeasy Midi (Qiagen) kit and protocol, separated by formaldehyde-borate agarose gel electrophoresis (Derman et al., 1981) and transferred to nylon hybridization membranes (Ausubel, 1987). Probes were generated by amplifying fragments of the hamster *GARS* and human cyclophilin cDNAs using polymerase chain reaction (PCR) in which α - P^{32} labeled dCTP was directly incorporated in the PCR reaction. Primers used were: *GARS*; 230F 5'-GATCACACTGTCCTGGCTCA-3', 662R 5'-CTTCTTCCCCGTCCAGAACTCT-3'; Cyclophilin; hCyclo23F 5'-GCCGAGGAAAACCGTGTACT-3', hCyclo183R 5'-CCTTTCTCTCCAGTGCTCAGA-3'. The *GARS* probe hybridizes to the *GARS* domain of both the *GARS* and *GART* mRNAs. Northern blots were performed using the Ultrahyb reagent and protocol (Ambion) and visualized using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.3 Western blot analysis of GARS and GART proteins

Generation of the *GARS* monoclonal antibody has been described (Brodsky et al., 1997). Protein isolations were performed as previously described (Barnes et al., 1994). Protein concentrations were determined using the BioRad Microassay reagent. Equal amounts (30-40 μ g) of total protein were resolved by gel electrophoresis and electroblotted onto nitrocellulose membranes. Immunoblotting and detection were performed essentially as previously described (Barnes et al., 1994; Brodsky et al., 1997). Protein bands were visualized using CDP-*Star* substrate according to the supplier's protocol (Applied Biosystems). Images of Western blots were captured using a Diana III camera system (Raytest), and analyzed using AIDA Image Analyzer software (Raytest).

2.3 Relative Specific Enzyme activities of GARS AIRS, and GART in CHO-K1, AdeC, and AdeG (55-1)

Enzyme assays of *GARS*, *AIRS*, and *GART* were carried out using our previously published procedures (Chang et al., 1991). All assays were linear with time and amount of protein and were run in duplicate.

2.4 Sequence analysis of CHO *GART* cDNAs

To determine the coding sequence of CHO-K1 *GART* and to identify mutations in AdeC and AdeG (55-1), *GART* cDNAs were isolated and cloned from CHO-K1 and the mutant cell lines. *GART* cDNA was amplified from total RNA using *GART* primers USOUT (5'-GCGGGTTTCATTTTGCTTC-3') and GARTDSIN2 (5'-GACAAGAGTTTGGGACAAGA-3') and the SuperScript™ One-Step RT-PCR for Long Templates (Invitrogen). PCR products were analyzed by agarose gel electrophoresis. These PCR products were ligated into the pGEM-T Easy TA cloning vector (Promega) and transformed into NovaBlue competent cells according to the supplier's protocol (Novagen). Insert-containing clones were isolated based on blue/white screening and positive restriction digestion of plasmid DNA (Promega Wizard Plus Miniprep Kit).

GART cDNA clones were sequenced using primers that allowed for complete sequence coverage in both directions with overlapping sequence results (Table 1). Sequencing reactions were carried out using the ABI Big Dye Terminator Cycle Sequencing kit. Purified products were sequenced using an ABI Prism 377 DNA sequencer. *GART* sequence data was analyzed using the Sequencher application (Gene Codes).

2.5 Sequence analysis of CHO *GART* genomic regions

Genomic DNA was isolated from CHO-K1, AdeC, and AdeG (55-1) with the PureGene Genomic DNA Purification Kit (Gentra Systems). Regions of the CHO *GART* gene corresponding to putative mutations in the *GART* cDNA were amplified with the Expand High Fidelity PCR Kit (Roche) using primers for GARS (230F 5'-GATCACACTGTCCTGGCTCA-3', 386R 5'-TGGACTCTAACTGGGCTGCT-3) and AIRS (1933F 5'-ATGGGTTTAGCCTTGTGAGGA-3', 2135R 5'-GGAGGACTCTGGGGATGTTTC-3'). PCR products were visualized on 1% agarose gels in 1X Tris-acetate-EDTA containing ethidium bromide. PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham) and sequenced and analyzed as described above, except 100 ng of PCR product was sequenced.

2.6 Fluorescence in situ hybridization (FISH) of *GART* in CHO cell-lines

CHO *GART* probe was produced from two pooled PCR amplified fragments of the genomic CHO *GART* sequence. A 7 kb fragment spanning exons 3 to 10 was PCR amplified using *GART* specific primers 230F 5'-GATCACACTGTCCTGGCTCA-3' and Exon10R 5'-TGCCATGACAACAGTTACGG-3'. A 5 kb *GART* genomic PCR fragment spanning exons 10 to 16 was amplified with the primers CHOGTE16F1 5'-TGGAGAACATCCCCAGAGTC-3' and 2764R 5'-TGACAAAAGGGCCAGAGAG-3'. These fragments were then TA cloned into the pGEM-Teasy vector (Promega). Fragments containing the entire insert were released by NotI digestion and 500ng of each fragment were combined and labeled using the Nick Translation Kit (Vysis) with the SpectrumOrange fluorophor (Vysis). Metaphase spreads were prepared for each CHO cell line using standard cytogenetic methods (McCarthy et al., 2004). Metaphase spreads were then co-denatured at 70°C for 5 minutes with 1 µl probe mixed with 4 µl Hybridization Buffer (Vysis). Slides were hybridized overnight at 37°C, washed for 1-2 minutes in 0.4XSSC at 73 ± 1°C and counterstained with DAPI. Hybridized metaphase spreads were visualized using an Olympus epifluorescence microscope and single, dual and triple band pass filters (DAPI, SpectrumOrange). A minimum of 10 metaphase spreads per slide were analyzed, and representative metaphase spreads were captured with the Cytovision imaging system (Applied Imaging Corp.).

2.7 Cloning and mutagenesis of CHO *GART* gene in a mammalian expression vector

The full-length *GART* cDNA from CHO-K1 was amplified and cloned into the G418^R TA cloning and mammalian expression vector pTarget (Promega). The ends of the insert in this clone (pCMV-*GART*-K1) were sequenced using vector T7 and Sp6 primers, confirming the presence of full-length CHO *GART* cDNA.

Site-directed mutagenesis was performed with the QuickChange II site-directed mutagenesis kit and protocol (Stratagene). The primers used to introduce point mutations into pCMV-*GART*-K1 are listed in Table 1. The presence of the introduced mutations was verified by DNA sequencing as described above. The successfully mutated *GART* expression constructs, the wild-type *GART* clone, and the empty pTarget vector were all used for transfection after purification with the Pure Yield Plasmid Midi Prep System (Promega) followed by ethanol precipitation and washing to remove contaminants.

2.8 Transfection of *GART* cDNA expression constructs into CHO *GART* mutants

The CHO mutants AdeC and AdeG (55-1) were transfected with the *GART* cDNA expression constructs using the Lipofectamine 2000 reagent according to the supplier's protocol (Invitrogen).

To select for the ability of transfected cells to grow in purine deficient media, cells were cultured in Ham's F12 without hypoxanthine supplemented with 5% (v/v) twice dialyzed fetal calf serum, antibiotic/antimycotic (Ham, 1965) and G418 (Geneticin[®], Gibco) at an active concentration of 400 µg/ml.

Cells were fixed and then stained with crystal violet. Plates were scanned using a digital photograph scanner and the image file of each plate was scored for colonies using the colony-counting tool in the TotalLab[™] TL100 software application (Nonlinear Dynamics).

3. Results

3.1 Cloning and sequencing the CHO-K1 *GART* cDNA

The full-length CHO *GART* cDNA sequence has an open reading frame of 3030bp (GenBank accession number [EU622913](#)) and codes for a protein with 1010 amino acids. The human and CHO protein sequences are 86% identical and 93% similar. The mouse and CHO protein sequences are 89% identical and 95% similar (Figure S1).

3.2 Sequencing of the mutant cDNAs and genomic DNAs

We sequenced the cloned full-length trifunctional *GART* cDNA and relevant *GART* genomic regions from CHO-K1 and the AdeC and AdeG (55-1) mutants. The mutation in AdeC is a G to A transition at nucleotide 223 of the *GART* coding region resulting in a glutamate (*glu*) to lysine (*lys*) substitution at amino acid position 75. The mutation in AdeG (55-1) is a G to A transition at position 2050 resulting in a glycine (*gly*) to aspartate (*asp*) substitution at amino acid 684.

To confirm the mutations in each mutant, we sequenced PCR amplification products from genomic DNA from each mutant. In both AdeC and AdeG (55-1), the mutations also appeared in genomic DNA with no evidence of the wild-type sequence.

3.3 *GART* gene mutations cause the purine auxotrophy

To verify that the mutant phenotypes in AdeC and AdeG (55-1) are caused by the mutations identified above, we tested the ability of mutant *GART* cDNAs to alleviate the purine auxotrophy in these cell lines. For this purpose, we introduced the relevant mutation into the wild-type CHO-K1 cDNA expression construct and transfected the *GART* mutants to determine whether expression of the mutant cDNAs could alleviate the purine auxotrophy of the various mutants. In each case, we found that the mutant cDNAs could not alleviate the purine auxotrophy in cells with the corresponding mutation. However, the AdeC mutation could alleviate the auxotrophy of the AdeG (55-1) mutant and vice-versa. Wild-type *GART* alleviated both the AdeC and AdeG (55-1) purine requirements (Figure 2).

3.4 Northern blot analysis of the CHO-K1 *GART* mutants

To assess whether the changes in the *GART* gene in these mutants affect *GART* transcript sizes or amounts, the mRNAs for monofunctional *GARS* and trifunctional *GART* were analyzed by Northern blot using a probe specific for the *GARS* region (Figure 3). CHO-K1 and the mutants all express significant levels of mRNA for the trifunctional *GART* and monofunctional *GARS* transcripts. The approximate lengths of the *GART* and *GARS* mRNAs in all cases are

3.2 and 1.7 kb, respectively, similar to the lengths seen in mice (Kan and Moran, 1995; Kan and Moran, 1997). Full length *GART* cDNAs were also obtained by RT-PCR from all the cell lines (data not shown). The levels of mRNA in CHO-K1 and the two mutants are qualitatively similar, although the Northern blot suggests that mutant AdeG (55-1) may have slightly reduced levels of the *GARS* transcript on the basis of the apparent differences in the relative intensity of the *GART* and *GARS* mRNAs in CHO-K1 and AdeG (55-1). Thus, neither mutation has major effects on *GARS* or *GART* mRNA levels.

3.5 Western blot and enzyme activity analysis of the CHO-K1 *GART* mutants

In a previous study (Brodsky et al., 1997), this laboratory detected *GARS* (~50kDa) and *GART* (~110 kDa) proteins in CHO-K1 and the two mutants by Western blotting. AdeC produces *GARS* protein with a consistently observed increased mobility (Fig. S2). Similar mobility shifts for *glu* to *lys* substitution have been observed previously (Yannoukakos et al., 1991). In general agreement with our previous studies (Oates et al., 1977; Irwin et al., 1979; Chang et al., 1991; Brodsky et al., 1997; Bleskan and Patterson, unpublished results) AdeC and AdeG (55-1) both contain at least as much *GARS* and *GART* protein as CHO-K1 lysates (Fig. S2). Most significantly, AdeC has undetectable levels of *GARS* enzyme activity, and AdeG (55-1) has undetectable levels of *AIRS* enzyme activity. AdeC has significant levels of *AIRS* and *GART* activity and AdeG (55-1) has significant levels of *GARS* and *GART* activity. In each mutant there is a trend towards reduced levels of the remaining enzyme activities.

Based upon the *E. coli* *AIRS* structure (Li et al., 1999) and the high level of sequence conservation at the *gly684* residue, the *gly684asp* mutation would alter a well-conserved glycine-rich loop at or near the *AIRS* active site. This substitution would also introduce a negative charge at this position. To explore whether the effects of this mutation are due to a structural change or a charge change, mutants were created in which amino acid 684 was substituted with *lys* or proline (*pro*). The former substitution introduces a positive charge as opposed to the negative charge introduced by aspartate, while the latter is expected to increase the rigidity of the loop and alter its three-dimensional structure.

The cDNA expression constructs with residue 684 mutations were transfected into AdeG (55-1) cells and selected for purine-free growth. When the original AdeG (55-1) mutation (*gly684asp*) or *gly684pro* were transfected into AdeG (55-1) cells there was no auxotrophy rescue, while the *gly684lys* mutation did rescue at levels comparable to the wild-type CHO-K1 cDNA. As expected, all 3 amino acid substitutions relieved the auxotrophy of AdeC cells (Figure 2).

3.6 *GART* gene copy number in CHO-K1 and AdeC and AdeG (55-1)

To complement our understanding of the relationship between the mutations and the genomic changes in each cell line we determined the number of *GART* chromosomal loci in metaphase chromosome spreads from CHO-K1 and from the AdeC and AdeG (55-1) mutants using FISH (Figure 4) (McCarthy et al., 2004).

CHO-K1 cells have two copies of the *GART* allele, both very close to the telomeres of two different chromosomes. The AdeC FISH results show two *GART* genes on the same chromosome and an apparent reduction in chromosome number, so rearrangement of the two *GART*-containing chromosomes with each other may have occurred in this cell line. AdeG (55-1) has two copies of *GART* on different mid-sized, metacentric chromosomes, again demonstrating rearrangement involving the *GART* containing chromosomes seen in CHO-K1. We cannot determine from this result whether the *GART* gene was altered by these chromosome rearrangements, but clearly, one copy of the entire gene was not lost during generation of the AdeC or AdeG (55-1) mutations.

4. Discussion

4.1 Identification of the mutations responsible for the purine auxotrophy in AdeC and AdeG (55-1) CHO-K1 mutants

There have been limited genetic analyses of the GART protein from mammals, mainly consisting of targeted mutagenesis and structural studies focused on the human GART domain. In this case, significant differences between the human GART protein and the *E. coli* protein have been observed (Manieri et al., 2007). These studies suggest that study of the human (or mammalian) GART and GARS proteins are important for their accurate characterization, although clearly comparisons of the mammalian and bacterial proteins will be useful, as described below. The mutations reported here are the first in which mutations with functional significance have been reported for the GARS and AIRS domains of mammalian GART. Our results demonstrate that the defects leading to the purine deficiency in these cells are due to coding region mutations in the relevant enzyme domains as opposed to epigenetic effects such as gene silencing as has been observed for other CHO cell mutants (Harris, 1984). They represent a start toward genetic analysis of these proteins.

The mutation in AdeC is a G to A transition at nucleotide 223 of the *GART* coding region resulting in a glutamate to lysine substitution at amino acid position 75. The structure of the *E. coli* GARS enzyme indicates that *glu73*, which is highly conserved and equivalent to *glu75* in CHO GARS, may be responsible for hydrogen bonding to the glycyl moiety of GAR, the product of GARS catalysis (Wang et al., 1998). This observation is consistent with the AdeC CHO mutant phenotype; namely, the *glu75lys* mutation abolishes cellular GARS enzyme activity but retains at least normal GARS and GART protein levels and more than half of wild-type levels of AIRS and GART activity. Thus, alteration of the *glu75* residue does not result in protein instability and degradation. Since this mutant retains at least 50% of AIRS and GART activities compared to CHO-K1, it is evident that the *glu* to *lys* mutation does not severely impact the function of the other domains.

The mutation in AdeG (55-1) is a G to A transition at position 2050 resulting in a glycine to aspartate substitution at amino acid 684. Thus, *gly684* is essential for AIRS enzyme activity but retains GARS and GART protein and enzyme activities. This finding was confirmed by showing that substitution of residue 684 with pro also inactivates AIRS activity as assessed by transfection studies and does not have a major effect on GARS or GART protein levels.

4.2 Mechanisms by which mutations in CHO-K1 cells can arise

It is of some interest to consider how recessive mutations such as those reported here could be generated in CHO-K1 cells, which should be diploid for the *GART* locus. The CHO-K1 karyotype has been extensively rearranged, so the possibility existed that CHO-K1 cells might contain only a single copy of the *GART* gene or that one copy of the gene might be lost in the mutants. However, our FISH analysis demonstrates two signals for *GART* in CHO-K1 cells. Recently, the inner mitochondrial folate transport protein gene that is mutated in CHO-K1 glyB mutants was found to be present in two copies in CHO-K1 cells but in one copy in the glyB mutant cells (McCarthy et al., 2004; Kao et al., 1969). The authors hypothesized that the single mutant allele in the glyB cells was due to the loss of one wild-type allele during selection of the glyB line. Both the AdeC and AdeG (55-1) mutants reported here apparently remain diploid for the *GART* locus, although the possibility of rearrangement or epigenetic silencing of one copy of the gene cannot be ruled out. However, both the AdeC and AdeG (55-1) mutations reported here appear to have altered chromosomal locations for one *GART* allele. Thus, the available evidence from the GlyB, AdeC, and AdeG (55-1) mutants is consistent with the hypothesis that chromosomal rearrangement of the relevant genes is a common feature in generation of mutations in CHO-K1 cells.

4.3 Implications of these results for the structure of mammalian GART

These data are the first to identify amino acids critical for the function of mammalian GARS or AIRS. X-ray crystal structures are not available for human GARS, AIRS, or trifunctional GART. Therefore, we compared the amino acid sequences for the GARS and AIRS domains of CHO GART with the crystal structures of *E. coli* GARS and AIRS (Wang et al., 1998; Li et al., 1999). The *E. coli* and CHO GARS domains are 51% identical and 65% similar while homology between the AIRS domains shows 49% identity and 65% similarity. Modeling of the CHO domains with SwissModel using the *E. coli* structures as templates produced models that were very similar to the *E. coli* enzymes except in some exposed loops (Guex and Peitsch, 1997).

The mutation in AdeC results in a *glu75lys* substitution. Mutations that cause human disease are thought to be most likely at amino acid residues that are conserved across species (Miller and Kumar 2001; Vitkup et al., 2003). *Glu* at this residue is completely conserved in all eukaryotic sequences currently available and in all bacterial sequences examined with the exception that two *Clostridia*, six *Streptococcus* strains, *Caldicellulosiruptor saccharolyticus*, and *Cloroflexus auranticans* have *asp* at this position. Of 34 Archaea sequences, all are *glu* except two, which are *asp*, a conservative substitution for *glu*. Two strains of bacteria, *Roseiflexus sp. RS-1* and *Chloroflexus aurantiacus* have serine at this position. Thus, this is a highly conserved amino acid position throughout evolution.

Although *glu* to *lys* substitutions are uncommon in interspecies comparisons, lysine is the most common substitution for glutamate in human genetic disease (Miller and Kumar, 2001; Vitkup et al., 2003). Thus, the mutation in AdeC fits the pattern expected for mutations that might be associated with genetic disease.

The *gly684asp* mutation in AdeG (55-1) is a non-conservative substitution at a remarkably conserved residue. All available eukaryotic AIRS sequences have a glycine at this residue. One bacterial sequence has a serine residue and two have an aspartate at this position. Mutations at *gly* residues are relatively common in human genetic disease, perhaps because glycine is important for protein structural stability (Vitkup et al., 2003). Thus, again, this mutation follows a pattern consistent with that expected for mutations that cause genetic disease in humans.

This residue corresponds to *gly251* in *E. coli* AIRS, which is the central residue in a highly conserved and short glycine-rich loop between strand $\beta 6$ and helix $\alpha 8$ of *E. coli* AIRS (Li et al., 1999). $\beta 6$ is a component of the proposed AIRS active site and contains histidine 247 and threonine 249, both highly conserved polar residues that could interact with metal ions in the active site. Glycine residues are often found in short loops and enzyme active sites due to the flexibility they allow (Li et al., 1999; Krieger et al., 2005; Yan and Sun, 1997). If this residue serves a similar function in CHO AIRS, substitution of CHO *gly684* with a larger, polar, and negatively charged aspartate could disrupt the structure of the AIRS active site. Alternatively, the negative charge introduced by the aspartate might interfere with the active site or introduce attractions or repulsions with other amino acids (of course these substitutions may also cause structural changes). The finding that substitution of a *pro* at residue 684 in the CHO-K1 enzyme prevents the protein from alleviating the purine auxotrophy of AdeG (55-1) strongly implies that the structural features of glycine are significant for activity. Introduction of a positively charged lysine at this site does not eliminate enzyme activity. Thus, the introduction of a charge itself does not prohibit enzyme function, a finding that is again consistent with this glycine being critical for the structure of the active site of AIRS. It is interesting to note that two bacterial AIRS proteins do have an aspartate at this residue, an observation consistent with this interpretation.

In general, missense mutations can impact proteins and their cellular role by either altering functional sites or decreasing active enzyme levels. Unstable proteins are often degraded, improperly localized, or form aggregates. The most straightforward interpretation of the data presented here is that the *glu75lys* mutation affects enzyme activity but not protein stability since our Western blot results show that AdeC has GARS and GART protein levels at least equivalent to those found in CHO-K1. The *gly684asp* mutation in AdeG (55-1) eliminates AIRS enzyme activity but does not appear to result in a decrease in GART (and GARS) protein levels.

The information gained from identifying and characterizing the consequences of the mutations in AdeC and AdeG (55-1) enhances the current knowledge of the mammalian GARS and AIRS enzymes. This is important since no genetic studies on the GARS and AIRS domains of the mammalian protein have been reported so far, and it is not clear how well studies on the bacterial GARS, GART, and AIRS proteins apply to the mammalian trifunctional protein. Although it has been hypothesized on the basis of the structural studies that the *E. coli* GARS, AIRS, and GART proteins might form a complex involving all three proteins, thus mimicking the trifunctional structure of the animal protein, and that this protein might interact with PRAT, the first committed enzyme of *de novo* purine synthesis to allow channeling of the unstable product of PRAT, PRA, to the GARS active site (Li et al., 1999), complex formation has not yet been detected experimentally in bacteria. A recent elegant study demonstrates that the human GART trifunctional protein does indeed interact with the PRAT in mammalian cells under conditions in which the cells need to synthesize purines (An et al., 2008).

4.4 Possible implications for inborn errors of metabolism and Down syndrome and future studies

Inborn errors have now been associated with 3 genes of *de novo* purine synthesis, *PRPS1*, *ADSL*, and *ATIC*. All of these inborn errors have neurological consequences and can lead to hypotonia, and many lead to sensorineural hearing loss. We speculate that deficiencies of one or more of the activities of the GART protein (or any other protein in the *de novo* purine biosynthetic pathway) may also lead to inborn errors in humans. We and others have published methods to detect most of the intermediates of purine biosynthesis (Hards and Patterson, 1988; Friedecký et al., 2007). Lack of GARS, AIRS, or GART activities might be expected to alter purine concentrations or profiles in bodily fluids and/or lead to accumulation of intermediates of *de novo* purine synthesis. Thus, it seems reasonable to test individuals with developmental delay of unknown origin with these methods. We therefore hypothesize that an analysis of individuals with developmental delay of unknown origin using these methods may uncover clinically relevant alterations in GARS, AIRS, or GART, revealing both basic enzymatic mechanisms and pharmacological targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GAR	phosphoribosylglycinamide
GART	phosphoribosylglycinamide transformylase
GARS	phosphoribosylglycinamide synthase
AIRS	phosphoribosylaminoimidazole synthetase
CHO	Chinese hamster ovary
PRPP	phosphoribosylpyrophosphate
PRPS1	PRPP synthase 1
CMTX5	Charcot-Marie Tooth syndrome
ADSL	adenylosuccinate lyase
ATIC	phosphoribosylaminoimidazolecarboxamide transformylase/IMP synthase
FCS	fetal calf serum
PCR	polymerase chain reaction
dNTP(s)	

	deoxynucleotide triphosphates(s)
DS	Down syndrome
FISH	fluorescence in situ hybridization
DAPI	4,6-diamidino-2-phenylindole
CMV	cytomegalovirus
RT-PCR	reverse transcription PCR
kDa	kiloDaltons
E. coli	Escherichia coli

Fig. 1.

The *de novo* purine biosynthesis pathway. Enzymes associated with pathology are underlined. The steps encoded by the GART gene are indicated. Abbreviations are: R-5-P, ribose-5-phosphate; PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; GAR, phosphoribosylglycinamide; FGAR, phosphoribosylformylglycinamide; FGAM, phosphoribosylformylglycineamidine; AIR, phosphoribosylaminoimidazole; CAIR, phosphoribosylaminoimidazole- carboxylate; SAICAR, phosphoribosylsuccinylaminoimidazole; AICAR, phosphoribosylaminoimidazolecarboxamide; FAICAR, phosphoribosylformylaminoimidazolecarboxamide; IMP, inosine monophosphate; SAMP,

adenylosuccinate; XMP, xanthosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate. Pathway enzymes are: (1) PRAT, phosphoribosylamidotransferase; (2) GARS, GAR synthetase; (3) GART, GAR transformylase; (4) FGARAT, FGAR amidotransferase; (5) AIRS, AIR synthetase; (6) AIRC, AIR carboxylase; (7) SAICARS, SAICAR synthetase; (8) and (12) ADSL, adenylosuccinate lyase; (9) AICART, AICAR transformylase; (10) IMPS, IMP synthetase; SAMPS, SAMP synthetase; IMPDH, IMP dehydrogenase; GMPS, GMP synthetase. PRPS1, phosphoribosylpyrophosphate synthase 1

Fig. 2.

Transfection of AdeC and AdeG (55-1) cells with wild-type and mutant cDNA constructs. The wild-type and mutant GART clones indicated were transfected into AdeC or AdeG (55-1) cells in F12 without purines and clones able to grow in the absence of purines were scored as described in Materials and Methods. Error bars indicate the standard deviation of the measurements. pT indicates transfection with the empty cloning vector.

Fig. 3. Northern blot of mRNA from wild-type and GART mutant CHO cells. Total RNAs were isolated from CHO cells, separated on 1% agarose-formaldehyde gel, transferred to nylon membrane and probed with (A) Labeled PCR fragment of GARS cDNA. (B) The membrane was stripped then reprobed with a control cyclophilin cDNA. 1 = CHO-K1; 2 = AdeC; 3 = AdeG (55-1).

Fig. 4. CHO-GART copy number in CHO mutants. Fluorescence *in situ* hybridization of metaphase chromosomes from CHO-K1, AdeC, and AdeG (55-1) cells. The left panel is CHO-K1, the middle panel is AdeC, and the right panel is AdeG (55-1). The probe was made from two fragments of the CHO GART gene that were purified and labeled with SpectrumOrange (Vysis Inc.).

Table 1

Oligonucleotides used to sequence and mutagenize the CHO GART cDNA.

Primer	Sequence 5'-3'
AK1	gggtgcgggttcattttgct
638R	ccacaaccactgtttctcca
AK4	gcgcagtggaagcctttac
CHOGT33	tccacctagatggcctgct
CHOGT34	ctgttatctctacacct
AIRSUSOUT	gtgtcagccctgaagaagc
DSIN	ttgtaagttagcccttg
CHOGT4	ttgccagctgtgcaacaag
1713REV	tccagcaacaacagcttcag
2056FWD	cagccattcactgttgctta
2153REV	ggaggactctgggaggttc
GARTUSOUT	gaagattcccctcgtgtcaga
CHOGT11	tggcctccatcagattctg
18US	gctggcctagacaaagcaga
14DS	gaatccagctttttctgct
GARTDSOUT	acgggtaagggtgaagtct
Site-Directed Mutagenesis Oligonucleotides	
Oligonucleotide	Sequence 5'-3'
AdeC_EtoK	GTGGTTGTTGGACCAAAGGCCCTCTAGCTG
AdeC_EtoK_R	CAGCTAGAGGGGCTTTGGTCCAACAACCAC
55-1_GtoD	GCTCACATTACTGGAGATGGACTGCTGGAGAAC
55-1_GtoD_R	GTTCTCCAGCAGTCCATCTCCAGTAATGTGAGC
Gly684toLys	GCCTTGGCTCACATTACTGGGAACGGGCTGCTGGAGAACATCC
Gly684toLys_R	GGATGTCTCCAGCAGCCGTTCCAGTAATGTGAGCCAAGGC
Gly684toPro	CTTGGCTCACATTACTGGGCTGGGCTGCTGGAGAACATC
Gly684toPro_R	GATGTTCTCCAGCAGCCAGGCCAGTAATGTGAGCCAAG

Table 2

Enzyme activities for steps catalyzed by GART gene products in CHO-K1, AdeC, and AdeG (55-1). Units are: GARS and GART, nmols mg⁻¹ min⁻¹; AIRS, pmols mg⁻¹ min⁻¹. ND = not detectable.

	CHO-K1	AdeC	AdeG (55-1)
GARS	82.4	ND (0)	53.6 (65)
GART	18.6	10.5 (56)	15.3 (82)
AIRS	10.3	7.5 (73)	ND (0)