How does prolyl hydroxylase-3 induce apoptosis in neuronal cells?

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ABSTRACT
Prolyl hydroxylase-3 (PHD3) is an important cellular oxygen-sensor that hydroxylates substrates in an oxygen-dependent manner. PHD3 activity is required during development for the induction of apoptosis in sympathoadrenal progenitor cells. Human PHD3 localizes to the nucleus and cytoplasm, while the highly conserved rat orthologue SM20 has been reported to localize to mitochondria. Given that protein localization is likely to determine the ability of PHD3 enzyme to hydroxylate specific substrate proteins, the aim of the project was to reevaluate the localization of both endogenous and transfected SM-20. Using Immunoblotting and immunofluorescence, it was found that despite presence of an N-terminal mitochondrial targeting sequence (MTS), transfected SM-20 localized to both nucleus and mitochondria. Human PHD3 lacks a MTS and localizes to nucleus and cytosol, but not to mitochondria. Localization analysis of endogenous SM20 in rat cells and tissue showed no clear evidence of mitochondrial localization. Interestingly, inclusion of the 5'untranslated region (5'UTR) in the SM-20 expression plasmid prevented the translation of the MTS, thus explaining the partial nuclear localization. It is hypothesized that the 5'UTR, via secondary structure in the mRNA, causes utilization of a second start codon downstream of the MTS, which corresponds to the start codon in the human PHD3.

INTRODUCTION
Pheochromocytoma is a cancer of the adrenal medulla, and these tumors comprise of chromaffin cells derived from the sympathetic neural progenitor cells (Lee et al., 2005). Appropriate removal of neuronal progenitor cells found in adrenal medulla, via apoptosis during development is important to prevent pheochromocytoma. In the absence of nerve growth factor, the cells undergo apoptosis that is dependent on c-Jun and its transcriptional target, PHD3 (Lee et al., 2005). PHD3, like its homologues PHD1 and PHD2, has the ability to hydroxylate HIF-1α in vitro. However, it has been shown that in vivo only the knockdown of PHD2, but not PHD3 or PHD1, causes an increase in HIF-1α (Berra et al., 2003). The physiological role of PHD3 is therefore not to regulate HIF-1α but may play an important role in regulating neural apoptosis. The human PHD3 orthologue was found in nucleus and cytoplasm (Metzen et al., 2003) whereas the rat orthologue was reported to be localized to mitochondria (Lipscomb et al., 2001). Interestingly, despite the differences in subcellular localization of human and rat PHD3, both have been reported to induce apoptosis in neural cells (Lipscomb et al., 1999). The subcellular localization of pro-apoptotic proteins is important for cell death. This is demonstrated in mitochondrial proteins such as cytochrome c, apoptosis inducing factor and endonuclease G.

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Given that both human PHD3 and the rat homologue SM-20 can induce apoptosis in neural cells, but appear to be localized to different intracellular compartments, the aim of this project was to reevaluate the localization of both endogenous and transfected forms of PHD3 and SM-20. A further goal was to characterize the mechanisms that govern the different localizations of human and rat orthologues of PHD3.

MATERIALS AND METHODS

Rat SM-20 cDNA was a gift from Dr. R. S. Freeman. Methods used in the experiments include cell culture and transfection techniques, immunoblotting, immunofluorescence and plasmid construct of SM-20-V5, 5'UTR-SM-20, 5'UTR-SM-20 ATG codon mutants, PHD3-HA and untagged PHD3.

RESULTS AND DISCUSSION

Transfected SM-20 is localised to both mitochondria and nucleus

To confirm that the rat SM-20 localizes to the mitochondria, SM-20 cDNA was PCR amplified and transfected into NIH-3T3 cells western blot analysis was carried out on fractionated nuclear and cytoplasmic fractions. Results showed that transfected SM-20 can translocate partially to the nucleus. Smaller V5-immunoreactive bands were also observed and may represent forms of SM20 after cleavage by the mitochondrial signal peptidase.

To confirm the unexpected nuclear localization, NIH-3T3 and HEK293 cells were transfected with SM-20-V5 and analyzed by immunofluorescence. Apart from mitochondrial localization, nuclear localization was also detected (Fig. 1).

We also determined the localization of transfected human PHD3-HA. Localization to both nucleus and cytosol, where it showed granular staining, is consistent with previous reports (Metzen et al., 2003). However, there was no mitochondrial staining, consistent with the absence of a MTS human PHD3.

![Figure 1: Immunofluorescence of transfected SM20-V5 in NIH-3T3 and HEK293.](image-url)
Localization of endogenous SM-20/PHD3 in cultured cells

Endogenous localization of rat SM-20 and mouse PHD3 proteins were investigated in PC12 and NIH-3T3 cells with PHD3 antibody (Novus Biological). Immunofluorescence results showed that in PC12, SM20 is localized to both nucleus and cytoplasm under normoxic conditions, with translocation to the nucleus under hypoxia (Fig. 2). Immunofluorescent staining of mouse PHD3 in NIH-3T3 showed similar results.

A 27kDa band was observed in human MCF7 and HEK293 cells western blot experiments. In rat PC12 cells, no protein of 39kDa was seen. However, the band of approximately 27kDa may correspond to the cleaved form of SM-20. All lysates showed multiple additional bands of higher molecular weight, which could be due to non-specific bands detected by the PHD3 antibody (Novus Biological) or modified forms of PHD3 (e.g. ubiquitinated PHD3).

PHD3-HA was transfected into HEK293 cells to determine whether the presence of multiple bands was due to modified forms of PHD3. Results showed a single 27kDa band. Addition of MG132, a proteasome inhibitor that prevents the degradation of ubiquitinator proteins did not lead to appearance of multiple bands. This suggests that the upper bands were not due to ubiquinated form of PHD3.

Confirmation of specificity of the PHD3 antibody was done by creating an untagged PHD3 plasmid. Results showed that there was increased abundance with increasing PHD3 plasmid concentration at 27kDa, indicating that the antibody can detect PHD3 proteins.

To determine specificities of the PHD3 antibody for bands corresponding to endogenous protein, siRNA experiments were conducted. However, none was able to reduce the PHD3 signal in MCF7 cells.

**Figure 2:** Immunofluorescence of endogenous PHD3 in PC12 cells under normoxic and hypoxic conditions

Localization of endogenous SM-20/PHD3 in mouse and rat liver tissue

Endogenous localization of PHD3 and SM-20 was also examined in mouse and rat liver tissue. Liver tissue was subfractionated into mitochondria, cytoplasm and nuclei fractions. Western blot analysis of mouse liver fractionation showed that there was nuclear localization
corresponding to 27kDa. Aviva System Biology and Santa Cruz Biotechnology PHD3 antibodies were used, in addition to the Novus Biological PHD3 antibody. However, multiple bands were also present, questioning the specificity of the antibody. In addition, the higher molecular weight bands detected with the various antibodies did not correspond to each other, suggesting that they are non-specific.

The 5′UTR of SM20 regulates the expression of mitochondrial targeting sequence

The 5′UTR can contain regulatory sequences which via secondary structures promote or prevent the initiation of translation. To test this hypothesis, we generated a SM-20 expression plasmid containing N-terminal 5′UTR sequence to test the size of the expressed protein by Western blot. Results showed that only a band of 25kDa was detected, without the 39kDa form. These data suggest that the presence of the 5′UTR prevents the translation of the MTS and therefore, the 39kDa protein was not seen.

Some proteins, such as pVHL, has internal translation from the second Methionine. Thus, we hypothesize that translation of SM-20 is at least in part initiated at the second start codon. Two mutants that have the start codons mutated were generated. The first start codon ATG was mutated to Threonine and the second to Alanine. These mutants are currently tested for the intracellular localization.

The experiments to determine the localization of endogenous PHD3 and SM20 proteins were complicated by the numerous non-specific bands detected with the various antibodies. In future studies, further experiments to validate these PHD3 antibodies will be necessary. This is as immunofluorescence results may not be reliable. In addition, western blot analysis is not conclusive as it is difficult to evaluate which bands are PHD3 and which bands are non-specific binding.

The physiological significance of SM20 nuclear translocation in hypoxia and mitochondrial versus nuclear localization is currently not known. Thus, it will be important to identify target proteins of PHD3/SM20 which mediate the proapoptotic effects of the enzyme. This is expected to give further insight into how subcellular localization of PHD3/SM20 can affect its ability to induce apoptosis in neural cells.

REFERENCES


