Differential Expression of S6K2 Dictates Tissue-Specific Requirement for S6K1 in Mediating Aberrant mTORC1 Signaling and Tumorigenesis

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Abstract

The S6K1 and S6K2 kinases are considered important mTOR signaling effectors, yet their contribution to tumorigenesis remains unclear. Aberrant mTOR activation is a frequent event in cancer that commonly results from heterozygous loss of PTEN. Here, we show for the first time a differential protein expression between S6K1 and S6K2 in both mouse and human tissues. Additionally, the inactivation of S6k1 in the context of Pten heterozygosity (Pten+/−) suggests a differential requirement for this protein across multiple tissues. This tissue specificity appears to be governed by the relative protein expression of S6k2. Accordingly, we find that deletion of S6k1 markedly impairs Pten+/−-mediated adrenal tumorigenesis, specifically due to low expression of S6k2. Concomitant observation of low S6K2 levels in the human adrenal gland supports the development of S6K1 inhibitors for treatment of PTEN loss-driven pheochromocytoma. Cancer Res; 71(10); 1–7. ©2011 AACR.

Introduction

Aberrant activation of the phosphatidylinositol-3 kinase (PI3K) pathway plays a key role in tumorigenesis through regulation of pivotal biological processes including proliferation, growth, survival, and migration (1). A major regulator of this pathway is the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene. PTEN negatively regulates the PI3K signaling cascade and is now established as one of the most frequently mutated, deleted, and silenced tumor suppressor genes in human cancer (2). We have previously established several faithful mouse models to elucidate the role of Pten in cancer (reviewed in ref. 3). Mice engineered to have only one allele of Pten (Pten+/−) mice die prematurely as a result of an autoimmune disorder characterized by massive lympho-splenomegaly (4) and are extremely susceptible to developing a wide spectrum of epithelial tumors (5).

Downstream of the PI3K/PTEN pathway, the mTOR complex 1 (mTORC1) has been shown to be an essential effector in promoting cell proliferation and susceptibility to cancer development (reviewed in ref. 6). mTORC1 becomes highly active in the absence of PTEN and promotes cell growth through phosphorylation of important regulators of protein translation including the well-characterized ribosomal protein S6 kinase (S6K) and the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1; reviewed in ref. 7). These 2 mTORC1 substrates represent 2 distinct signaling pathways downstream of mTORC1. Phosphorylation of 4EBP1 leads to its uncoupling from the eukaryotic initiation factor 4E (eIF4E), whereas activation of the S6K protein family results in the phosphorylation of additional downstream targets including the 40S ribosomal protein S6 (Rps6; ref. 8), eIF4B (9), eEF2 kinase (10), and the tumor suppressor PDCD4 (11). Release of eIF4E from 4EBP1 and the phosphorylation of Rps6 result in the upregulation and activation of the translation machinery (7).

Downstream of mTOR, the role of the 4EBP1/eIF4E branch of the pathway in tumorigenesis has been extensively studied both in vivo and in vitro (12, 13, and reviewed in ref. 14).

In contrast, the contribution of the S6K arm of the mTOR signaling cascade in oncogenesis has been less well investigated. The S6K family consists of 2 kinases, S6K1 and S6K2 (15–18). To date, both kinases are reported to be ubiquitously expressed, with mRNA expression detected in all mouse and human tissues examined (15–17). In addition, knockout mice for S6k1 or S6k2 alone show that there is redundancy between both genes, and it has also been suggested that inactivation of one may be compensated for through upregulation of the other (15, 19). This has made it difficult to properly understand...
the role of the S6K signaling arm in cancer. However, over-expression of both S6K1 and S6K2 have been reported in breast cancer (20), whereas a recent report showed that only S6K1 is required for insulinoma formation induced by expression of constitutively active Akt1 in the mouse pancreas (21). Thus, the tissue-specific requirement of both S6K1 and S6K2 in oncogenesis remains an open question, and one that is very relevant for the therapeutic targeting of this arm of the pathway.

Here we show that S6K1 genetic deletion can suppress phenotypes mediated by Pten haploinsufficiency in an exquisitely tissue-specific fashion. This tissue specificity appears to be dictated by the abundance of S6K2 protein levels. Indeed, we report for the first time that, in contrast to S6K1, S6K2 protein levels vary profoundly among different tissues.

Materials and Methods

S6k1 and Pten mice

S6k1−− and Pten+/− mice were previously generated (15, 22) and were crossed through several generations of breeding to ensure normalization of the background strains. All mouse work was carried out in accordance with our Institutional Animal Care and Use Committee approved protocol. For genotyping, tail DNA was subjected to PCR following the protocols previously described (15; 22).

Histopathology and immunohistochemistry

Mice were autopsied, and tissues were extracted and fixed in 10% neutral-buffered formalin (Sigma) overnight, subsequently washed once with PBS, transferred into 50% ethanol, and stored in 70% ethanol. After that, the tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) in accordance with standard procedures. Sections were stained with the following antibodies: B220 (BD Pharmingen); CD3 (Dako); synaptophysin (Dako); phospho-S6 (S235/S236; Cell Signaling Technology); Ki-67 (Novacasta); and S6K1 (Cell Signaling Technology; 49D7 Rabbit mAb).

Quantitative real-time PCR

Total RNA was prepared from mouse tissues using the Trizol method (Invitrogen). cDNA was obtained with iScriptTM cDNA Synthesis Kit (Bio-Rad). Taqman probes were obtained from Applied Biosystems. Amplifications were run in a 7900 Real-Time PCR System (Applied Biosystems). Each value was adjusted by using Hprt1 levels as reference.

Western blot analysis on murine tissues

Cell lysates were prepared with RIPA buffer [1× PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche)] and cleaned by centrifugation. The following antibodies were used for Western blot analysis: glyceraldehyde 3 phosphate dehydrogenase (GAPDH; 1:400 Cell Signaling Technology); S6K1 (Cell Signaling Technology; 49D7 Rabbit mAb); S6K2 [p70 S6 kinase β (C-19) Santa Cruz Biotechnology]; phospho-S6 (S235/S236; Cell Signaling Technology); total S6 (Cell Signaling Technology); and Pten (Cell Signaling Technology).

Human sample analysis

Human tissue lysates (Leinco Technologies) were probed with S6K1 (Cell Signaling Technology; 49D7 Rabbit mAb); S6K2 (Bethyl Laboratories); and GAPDH (1:410 Cell Signaling Technology). Human sections were analyzed by immunohistochemistry (IHC) with S6K2 antibody (Bethyl Laboratories).

Original H&E sections from 16 postdiagnostic cases of human pheochromocytoma were reviewed to verify the histologic diagnosis. S6K1 immunostaining (Cell Signaling Technology; 49D7 Rabbit mAb) was evaluated independently by 2 pathologists (M. Loda and G. Fedele) and scored as negative (0), weak (1), moderate (2), or strong (3). All human sections were collected following Institutional Review Board approval at the Brigham and Women’s Hospital.

Phylogenetic analysis

Multiple alignments of amino acid sequences were carried out with ClustalW (23). Amino acid sequences for human (Homo sapiens); RP56K (NP_003152.1); RP56K (NP_003943.2); cow (Bos taurus); RP56K (NP_991385.1); RP56K (XP_582478.4); rat (Rattus norvegicus); RP56K (NP_114191.1); RP56K (NP_00101962); mouse (Mus musculus); RP56K (NP_001107806); RP56K (NP_067460.1); rabbit (Oryctolagus cuniculus); RP56K (NP_001095160); chicken (Gallus gallus); RP56K (NP_001025982.1); zebrafish (Danio rerio); RP56K (NP_998241.1); frog (Xenopus laevis); RPS6Kb-1 (NP_001080935).

Results

S6k1 deletion impacts Pten heterozygous–driven phenotypes in a tissue-specific manner

To explore the contribution of the S6K1 arm of mTORC1 signaling to the phenotype dictated by Pten heterozygosity, we crossed Pten+/− mice with Rps6k1-null (S6k1−−) mice. In particular, we have focused on 4 specific genotypes for our study: wild type (wt), S6k1−−/− Pten+/−, and S6k1−−/− Pten+/− mice. Initially, these cohorts were evaluated for long-term survival. Surprisingly, genetic deletion of S6k1 had no effect on the lifespan of Pten+/− mice (Fig. 1A). We have previously reported that Pten+/− mice die from an autoimmune disorder, characterized by a severe lymphadenopathy affecting mainly the submandibular, axillary, and inguinal lymph nodes (4). Thus, we examined the S6k1−−/− Pten+/− mice to determine whether this was also the case for this population of mice. Indeed, the lymph nodes from Pten+/− and S6k1−−/− Pten+/− mice were found to be indistinguishable in composition, with lymph nodes from both genotypes exhibiting an expansion of B- and T-lymphocytes as characterized by IHC with the B-lymphocyte–specific marker B220 and the T-lymphocyte marker CD3 (Fig. 1B). Furthermore, lymph nodes from Pten+/− and S6k1−−/− Pten+/− mice showed no significant differences in positive staining for the cell proliferation marker Ki-67 (Fig. 1C, top). Thus, these data suggest that S6k1 deletion does not impact the increased lymph node proliferation triggered by Pten heterozygous loss. Additionally, staining of S6k1−−/− Pten+/− lymph nodes for phosphorylation of the
S6K1 downstream target Rps6 (phospho-S6) showed only a slight reduction in phosphorylation as compared with lymph nodes from Pten+/− mice (Fig. 1C, bottom). This observation was further confirmed by Western blot analysis and its quantification (Supplementary Fig. S1A).

Taken together, our data suggest that S6K1 deletion has little impact on the proliferative advantage conferred by Pten heterozygous loss, consistent with the comparable survival rates between Pten+/− and S6K1−/−:Pten−/− cohorts of mice. Furthermore, the modest decrease in phospho-S6 staining and equivalent proliferation in the lymph nodes suggests redundancy between S6K1 and S6K2, as previously reported for the S6K1-deficient mice (15).

To further characterize the S6K1−/−:Pten−/− mice we examined the impact of S6K1 genetic deletion in tumorigenesis driven by Pten heterozygous loss. Pten−/− mice are highly prone to develop spontaneous epithelial tumors in a variety of tissues (5). However, some of the tumors driven by Pten heterozygosity occur after a long latency and at incomplete penetrance. Thus, we focused on pheochromocytoma as it is the most penetrant phenotype at earlier time points in our genetic background (5). As we have previously reported, Pten−/− mice develop a marked expansion and proliferation of chromaffin cells in the medulla of the adrenal gland. This expansion is typical of pheochromocytoma, with 100% of Pten−/− mice showing this phenotype by...
9 months of age (5). Strikingly, the S6k1-/-;Pten+/− mice showed a dramatic reduction in the proliferation of the chromaffin cells as compared with Pten+/− mice (Fig. 1D, left). This is confirmed by staining the adrenal glands with both synaptophysin and chromogranin A, specific markers of the adrenal medulla (Fig. 1D, right and Supplementary Fig. S1B, respectively). Pathologic analysis of the S6k1-/-; Pten+/− mice at 8 to 12 months of age revealed a 20% incidence of phaeochromocytoma when compared with complete penetrance in age-matched Pten+/− mice (Fig. 1E). At 13 to 15 months of age the S6k1-/-; Pten+/− still showed a markedly lower penetrance of the tumor phenotype compared with Pten+/− mice (40% in S6k1-/-; Pten+/− versus 100% in Pten+/−; Fig. 1E). We also identified a profound suppression of proliferation as characterized by Ki-67 staining in the adrenal medullas of the S6k1-/-; Pten+/− versus those of the Pten+/− mice (Fig. 1F, top). In addition, deletion of S6k1 in the Pten+/− background resulted in a notable reduction in phospho-S6 in these adrenal glands (Fig. 1F, bottom and Fig. 2C), highlighting the relevance of the S6k1 arm of mTORC1 signaling in driving proliferation triggered by Pten heterozygous loss in this tissue. Thus, the marked impairment in Pten+/−-driven pheochromocytoma indicates a lack of redundancy between S6k1 an S6k2 in this tissue and, in conjunction with the contrasting results obtained in the lymph nodes, clearly shows that the genetic deletion of S6k1

Figure 2. S6K1 and S6K2 proteins are differentially expressed in mouse tissues. A, TaqMan RT-PCR analysis of the RNA from a panel of tissues from wt mice with S6k1- and S6k2-specific probes. Error bars show SD from 3 independent experiments. B, Western blot analysis from the same tissues showed in A. The asterisk indicates S6k2 found in the lymph node loaded as a control. C, Western blot analysis from the adrenal gland of 3 different 13-month-old mice of the indicated genotypes.
has a differential impact on specific phenotypes and in a tissue-specific fashion.

Interestingly, an analysis of the effect of S6k1 inactivation on uterine tumor formation in Pten+/− female mice, a less penetrant phenotype in this background (5), leads to a mildly reduced penetrance when compared with Pten+/− females alone (Supplementary Fig. S2). However, the reduced penetrance is not as striking as that observed for the adrenal glands. These data suggest that there may be incomplete redundancy between S6k1 and S6k2 in this tissue, which is in contrast to the complete redundancy observed with the lymph node phenotype of S6k1+/−:Pten+/− mice, and again supports a tissue-specific role for the S6k1 and S6k2 proteins.

Differential expression of S6k1 and S6k2 proteins in mouse tissues

To understand the molecular basis underlying the tissue-specific differential response to deletion of S6k1, we decided to analyze in depth the expression status of both S6k1 and S6k2 across a panel of tissues. Previously published data have reported the mRNA for S6k1 and S6k2 to be ubiquitously expressed in all tissues analyzed (15–17). Indeed, using TaqMan real-time PCR assays we confirmed the expression of mRNA for both genes in tissues obtained from wt mice, indicating that they are actively transcribed in all organs tested (Fig. 2A). Surprisingly however, Western blot analysis revealed a differential expression of the 2 proteins, with S6k2 levels found to vary profoundly (Fig. 2B and Supplementary Fig. S3). For example, comparing the amount of S6k2 protein between the lymph node and the adrenal gland shows strong differences in expression levels, whereas in contrast the S6k1 protein levels are comparable (Fig. 2B, left). In addition, it should be noted that S6k2 showed clear protein expression in the uterus. These data suggest the existence of a posttranscriptional mechanism that fine-tunes the levels of the S6k2 protein in a tissue-specific manner. Additionally, we carried out Western blot analysis on adrenal glands from wt, Pten+/−, and S6k1+/−:Pten+/− mice and observed that S6k2 protein levels did not increase in the S6k1+/−:Pten+/− glands (Fig. 2C).

Figure 3. S6K1 and S6K2 protein expression in human tissues. A, left, Western blot analysis on human tissues lysates. As a control we have used a lysate from MCF7 human cell line. Right, IHC with anti-S6K2 on normal human tissues. B, staining with anti-S6K on 16 cases of human pheochromocytoma. S6K1 immunostaining was scored as negative (0), weak (1), moderate (2), or strong (3). C, phylogenetic tree indicates the relationship and evolutionary descent of various species based on protein sequence of S6K1 and S6K2. Specifically, RPSKβ-1 refers to S6K1 protein and RPSKβ-2 to S6K2.
This finding indicates that S6k2 does not compensate for the loss of S6k1 expression in this tissue. Taken together, these data offer an explanation for why S6k1 deletion has a differential impact on the phenotypes dictated by Pten heterozygosity. The minimal effect of S6k1 loss on uterine tumor formation and the lack of an effect on lymph node proliferation may be accounted for through compensation and redundancy with S6k2, whereas S6k1 deletion has a pronounced effect on the pheochromocytoma incidence due to low protein expression of S6k2 in the adrenal gland.

Analysis of S6K1 and S6K2 protein expression in human tissues

Given the surprising finding that S6k2 protein levels were differentially expressed in the mouse, we sought to uncover if this was also the case for the human adrenal gland and kidney, the 2 mouse tissues that showed dramatically reduced S6k2 expression. It has been previously reported that both S6k1 and S6k2 are ubiquitously expressed in humans at the mRNA level (16). However, as we showed in the mouse, Western blot analysis of human adrenal gland revealed dramatically reduced S6k2 protein expression (Fig. 3A, left). In contrast, the human kidney tissue showed a relatively high S6k2 protein level, unlike our findings in mouse. This result was further confirmed by IHC (Fig. 3A, right). From these data it appears that not only S6k2 protein expression can vary dramatically between different tissues but also the tissue-specific pattern of expression can change between organisms. Although S6k2 protein levels were markedly different among the human tissues tested, we again found S6k1 protein expression to be more consistent (Fig. 3A). This finding further highlights the key role of S6k1 in signaling downstream of TSC1 in the human adrenal gland, as is the case for the mouse.

Given that our data in mouse indicate a pivotal role for S6k1 in the proliferation of the adrenal medulla, and the fact that the human adrenal gland expresses mainly S6k1, we decided to analyze the protein expression status of S6k1 in human pheochromocytoma. We carried out IHC analysis on a number of human pheochromocytoma cases. Staining of S6k1 overexpression was quantified using a scale going from negative (0), weak (1), moderate (2) to strong (ref. 3; Fig. 3B). The vast majority of pheochromocytoma cases expressed S6k1 at various levels (Fig. 3B), whereas the normal adjacent tissue scored as very weak (i.e., 0–1) for S6k1 expression (Supplementary Fig. S4). Although these data may be limited by a lack of additional information regarding the genetic determinants underlying these tumors, it is important to note that 25% (4 of 16) of the pheochromocytomas showed very high levels of S6k1 (Fig. 3B), suggesting that targeting of this kinase may represent a potential avenue for treatment of this tumor.

Discussion

Overall, our data are in agreement with a model whereby the response of Pten heterozygous phenotypes to the genetic deletion of S6k1 is tissue specific. This specificity may be dictated by the relative protein expression of S6k2. Importantly, we have shown that S6k2 protein levels vary dramatically between different tissues, in humans as well as in mice, whereas S6k1 is less susceptible to such extreme differences in protein expression. A phylogenetic analysis shows that S6k1 is the ancestral kinase (Fig. 3C) and that S6k2 is only found in mammalian organisms (15). This suggests that S6k2 may have arisen at a later time and underscores our observation regarding the protein expression level of S6k1 relative to that for S6k2 across multiple tissues, highlighting the importance of S6k1 for therapeutic targeting.

Specifically, our data constitute the rationale to develop an S6k1-specific inhibitor to target tumors triggered by loss of Pten in tissues having low expression of S6k2. The precise targeting of S6k1 in such tissues allows for inhibition of signaling downstream of mTORC1, while avoiding a general toxicity due to inhibition of both in normal tissues, as suggested by the perinatal lethality of the S6k1−/−;S6k2−/− mice. An excellent example for such an S6k1 targeted therapy is represented by the treatment of pheochromocytoma, as is clearly shown through our genetic inactivation of S6k1 in the adrenal gland of Pten+/− mice and our concomitant human data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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