Asparagine synthetase: regulation by cell stress and involvement in tumor biology

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Asparagine synthetase: regulation by cell stress and involvement in tumor biology. Am J Physiol Endocrinol Metab 304: E789–E799, 2013. First published February 12, 2013; doi:10.1152/ajpendo.00015.2013.—Asparagine synthetase (ASNS) catalyzes the conversion of aspartate and glutamine to asparagine and glutamate in an ATP-dependent reaction. The enzyme is ubiquitous in its organ distribution in mammals, but basal expression is relatively low in tissues other than the exocrine pancreas. Human ASNS activity is highly regulated in response to cell stress, primarily by increased transcription from a single gene located on chromosome 7. Among the genomic elements that control ASNS transcription is the C/EBP-ATF response element (CARE) within the promoter. Protein limitation or an imbalanced dietary amino acid composition activate the ASNS gene through the amino acid response (AAR), a process that is replicated in cell culture through limitation for any single essential amino acid. Endoplasmic reticulum stress also increases ASNS transcription through the PERK-eIF2-ATF4 arm of the unfolded protein response (UPR). Both the AAR and UPR lead to increased synthesis of ATF4, which binds to the CARE and induces ASNS transcription. Elevated expression of ASNS protein is associated with resistance to asparaginase therapy in childhood acute lymphoblastic leukemia and may be a predictive factor in drug sensitivity for certain solid tumors as well. Activation of the GCN2-eIF2-ATF4 signaling pathway, leading to increased ASNS expression appears to be a component of solid tumor adaptation to nutrient deprivation and/or hypoxia. Identifying the roles of ASNS in fetal development, tissue differentiation, and tumor growth may reveal that ASNS function extends beyond asparagine biosynthesis.

amino acid metabolism; asparaginase; ATF4; nutrient sensing; unfolded protein response

Asparagine Synthetase Enzyme Function

THE MAMMALIAN ASPARAGINE SYNTHETASE (ASNS) enzyme catalyzes the ATP-dependent conversion of l-aspartate and l-glutamine into l-asparagine and l-glutamate (reviewed in Ref. 73). The biochemical characterization of ASNS activity has been studied following purification from mammalian cells and recombinant systems of both bacterial and mammalian origin. Early investigation revealed that the reaction requires magnesium ions and ATP and involves formation of a β-aspartyladenylate intermediate (68). The β-aspartyladenylate intermediate is converted to l-asparagine by transfer of ammonia from l-glutamate to yield l-glutamate and AMP as the remaining products (74). There are two bacterial forms of ASNS, one that uses ammonia (AS-A) as the nitrogen donor and a second that uses glutamine as the nitrogen donor (AS-B) (73). The latter reaction is analogous to that performed by the mammalian enzyme, and structural studies on the Escherichia coli glutamine-dependent AS-B have revealed the presence of two distinct catalytic domains, an NH2-terminal amidotransferase domain and a COOH-terminal ATP-pyrophosphatase domain bridged by an intramolecular tunnel that allows for ammonia to shuttle between the two domains (56). Whereas the protein’s name directs focus on its function in asparagine synthesis, the reaction it catalyzes may impact the cellular levels of the other three reactants as well. Given the critical function of glutamine as an oxidizable energy source, a key interorgan nitrogen carrier, and a mammalian target of rapamycin (mTOR) regulator, the possible impact of ASNS activity should also be considered when evaluating glutamine homeostasis.

The level of ASNS expression among tissues in adult animals varies considerably. Based on a direct comparison of enzyme specific activity in many tissues, the pancreas was shown to exhibit much greater expression than any other tissue analyzed (63, 64). This distribution is consistent across many species, including humans, rodents, birds, and ox (63), and the prevalence of higher pancreatic ASNS expression has been confirmed at the protein level by immunoblotting using both polyclonal (47) and monoclonal antibodies (Fig. 1; R. Hutson and M. Kilberg, unpublished results). As illustrated by immunohistochemistry of human pancreatic tissue, pancreatic ASNS protein expression is largely associated with the exocrine cells (33). After fasting mice for 54 h or feeding them an asparagine-free diet for 10 days, their pancreatic ASNS activity was unaltered (64), in apparent contrast to the nutritional regulation of ASNS observed for other tissues, as discussed below. The pancreas does not release significant
amounts of asparagine into the circulation, and radioactive incorporation studies have suggested that the bulk of newly synthesized asparagine is used for protein synthesis (64). It is tempting to speculate that serum ASNS activity may be a valuable marker for pancreatic exocrine cell lysis, as Cooney et al. observed release of ASNS protein from murine primary tumors into the serum at a rate proportional to tumor growth (27).

The Mammalian Asparagine Synthetase Gene

The human ASNS gene, a schema of which is illustrated in Fig. 2A, was first assigned to chromosome 7 by analyses of somatic cell hybrids (9). Greco et al. (38) established that the ASNS locus is derived from the long arm of human chromosome 7, and in situ hybridization mapped the locus more precisely to chromosome region 7q21.3 (44). An ASNS cDNA was first cloned from Chinese hamster ovary (CHO) cells (71), and subsequently, human clones containing the complete ASNS coding sequence were obtained (6). Zhang et al. (100) determined that the human genomic sequence for ASNS was ~35 kb long and organized into 13 exons. Those authors assigned the transcriptional start site of ASNS to be 203 nucleotides (nt) upstream of the translation start site. Groco et al. discovered that a temperature-sensitive hamster gene that controlled cell cycle progression, called ts11, was ASNS and characterized the human ASNS counterpart to show that the first two exons were noncoding, with translation starting in the third exon (38, 39). Both the Andrulis and Basilico groups determined that the region surrounding the transcription start site of the human ASNS gene has a high CG content, a circumstance that we now recognize as a promoter-associated CG island (38, 100). Greco et al. (38) discovered that the human ASNS gene exhibited multiple transcription start sites, with one major site at 117 nt upstream of the translation start site. Chen et al. further characterized the multiple transcription initiation sites for the human ASNS gene during amino acid (AA) deprivation of HepG2 human hepatoma cells (24). Those investigators used both 5’ rapid amplification of cDNA ends and a ribonuclease protection assay to establish that multiple transcription initiation sites were spread across a 70 nt region for cells cultured in AA complete medium. However, following AA limitation, which induces ASNS transcription, there was a shift toward a predominant utilization of the transcription initiation site 117 nt upstream of the translation start site originally identified by Greco et al. This transcription start site will be used as nt +1 to designate all genomic sequences in this review.

Regulation of ASNS by Nutrient Limitation and Other Forms of Cell Stress

Protein or AA limitation: the AA response. Mammals exhibit a wide spectrum of adaptive processes that serve to sense and respond to fluctuations in the diet, including dietary protein or...
mRNA was induced by AA limitation of mammalian cells, not be reversed by asparagine supplementation, Basilico and cycle defect of cells harboring the ts11/ASNS mutation could GCN2 as the sensor of AA availability by monitoring the level nature. Those data are now understood to reflect the role of thesis mutant were transferred to the nonpermissive temper sensitive asparaginyl-, leucyl-, methionyl-, or lysyl-tRNA syn- bated in asparagine-free medium exhibit reduced aminoacyla- analysis, Arfin et al. (10) demonstrated that CHO cells incu- the transcription factors ATF4 and ATF5 (62, 91, 102). Activated GCN2 phosphorylates eIF2 on its 2-eukaryotic initiation factor 2-activating transcription factor 4) ap- translation rates, mRNA splicing, RNA export, RNA turnover, and translation initiation (reviewed in Ref. 52). Among the AAR pathways, GCN2-eIF2-ATF4 (general control nondepressible transcription factor) appears to be the predominant signaling mechanism that activates transcription from the ASNS gene during the AAR, as induction of ASNS expression is blocked in ATF4-deficient cells (78, 81, 87). GCN2 is a well-characterized cellular AA monitoring mechanism that is conserved from yeast to humans. The GCN2 protein has an inherent kinase activity that is increased when the protein binds any one of the uncharged tRNA molecules; thus, GCN2 has the ability to sense the level of all 20 of the AA typically associated with protein synthesis. Activated GCN2 phosphorylates eIF2 on its α-subunit, which suppresses general protein synthesis (reviewed in Refs. 45, 53, 95, 99), but promotes a paradoxical increase in translation of selected mRNA species. As discussed in more detail below, in mammalian cells this translational control mechanism includes the transcription factors ATF4 and ATF5 (62, 91, 102). Long before molecular probes were available for ASNS analysis, Arfin et al. (10) demonstrated that CHO cells incubated in asparagine-free medium exhibit reduced aminoacylation of asparaginyl-tRNA and an increased level of ASNS enzymatic activity. Likewise, Andrulis (7) showed that ASNS activity was elevated when cells containing a temperature-sensitive asparaginyl-, leucyl-, methionyl-, or lysyl-tRNA synthetase mutant were transferred to the nonpermissive temperature. Those data are now understood to reflect the role of GCN2 as the sensor of AA availability by monitoring the level of each of the uncharged tRNAs. After discovering that the cell cycle defect of cells harboring the ts11/ASNS mutation could be reversed by asparagine supplementation, Basilico and colleagues [Greco et al. (37)] were the first to show that ASNS mRNA was induced by AA limitation of mammalian cells, not only for asparagine but also for leucine, isoleucine, or glutamine. Hutson and Kilberg (48) extended those studies by reporting that ASNS mRNA level increased following limitation for all AA or for a single essential AA. Consistent with the earlier reports that asparagine levels regulated ASNS enzymatic activity (10), after AA limitation there is increased association of ASNS mRNA with polysomes (50) and increased ASNS protein production, as revealed by pulse-chase labeling (49).

Endoplasmic reticulum stress: the unfolded protein response. GCN2 is only one of four eIF2 kinases that mediate the response to a variety of cellular stresses (reviewed in Ref. 95). A second eIF2 kinase that impacts ASNS expression is the double-stranded RNA-activated protein kinase-like endoplasmic reticulum (ER) kinase (PERK) (Fig. 4). The PERK signaling pathway, together with the ATF6 and inositol-requiring enzyme (IRE)-1 pathways, make up what is referred to as the unfolded protein response (UPR), which is activated by cellular insults such as ER protein synthesis overload, oxidative stress, or other circumstances that perturb ER function. Like GCN2, activation of PERK leads to phosphorylation of eIF2, suppression of global translation, and increased ATF4 synthesis (42). Consequently, the ATF4-dependent downstream transcriptional programs for the AAR and UPR overlap. Thus, in many instances, either AA limitation or ER stress will lead to transcriptional activation of common ATF4-responsive target genes, including ASNS (14–16). However, there are also differences in the gene profile activated by GCN2 and PERK signaling, indicating that factors other than ATF4 add specificity to each pathway (29).

Genomic Sequences that Control ASNS Transcription During the AAR and UPR

C/EBP-ATF response element. After discovering that the ASNS gene was regulated by AA availability, the Basilico group analyzed the promoter region to identify the essential cis-regulatory elements (40). A region around nt −68 was indispen- sible for the response and was designated an “amino acid response element” (AARE). This was the first description of an AA-responsive genomic element in a mammalian cell. To further dissect the ASNS promoter, Barbosa-Tessmann et al. (15) used a combination of in vivo footprinting, deletion analysis, and site-directed mutagenesis to identify the specific nucleotide sequences that mediated the AAR-induced transcription (Fig. 2 B). Those authors determined that two distinct regions within the human ASNS promoter were both necessary for AAR-sensitivity, one from nt −68 to −60 with the sequence 5′-TGATGAAAC-3′ (initially called “nutrient-sensing response element 1”, NSRE1) was the same as that identified by Basilico and colleagues, and a second previously undetected site from nt −48 to −43 with the sequence 5′-GTACAA-3′ (NSRE2). The NSRE1 sequence is now referred to as a CCAAT/enhancer-binding protein (C/EBP)-ATF response element (CARE). As is the hallmark of enhancer elements, the NSRE1/NSRE2 “unit” functions independently of location or orientation, and is also functional when linked to heterologous promoters. However, Zhong et al. (101) showed that the 5′ to 3′ orientation of NSRE1 relative to NSRE2 is not reversible and that the 11 nt spacer region between the NSRE1 and NSRE2 sequences is required for enhanced transcription. The requirement for −10 bp between the NSRE1 and NSRE2 sug-
Fig. 3. Multiple signal pathways that make up the amino acid response (AAR) in mammals. The AAR is a collection of signaling pathways that result in an integrated transcriptional program. Whereas uncharged tRNA activation of the GCN2 kinase has been documented to be the AA sensor for the pathways leading to increased NF-κB activity (51) and ATF4 synthesis (42), the sensor that leads to MEK (90) and GPCR12 (22) activation has not been identified (indicated by dashed lines). AA transporters have been proposed as possible sensor molecules (89).
gests that it is functionally necessary for these sites to face the same side of the DNA helix.

Although the trans-acting factors that bind to the NSRE2 site have not been identified, some of those that bind to the NSRE1 sequence have been established, and they include several members of the ATF and C/EBP subfamilies of the basic-leucine zipper (bZIP) superfamily of transcription factors (81, 82). The ASNS NSRE1 sequence, composed of a half-site for the C/EBP family and a half-site for the ATF family of transcription factors, is just one example of a family of related sequences with the consensus sequence of 5'-TGATGXAAX-3' that are present in a wide spectrum of genes activated by the AAR and/or the UPR (52). This type of “C/EBP-ATF composite site” element was first identified by Wolfgang et al. within the C/EBP-homology protein (CHOP) gene (96), and the same laboratory showed that, following arsenite treatment, ATF4 binds to the element as an activator but is later displaced by ATF3, which represses transcription (35).

To continue the use of the “C/EBP-ATF” nomenclature, we refer to this family of sequences, including the ASNS NSRE1 site, as C/EBP-ATF response elements (CARE). Following the determination that ER stress, initiated by either carbohydrate deprivation or disruption of protein folding, leads to induction of ASNS transcription (14, 16), analysis of the promoter established that the same two elements that were responsible for the response to AA limitation, NSRE1 and NSRE2, were also necessary for the transcriptional response to the UPR (15). The sensitivity of the gene to limitation of either AA or glucose is the reason that those investigators coined the term NSRE. Although these genomic sequences exhibit “AARE activity”, their responsiveness to a range of other cellular stress signals is also clear.

The in vivo footprinting studies of the human ASNS gene also revealed constitutive protein binding to three GC boxes located upstream of the promoter-localized CARE (52). These sites are spread across the region covered by nt −153 to −93 and were discovered to be critical for maintenance of basal transcription (Fig. 2B), but were also required for a maximal response to AA deprivation. Upon further characterization of the three GC boxes, it was established that exogenous expression of Sp1 supported basal ASNS promoter activity only, whereas expression of Sp3 enhanced the basal activity and permitted AAR-induced ASNS transcription (57).

The network of transcription factors that control the ASNS gene. Chen et al. employed chromatin immunoprecipitation (ChIP) assays to show that within 30–45 min after initiating transcription (14, 16), analysis of the promoter established that the same two elements that were responsible for the response to AA limitation, NSRE1 and NSRE2, were also necessary for the transcriptional response to the UPR (15). The sensitivity of the gene to limitation of either AA or glucose is the reason that those investigators coined the term NSRE. Although these genomic sequences exhibit “AARE activity”, their responsiveness to a range of other cellular stress signals is also clear.

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AA limitation in HepG2 hepatoma cells, newly synthesized ATF4 rapidly translocated to the nucleus and was associated with the ASNS proximal promoter region (25). The amount of ATF4 binding continuously increased up to 4 h, coinciding with increased histone acetylation, recruitment of the general transcription machinery, and the peak of ASNS transcription. The authors observed that a subsequent decline in ASNS transcription between 4 and 24 h of continued AA limitation is mirrored by a gradual decline in ATF4 binding and a parallel increase in ATF3, C/EBPβ, and CHOP recruitment to the CARE site (25, 85). This auto-regulatory feedback cycle results from ATF4-dependent induction of the C/EBPβ, ATF3, and CHOP transcription followed by a subsequent recruitment of the corresponding proteins to the ASNS promoter (23, 66, 85). Subsequently, this self-limiting cycle for ATF4-dependent transcriptional activation was also observed for numerous CARE-containing genes (58, 66). While the transcriptional induction of other AA-responsive genes, such as CHOP, requires the histone acetyltransferase ATF2, ASNS exhibits less dependence on this ATF family member (18, 36). Therefore, although histone acetylation is an important component of the transcriptional activation of the ASNS gene following AA limitation, additional acetyltransferases appear to be involved (13, 25).

The ATF5 protein is homologous to ATF4 (3), and its synthesis is also subject to AA-dependent control through the GCN2-eIF2 translation mechanism (93, 102). Overexpression of ATF5 activates transcription driven by the ASNS promoter through the CARE site, and, like CHOP’s antagonism of ASNS induction by ATF4 (85), CHOP also counteracts ATF5 with regard to ASNS regulation (3). Interestingly, Al Sarraj et al. (3) showed that ATF4 was required to induce ATF5 expression and to support the ATF5-dependent transcription of ASNS. Although the ATF4 dependence for induction of ATF5 expression was confirmed by Su et al. (87), they did not observe a decrease in ASNS induction by AA limitation following ATF5 knockdown. Thus, the exact role of ATF5 in regulating the ASNS gene remains unresolved. However, an additional link between ASNS expression and ATF5 arises from the observation that ATF5 polymorphisms have been linked to an altered response to childhood acute lymphoblastic leukemia (ALL) therapy. During treatment for childhood ALL, patients are given asparaginase (ASNase), a component of combination chemotherapy (see below). ASNase therapy causes depletion of plasma asparagine followed by the loss of intracellular asparagine (26). Due to the lack of a rapid upregulation of ASNS protein content in ALL cells, they are preferentially sensitive to ASNase (11, 86). Rousseau et al. (77) observed that ALL patients with a T1562C polymorphism within the ATF5 gene have decreased event-free survival times after ASNase therapy. The T1562C polymorphism results in higher ATF5 promoter activity, and the investigators proposed that elevated ATF5-driven expression of ASNS within the leukemia cells causes decreased sensitivity to ASNase therapy.

Clinical Relevance of ASNS

ASNS and ASNase treatment of childhood ALL. Asparagine metabolism in transformed cells has received considerable attention after it was determined that certain types of tumors are susceptible to ASNase treatment (17). Primary ALL cells and many ALL cell lines exhibit a particularly low level of ASNS expression (12, 75) and are therefore unusually sensitive to asparagine depletion. Conversely, drug-selected ASNase-resistant ALL cell lines exhibit elevated expression of ASNS (11, 30), and overexpression of exogenous ASNS protein alone results in an ASNase-resistant phenotype in the absence of drug selection (11). Clinically, the relationship is less clear. In a survey of 173 patients, Holleman et al. observed a correlation between in vitro ASNase sensitivity and ASNS mRNA abundance measured by oligonucleotide arrays (46). Although ASNS was not one of the top genes associated with ASNase resistance, ASNS mRNA levels were about threefold higher in the resistant patients. However, several clinical studies have shown a lack of correlation between ASNS mRNA levels and ASNase sensitivity in ALL patients (8, 46, 55, 83, 84). One feature of all of these investigations was that ASNS mRNA was analyzed, rather than ASNS protein content or enzymatic activity. Su et al. (86) addressed this apparent conflict by demonstrating that ASNS protein level, not mRNA content, serves as a prognostic indicator of ASNase sensitivity in ALL cells. The molecular basis for this observation appears to be a significant time delay between upregulation of ASNS mRNA following ASNase treatment and the translation of that mRNA into ASNS protein. Interestingly, the correlation of ASNase sensitivity to ASNS protein, rather than mRNA, was later observed for several human ovarian tumor lines as well (59). The molecular mechanism for the delay between mRNA synthesis and protein expression has not been established, nor is there any published evidence for posttranslational modification of the ASNS protein.

One aspect of ASNase treatment that has not been fully explored is the effect of the drug on normal tissues, despite the fact that ASNase therapy is associated with toxicity. Bunpo et al. (20) demonstrated that ASNase treatment leads to the induction of the AAR in the liver, as illustrated by induction of hepatic ASNS and downregulation of mTOR. Using a Gcn2 knockout mouse model, the authors showed that the ASNase action was mediated through a Gcn2-dependent mechanism. In a subsequent study, they also documented that the Gcn2 dependence extended to the immunosuppressive action of ASNase on the thymus and spleen (19). The studies in Gcn2 knockout mice suggest that if there are humans with a deficiency in GCN2 activity they may be even more susceptible to ASNase toxicity. Collectively, these results provide mechanistic insight into the consequences of ASNase antitumor therapy on normal tissues and suggest that cotreatment with ASNase and GCN2-specific inhibitors may be even more effective than ASNase alone.

Methylation of the ASNS gene. A number of investigations have focused on a possible correlation between ASNase sensitivity and the DNA methylation status of the ASNS gene. Sugiyama et al. (88) proposed that the ASNS gene in asparagine-auxotrophic Jensen rat sarcoma cells was silenced by DNA hypermethylation because ASNS expression was detectable after 5-azacytidine (5-Aza-C) treatment, which leads to hypomethylation of the genome. Subsequently, several studies in asparagine-dependent and asparagine-independent cell lines have revealed a correlation between the DNA methylation within the ASNS gene locus and ASNS expression (5, 70, 97). In the context of human leukemic cell lines, Ren et al. (72) also observed that a high degree of ASNS promoter methylation correlated with lack of ASNS expression and that 5-Aza-dC treatment enhanced ASNS expression. Furthermore, by electrophoretic shift analysis with nuclear extracts from the leuke-
mia cells, the authors detected the presence of a “methyl-binding protein” that was bound to an oligonucleotide that contained a methylated CpG site near the ASNS CARE sequence. Interestingly, those authors observed that binding of ATF4 and several C/EBP members was significantly diminished when the methylated oligonucleotide was compared with the same sequence in the unmethylated state (72). They postulated that in vivo the methyl-binding protein might compete with transcriptional activators of ASNS expression, including ATF4. Ding et al. (31) cocultivated asparagine-dependent human leukemia cells with mouse peritoneal macrophages and observed subsequent demethylation of the ASNS promoter, enhanced ASNS expression, and acquisition of asparagine-independence. In ALL bone marrow samples, 74% of B cells and 83% of T cells displayed methylation of the ASNS promoter, in contrast with a lack of methylation observed in brain and breast tumors (2). On the basis of these studies, Akagi et al. (2) hypothesized that ASNS methylation may underlie the susceptibility of ALL cells to ASNase chemotherapy.

**ASNS and solid tumor growth.** In contrast to ALL, the potential relationship between ASNS expression and solid tumor initiation/promotion has not been as extensively studied. Koumenis and colleagues investigated the role of ATF4 in tumor cell survival and proliferation using an ATF4 shRNA knockdown strategy (98). They observed that, after ATF4 knockdown in HT1080 fibrosarcoma and DLD1 colorectal adenocarcinoma cells, survival was reduced in the absence of nonessential AA. The authors showed that reduced proliferative capacity and increased apoptosis were correlated with decreased ASNS expression in the ATF4-deficient cells. Supplementation of shATF4-expressing tumor cells with asparagine, but no other individual AA, reversed the increased apoptosis and autophagy, leading to increased cell survival. Ye et al. (98) sought to further define the mechanism of ASNS activation in transformed cells and discovered that GCN2 was activated in response to shATF4 knockdown, presumably due to lower ASNS expression and therefore, asparagine deficiency. Once again, supplementation of the cells with asparagine repressed the activation of GCN2. When the authors probed for the ability of transformed GCN2+/− and GCN2−/− cells to form tumors in a xenograft model, they observed that tumor growth was impeded in the GCN2−/− cells. As eIF2 is the sole known substrate for GCN2, the researchers concluded that activation of the GCN2-eIF2-ATF4 pathway 1) increases the survival and proliferative capabilities of tumor cells undergoing nutrient limitation, 2) is required for starvation-activated autophagy in transformed cells, and 3) induces ASNS as a key factor in tumor initiation and growth under AA limiting conditions (98). The exact role of ASNS and its product asparagine in modulating tumor growth are unknown. The most obvious explanation, protein synthesis, seems too simplified, as other AA synthesis pathways are not as highly regulated and other AA do not appear to play as critical a role.

Dufour et al. (33) used immunohistochemistry to screen 98 human pancreatic ductal carcinomas and determined that ASNS expression was low or below detection in about 70% of the patients. These results suggest that some pancreatic tumors may be susceptible to ASNase therapy, although clinical trials using ASNase were disappointing because of toxicity. Cui et al. (28) used expression array analysis to identify genes that were induced in pancreatic tumor cells exposed to low glucose, and one of those identified was ASNS. As mentioned above, ASNS transcription is induced via the UPR pathway in response to glucose deprivation (14–16). Cui et al. proposed that the function of ASNS upregulation in response to low glucose is to protect the pancreatic cancer cells from apoptosis, based on the observation that ASNS overexpression suppressed JUN NH2-terminal kinase (JNK) activation and reduced apoptosis, whereas ATF4 knockdown increased the susceptibility of the cells to apoptosis. In addition to greater tolerance for glucose limitation, pancreatic cancer cells overexpressing ASNS exhibited increased resistance to apoptosis induced by cis-diamine-dichloroplatinum (CDDP), a result also linked to the suppression of JNK activation by ASNS (28).

In addition to JNK, extracellular signal-regulated kinase (ERK) is activated by AA limitation and influences downstream signaling in the AAR (1, 22, 36, 65, 69, 90). Among the AAR-associated targets of JNK and ERK are ATF2 (22) and cJUN (36). The AP-1 family of transcription factors can form complexes composed of homo- or heterodimers of cJUN, JUN-B, JUN-D, cFOS, FOS-B, Fra1, and Fra2 (80), and of these, the cJUN, cFOS, JUN-B, and FOS-B genes are activated by the AAR in HepG2 human hepatoma cells, whereas those for JUN-D, Fra-1, and Fra-2 are not (36, 79). Fu et al. (36) showed that for some, but not all, cell lines from several human tissues, the relative induction of cJUN expression was greater in transformed cells compared than in nontransformed cells, independent of cell growth rate. Those authors also showed that overexpression of cJUN exhibited a concentration-dependent activation of both the basal and AAR- or ATF4-induced ASNS-driven transcription, whereas a dominant negative cJUN form suppressed the increased ASNS transcription. The results of Fu et al. also revealed that existing cJUN protein is phosphorylated through a cascade that involves both ERK and JNK, and subsequently, cJUN-ATF2 dimers induce transcription from the cJUN gene itself. Presumably, homo- or heterodimers containing cJUN then activate additional downstream genes. Given that cJUN promotes cell growth by increasing cyclin D expression (92), induction of cJUN by the AAR may contribute to tumor cell survival in the presence of a limited AA supply.

Lorenzi et al. (60) screened the NCI-60 human cancer cell panel using microarray assays and noted a negative correlation between ASNS mRNA expression and susceptibility to ASNase treatment in several ovarian cancer cells. They also observed increased ASNase sensitivity after siRNA knockdown of ASNS expression. In a second study, with a larger number of ovarian cell lines, the same group determined that, whereas the correlation between ASNase efficacy and ASNS mRNA expression was weak, there was a stronger correlation between ASNase efficacy and ASNS protein levels (59). As mentioned above, this result confirmed previous observations in human MOLT4 leukemia cells (86). These ovarian cell culture studies may lead to the use of ASNS as a biomarker in ovarian cancer screening (61).

**ASNS and tumor metastasis.** Metastasis is a complicated and incompletely understood process, but some evidence suggests that ASNS may be important in certain metastatic mechanisms. For cancer to spread from the primary tumor to distant sites, a cell or group of cells must detach from the primary tumor and enter the bloodstream, where they exist in suspension until they reach the metastatic site. Patrikainen et al. (67) mimicked this transition by adapting PC-3 prostate cancer cells from adherent to suspension culture and then examined changes in gene expression concomitant with suspension-adaption. They discovered that the ASNS expression was sixfold greater in the
suspension-adapted PC-3 cells than in the adherent cells. Subsequently, Ameri et al. (4) created orthotopic xenografts by using human MDA-MB-231 breast cancer cells in an established metastatic mouse model. The authors observed that the protein abundance for ATF4 and ATF3, as well as their target gene ASNS, were elevated in circulating tumor cells isolated from mouse blood compared with the parental MDA-MB-231 cell line. When returned to in vitro culture and exposed to hypoxia, the circulating tumor cells showed higher basal expression and greater induction of ATF4 and ASNS than the parental MDA-MB-231 cell line. Furthermore, the circulating tumor cells had an increased capacity for colony formation in a soft agar assay under hypoxic conditions and grew faster when reimplanted as xenografts. The authors speculated that circulating tumor cells may represent a subpopulation that is selected for its ability to survive insults such as hypoxia and nutrient deprivation as the result of upregulation of factors such as ATF4, ATF3, and ASNS. With specific regard to ASNS, its increased abundance in metastasizing cells suggests that ASNS activity is beneficial for cancer cell survival once they detach from the primary tumor and enter the bloodstream.

These studies documenting the upregulation of ATF4 and its target genes ATF3 and ASNS in metastasis reinforce studies revealing the importance of increased ASNS expression in tumor glycoprotein secretion with what appears to be much less ASNS synthesis and secretion, but the liver also has high rates of further exploration. The high ASNS content may simply be the in vivo impact during embryonic development or physiological circumstances that trigger each of these kinases are known, such as AA limitation for GCN2 and ER stress/hypoxia for PERK, others may yet to be discovered (54, 94). Second, while both PERK and GCN2 catalyze the phosphorylation of eIF2, which leads to ATF4 synthesis, the translational and transcriptional output for the two kinases differs, suggesting that additional modulating signals have yet to be discovered. (29).

Examples of the Many Remaining Questions

Although many facets of ASNS regulation and function have been characterized over the past several decades, there remain major gaps in our knowledge. The exact roles and physiological impact that this enzyme activity plays in maintaining homeostasis of two substrates and two products remain largely unexplored. For example, the name implies a focus on asparagynyl-tRNA in the regulation of asparagine synthetase in a mammalian cell line. (98) and PERK-ATF4 pathways, as well as their downstream target ASNS, in transformed cells. As illustrated in Fig. 4, activation of either eIF2 kinase GCN2 or PERK will lead to ATF4 production and the subsequent downstream transcriptional programs. However, two points are noteworthy and indicate that further investigation is required to fully understand the roles of these kinases in tumor biology. First, while some of the cell stress circumstances that trigger each of these kinases are known, such as AA limitation for GCN2 and ER stress/hypoxia for PERK, others may yet to be discovered (54, 94). Second, while both PERK and GCN2 catalyze the phosphorylation of eIF2, which leads to ATF4 synthesis, the translational and transcriptional output for the two kinases differs, suggesting that additional modulating signals have yet to be discovered. (29).

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Examples of the Many Remaining Questions

Although many facets of ASNS regulation and function have been characterized over the past several decades, there remain major gaps in our knowledge. The exact roles and physiological impact that this enzyme activity plays in maintaining homeostasis of two substrates and two products remain largely unexplored. For example, the name implies a focus on asparagine biosynthesis, but the activity could have significant effects on cellular glutamine content, especially during periods of long-term upregulation. Knockout animals have not been investigated to address the in vivo impact during embryonic development or physiological stress states during adulthood. The striking level of ASNS expression in the exocrine pancreas is an interesting opportunity for further exploration. The high ASNS content may simply be related to the need for asparagine availability for glycoprotein synthesis and secretion, but the liver also has high rates of glycoprotein secretion with what appears to be much less ASNS activity. The importance of increased ASNS expression in tumor proliferation in solid tumors and development of resistance to ASNase chemotherapy in leukemia illustrates that a better comprehension of ASNS regulation, as well as the AAR in general, is needed in transformed cells. Additional investigation of the impact of tumor-associated changes in ASNS regulation, such as the polymorphisms in the ATF5 gene, are likely to contribute to our understanding of the interesting links between aberrant ASNS expression and transformed cell growth that have been observed for many tumors.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: M.N.B., E.A.B., and M.S.K. conception and design of research; M.N.B., E.A.B., and M.S.K. drafted manuscript; M.N.B., E.A.B., and M.S.K. approved final version of manuscript; E.A.B. and M.S.K. prepared figures.

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