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Report

Conditional *JAG1* Mutation Shows the Developing Heart Is More Sensitive Than Developing Liver to JAG1 Dosage

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Mutations of Jagged 1 (*JAG1*), a ligand in the Notch signaling pathway, cause Alagille syndrome (AGS). AGS is an autosomal dominant, multisystem disorder with variable expressivity, characterized by bile duct paucity and resultant liver disease in combination with cardiac, ocular, skeletal, and facial findings. *JAG1* mutations in AGS include gene deletions and protein truncating, splicing, and missense mutations, suggesting that haploinsufficiency is the mechanism of disease causation. With limited exceptions, there is no genotype-phenotype correlation. We have studied a *JAG1* missense mutation (JAG1-G274D) that was previously identified in 13 individuals from an extended family with cardiac defects of the type seen in patients with AGS (e.g., peripheral pulmonic stenosis and tetralogy of Fallot) in the absence of liver dysfunction. Our data indicate that this mutation is "leaky." Two populations of proteins are produced from this allele. One population is abnormally glycosylated and is retained intracellularly rather than being transported to the cell surface. A second population is normally glycosylated and is transported to the cell surface, where it is able to signal to the Notch receptor. The JAG1-G274D protein is temperature sensitive, with more abnormally glycosylated (and nonfunctional) molecules produced at higher temperatures. Carriers of this mutation therefore have >50% but <100% of the normal concentration of JAG1 molecules on the cell surface. The cardiac-specific phenotype associated with this mutation suggests that the developing heart is more sensitive than the developing liver to decreased dosage of JAG1.

Alagille syndrome (AGS [MIM 118450]) is an autosomal dominant disorder, characterized by bile duct paucity (a reduction in the number of bile ducts seen on liver biopsy) that leads to cholestasis (the obstruction of biliary flow) in association with other clinical abnormalities. These include cardiac, musculoskeletal, ocular, facial, and, less frequently, renal and neurodevelopmental abnormalities (Watson and Miller 1973; Alagille et al. 1975, 1987). Cardiac defects most often involve the pulmonary vasculature, but other structural defects can be seen (Silberbach et al. 1994; Emerick et al. 1999; Krantz et al. 1999). Other clinical findings include cleft-ing of the vertebral bodies (butterfly vertebrae) and posterior embryotoxon (a defect of the anterior chamber of the eye). Affected individuals may have characteristic facial features, including a broad forehead, widely spaced, deep-set eyes, and a pointed chin. The clinical findings in AGS can be highly variable, even within the same family (Shulman et al. 1984; Elmslie et al. 1995; Emerick et al. 1999).

AGS is caused by mutations in Jagged 1 (*JAG1* [MIM 601920]), a cell surface ligand for the Notch transmembrane receptors (Lindsell et al. 1995; Li et al. 1997; Oda et al. 1997). The evolutionarily conserved Notch signaling pathway functions to regulate cell fate decisions during development (Artavanis-Tsakonas et al. 1999; Kadesch et al. 2000). *JAG1* mutations in patients with AGS are distributed throughout the gene and include protein truncating, splicing, and missense mutations, as well as total gene deletions (Krantz et al. 1998; Crosnier et al. 1999; Spinner et al. 2001). Since the same phenotypic features can be present in individuals with total gene deletions, missense mutations, and protein-trun-

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cating mutations, we propose that haploinsufficiency for *JAG1* is one mechanism causing AGS.

Missense mutations have been found in patients with AGS, although the mechanism by which they act is not as clearly understood as that for gene deletions or truncating mutations. We previously studied four JAG1 missense mutations (L37S, R184H, P163L, and P871R) (Morrissette et al. 2001). JAG1-R184H has been identified in multiple patients with AGS; JAG1-L37S and JAG1-P163L have been reported in single patients with AGS; and P871R has been found in patients and unaffected individuals, suggesting it is a polymorphism (Spinner et al. 2001). Expression studies of JAG1-R184H and JAG1-L37S demonstrated loss of Notch signaling in two functional assays, whereas the other two mutations (P163L and P871R) appeared to have no effect on activity (Morrissette et al. 2001). On the basis of these data, we have hypothesized that P163L is a polymorphism and that its identification in a patient with AGS was fortuitous. Analysis of JAG1-R184H and -L37S protein products revealed incomplete N-linked glycosylation modifications. The mutant proteins were not found on the cell surface; instead, they were found to accumulate in the endoplasmic reticulum. These experiments demonstrated that the R184H and L37S mutations result in functional haploinsufficiency.

No genotype-phenotype correlation has been noted among the mutations identified in patients with AGS to date. Within families, the same JAG1 mutations have been found to be associated with very different clinical features, ranging from severe heart and liver disease to very mild findings in a single organ (Watson and Miller 1973; LaBreque et al. 1982; Alagille et al. 1987; Krantz et al. 1998). However, in a recent report, the first potential example of a *JAG1* mutation with a specific phenotypic consequence was identified (Eldadah et al. 2001). A *JAG1* missense mutation, JAG1-G274D, was found to segregate with a cardiac-specific phenotype. This mutation was found in 13 individuals within a multigeneration family with isolated cardiac disease. Although some of these individuals were thought to have facial features consistent with AGS, none of them had a history of liver disease. Of 11 individuals who were available for clinical study, 2 were affected with tetralogy of Fallot, 6 had peripheral pulmonic stenosis, and 1 had ventricular septal defect with aortic dextroposition. Two individuals had no overt cardiac abnormalities; however, one of the unaffected individuals was the mother of two children with cardiac disease (tetralogy of Fallot and pulmonic stenosis), suggesting she is an obligate carrier. The JAG1-G274D mutation changes a highly conserved glycine residue within the epidermal growth factor–like repeats and has not been seen in any of the $\geqslant$200 patients with AGS who were studied or in the $\geqslant$80 population control individuals (Li et al. 1997; Oda et al. 1997). The limited manifestations of this mutation led us to hypothesize that it might be different from the *JAG1* missense mutations reported in patients with AGS. In the present report, we describe the results of expression studies of the *JAG1* missense mutation JAG1-G274D.

To study the expression of the *JAG1* missense mutation JAG1-G274D, the mutant was cloned into the pBABE retroviral expression vector and was expressed in NIH-3T3 cells (Morgenstern and Land 1990). The JAG1-G274D mutant construct was made by site-directed mutagenesis, using wild-type JAG1 as a template, as described elsewhere (Morrissette et al. 2001). Wild-type JAG1 and JAG1 that contains missense mutations (L37S or G274D) were introduced into NIH-3T3 cells, using the retroviral expression vector, pBABE-puro, into which *JAG1* or JAG1 mutant was cloned. Western blot analysis of untreated lysates from the cells expressing JAG1 and JAG1 mutants, using a polyclonal antibody to the C-terminal region (H-114; Santa Cruz, Inc.), revealed that the JAG1-L37S mutant protein has a slightly lower mobility than wild-type JAG1. This difference was shown to be due to differences in posttranslational glycosylation (Morrissette et al. 2001). In contrast, the JAG1-G274D protein appeared as a doublet, comigrating with both the wild-type and JAG1-L37S bands, suggesting that more than one species of protein is present (fig. 1). To begin to analyze posttranslational modification of the JAG1-G274D protein, cell lysates were subjected to western blot analysis before and after treatment with endonuclease H (Endo H), which removes high-mannose or hybrid N-linked carbohydrate structures but cannot remove complex carbohydrates. Wild-type JAG1 is Endo H resistant, presumably as a result of its modification with complex carbohydrates. The JAG1 mutants R184H and L37S, identified in patients with AGS, have been shown to be Endo H sensitive. We hypothesize that they are not properly glycosylated and are not properly targeted to the Golgi for processing, and they are therefore retained intracellularly rather than being trafficked to the cell surface. (Morrissette et al. 2001).

Endo H treatment revealed different sensitivities for wild-type JAG1, JAG1-L37S, and JAG1-G274D. As discussed above, wild-type JAG1 was resistant to Endo H, and JAG1-L37S was sensitive to Endo H. JAG1-G274D demonstrated two bands after Endo H treatment, indicating both resistant and sensitive protein populations (fig. 1). We hypothesized that these two populations differ in conformation, resulting in the correct processing of a subset of the JAG1-G274D protein. The JAG1-G274D-Endo H–resistant population is fully glycosylated (with complex modifications, similar to wild-type JAG1) and is targeted to the cell membrane, whereas the sensitive fraction is incompletely glycosylated and does not appear on the cell surface.

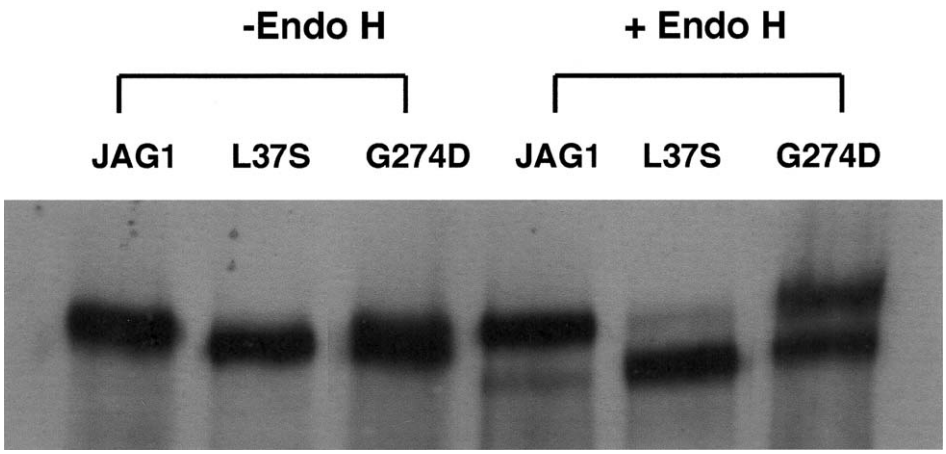

Figure 1 Western blot analysis of cell lysates containing the JAG1-G274D protein. Comparison of lysates before and after treatment with Endo H demonstrates two populations of proteins. NIH-3T3 cells expressing wild-type JAG1 or JAG1-L37S or JAG1-G274D *JAG1* mutants were grown at 37°C overnight. Cell lysates were run on a 7.5% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane as described in the text. Western analysis was performed on cell lysates, using H114 (a *JAG1* C-terminal antibody [Santa Cruz]) and were visualized by chemiluminescence. Cell lysates were untreated (−Endo H) or were treated (+Endo H) for 60 min, demonstrating partial sensitivity of JAG1-G274D to Endo H.

To determine if JAG1-G274D is localized to the cell surface, we treated NIH-3T3 cells that express wild-type or mutant JAG1 with the protease trypsin, such that only proteins on the cell surface would be degraded. When wild-type JAG1 was treated with trypsin, the full-length product decreased, and lower-molecular-weight bands, consistent with proteolytic products, appeared. Conversely, the mutant JAG1-L37S was insensitive to trypsin, consistent with its absence from the cell surface (fig. 2*A*). We hypothesized that the two JAG1-G274D protein populations differ in conformation, with only one of these populations reaching the cell surface. Therefore, this JAG1 protein would be partially resistant to trypsin. We further predicted that the JAG1-G274D protein would be more stable at lower temperatures, as has been demonstrated for other mutant proteins, such as cystic fibrosis transmembrane conductance regulator (CFTR) (Sharma et al. 2001). We assayed trypsin sensitivity of JAG1-G274D at two different temperatures (33°C and 39°C) (fig. 2*B*). JAG1-G274D was partially sensitive to trypsin at both temperatures, with some but not all of the protein cleaved on trypsin treatment. When cells expressing JAG1-G274D and grown at 33°C were analyzed by densitometry, the ratio of the two primary degradation products (marked with an asterisk in fig. 2*B*) to full-length JAG1 was 3.74. When cells were grown at 39°C, the ratio was 1.096. This is consistent with the appearance of more protein at the cell surface—and therefore with more degradation of JAG1-G274D—when cells were grown at the lower temperature (33°C). These results were also consistent with immunofluorescence experiments, in which the cellular localization of JAG1-G274D was studied after expression in NIH-3T3 cells (data not shown). These experiments demonstrated that JAG1-G274D was both intracellular and on the cell surface of cells grown at 39°C (data not shown), whereas cells grown at 33°C demonstrated more protein on the cell surface and transiting through the Golgi. No difference was observed in the subcellular localization of wild-type JAG1 at either temperature.

To examine the activity of the JAG1-G274D mutant, we assayed its ability to interact with Notch and to activate a downstream promoter at 32°C or 37°C. JAG1 activity was analyzed using a transcriptional reporter assay that detects Notch activation by JAG1. For these studies, we used a reporter plasmid (4XwtCBFLUC) that contained a luciferase construct cloned downstream of a Notch-sensitive promoter (Hsieh et al 1996). Wild-type JAG1 demonstrated a similar level of Notch activation at both 32°C and 37°C (fig. 3). JAG1-G274D demonstrated ~3.5-fold greater activity at 32°C (~59% of wild type), compared with 37°C (~17% of wild type), consistent with an increase in the stability of the mutant protein due to a temperature-sensitive conformational change ($P = .004$, by independent sample t test). The activity of the AGS-associated mutant protein JAG1-L37S, was slightly greater at 32°C, but this difference was not statistically significant ($P = .14$).

The results presented here suggest that the *JAG1* missense mutation JAG1-G274D is "leaky," in that both normal and abnormal protein species are produced. The mutant allele produces a conformationally sensitive protein existing in two forms. One form is correctly modified posttranslationally, reaches the cell surface, and is

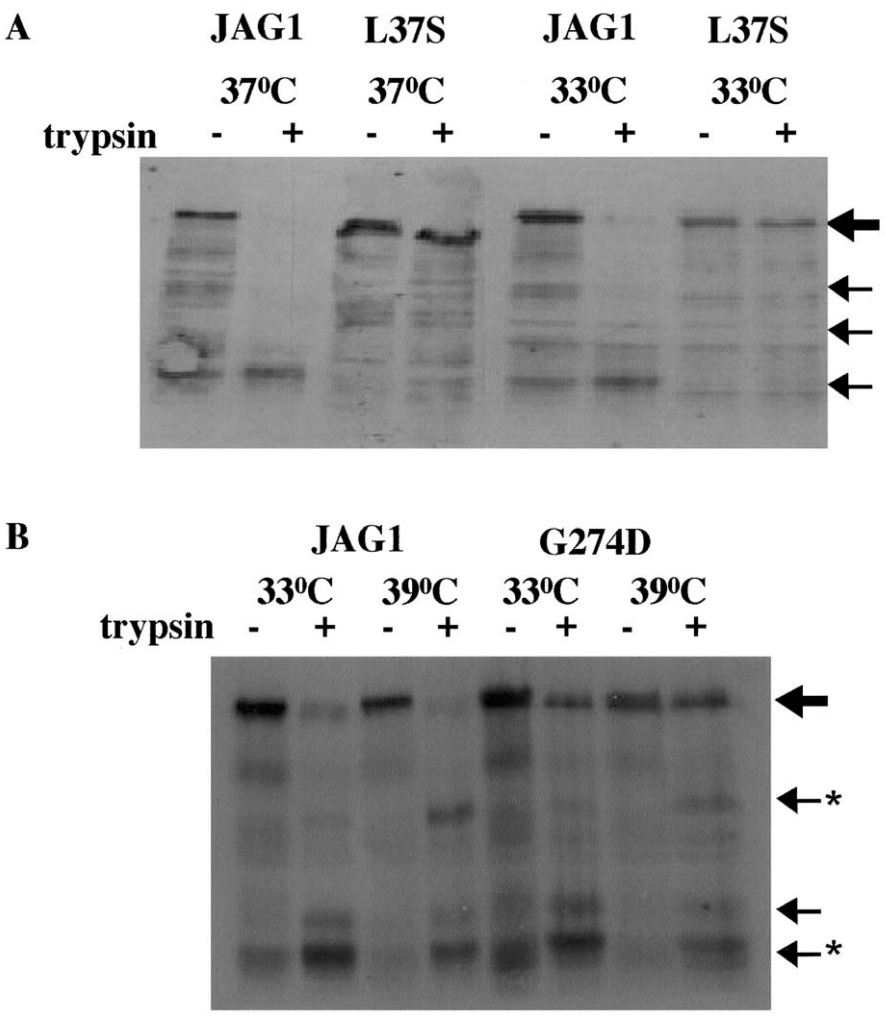

Figure 2 Sensitivity of wild-type and mutant JAG1 to trypsin. *A,* Wild-type JAG1. Wild-type protein, expressed in NIH-3T3 cells grown at 33°C or 37°C, is sensitive to mild trypsin treatment, consistent with its being expressed on the cell surface. JAG1-L37S expressed in NIH-3T3 cells grown at 33°C or 37°C is not sensitive to trypsin, consistent with its absence from the cell surface. Arrows indicate degradation products seen for wild-type JAG1. *B,* JAG1-G274D. Mutant protein, expressed in NIH-3T3 cells grown at 33°C or 37°C, is partially sensitive to trypsin, although there is increased degradation at 33°C, consistent with a higher proportion of protein being transported to the cell surface at this temperature. For all experiments, cells were exposed to trypsin for 0 or 10 min, after which the trypsin was inactivated. The cells were lysed in NP40 buffer and were analyzed by western blot. Asterisks indicate primary degradation products.

functionally normal. The second form is improperly modified, does not reach the cell surface, and is therefore not active. Temperature sensitivity has been demonstrated in other mutant protein molecules that require proper folding and posttranslational modification for targeting to the cell membrane (Michalovitz et al. 1990; Sharma et al. 2001).

This conditional mutant, JAG1-G274D, is associated with a cardiac-specific phenotype, providing the first example of a *JAG1* genotype-phenotype correlation. Haploinsufficiency for JAG1 is associated with the well-characterized (but variably expressed) phenotype of AGS. Previously studied *JAG1* missense mutations associated with the classic AGS phenotype displayed functional haploinsufficiency, in that they were unable to reach the cell surface, resulting in cell surface concentrations that were 50% of wild-type levels. In contrast, individuals with a JAG1-G274D allele have >50% but <100% of wild-type concentrations. Because the phenotype of the JAG1-G274D mutant appears to be cardiac specific—no individuals with this mutation have been found to have liver disease (Eldadah et al. 2001)—we hypothesize that the developing heart requires a higher dosage of JAG1 for normal development, compared with the liver. Alternatively, this mutation could involve differences in posttranslational processing of JAG1 during development of liver and cardiac cells.

Phenotypes have been shown to vary in many families

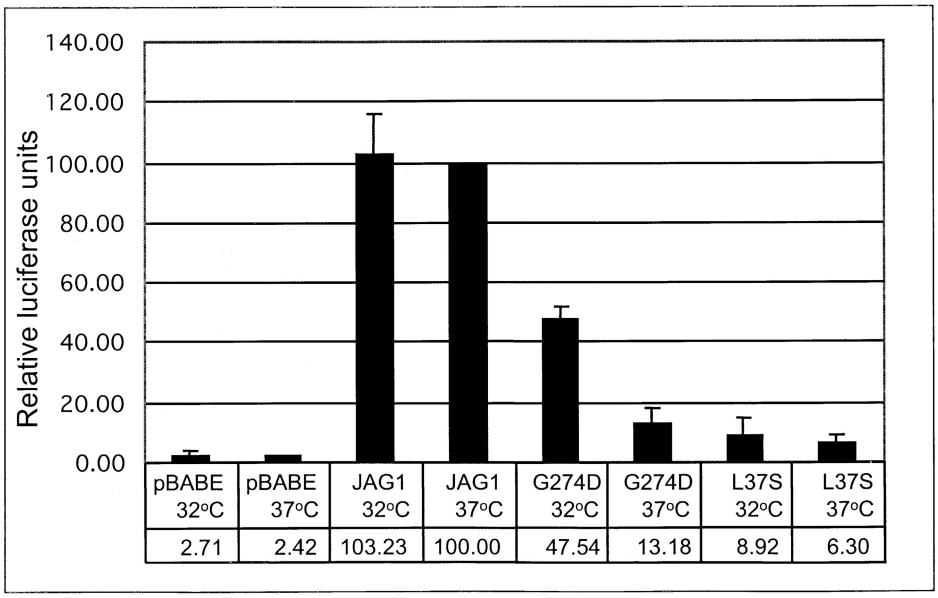

Figure 3 Temperature-dependent Notch activation by JAG1-G274D. NIH-3T3 cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Cells were transfected with the Notch-responsive plasmid 4XwtCBF1Luc. Transfected cells were seeded with NIH-3T3 cells expressing wild-type JAG1, JAG1-L37S, or JAG1-G274D and were incubated at either 32°C or 37°C for 72 h. Luciferase activity was measured, and transfections were normalized for the amount of total cellular protein (Biorad) and were repeated in triplicate. Transcription from 4XwtCBF1Luc was normalized to wild-type JAG1, having 100% activity at 37°C. The mutant JAG1-L37S had very low activity, with no significant difference between values at 32°C or 37°C ($P = .14$). JAG1-G274D demonstrates a threefold increase in Notch activation at 32°C compared with 37°C ($P = .004$), suggesting a conditional intermediate phenotype.

segregating *JAG1* mutations, suggesting the presence of modifying factors. On the basis of the work presented here, we hypothesize that polymorphisms in any of the proteins that modulate JAG1-induced Notch signaling might serve as modifiers of the AGS phenotype. JAG1 functions by binding to one of the Notch receptors, triggering a ligand-induced proteolytic cleavage of the receptor, with nuclear localization of the intracellular portion of the receptor. The Notch receptor fragment in the nucleus then binds to other proteins to direct transcription of Notch-responsive genes. Therefore, any of the many proteins involved in Notch cleavage, nuclear translocation, or Notch-mediated transcription might serve as potential modifiers of the AGS phenotype (Weinmaster 2000). Recent work has demonstrated that Notch2 is a modifier for AGS features in the mouse (McCright et al. 2002). Further studies will show whether Notch2 is a modifier of JAG1 mutations in patients with AGS or isolated cardiac disease, as was seen in the family with the JAG1-G274D mutation.

We have studied a single, unusual family with a cardiac-specific phenotype associated with a *JAG1* missense mutation. Although we recognize that it may be premature to draw conclusions from studies of a single mutation, we feel that the evidence is compelling that this mutation produces a higher proportion of functional JAG1 protein and that individuals with this mutation do not demonstrate liver disease, which is usually seen in patients with AGS. We therefore propose that the developing liver requires less JAG1 (>50% but <100% of normal levels) than the heart. Recently, another family was described that had a JAG1 missense mutation (C234Y) and congenital heart disease, hearing loss, and posterior embryotoxon (Le Caignec et al. 2002). Studies of this mutation are under way, and proof of our hypothesis will depend on whether other families are identified that have the same or similar mutations.

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Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AGS and JAG1)

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