Apr 17th, 11:00 AM - 12:00 PM

Effect of PAG1 on Src-Family Kinase Trafficking in Neuroblastoma Exosomes

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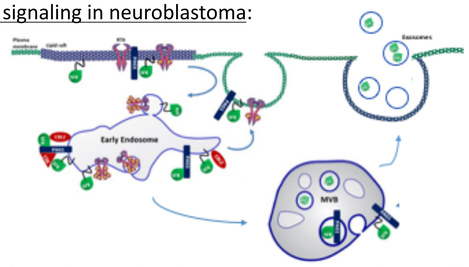
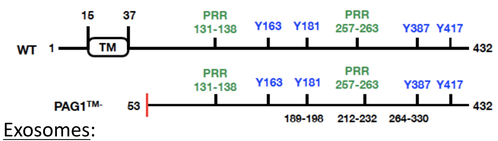
Question: Is PAG1 important for trafficking of SFKs into exosomes?

Background

SFK signaling in neuroblastoma:



SRC-family kinases (SFKs) are important in regulating cell-fate in neuroblastoma cells. PAG1 binds SFKs and helps regulate their activity by directing their movement within the cell. To better understand the role of PAG1, we created a line of mutant SH-SY5Y cells, called PAG1^{TM-} cells, which express a truncated PAG1 protein that lacks the transmembrane domain. These cells still express high levels of mutant, cytosolic PAG1. We have found that PAG1^{TM-} cells exhibit a hyper-cancerous phenotype and have higher global levels of active phosphorylated SFKs (pSFKs). The role of pSFKs in PAG1^{TM-} cells is still unclear. Protease protection experiments suggest that PAG1^{TM-} cells may accumulate more pSFKs in multivesicular bodies (MVBs), however fractionation experiments have indicated that pSFKs primarily localize to early endosome and cytosol fractions.



Exosomes:

Exosomes are small extracellular vesicles (50-100nm in diameter) that are released by fusion of MVBs with the plasma membrane. Neuroblastoma cells, including SH-SY5Y cells, have been shown to release exosomes containing SFKs, including FYN and LYN. To determine if PAG1 is important for trafficking of SFKs into exosomes, I isolated exosomes from both wild-type (WT) and PAG1^{TM-} SH-SY5Y cells to compare levels of SFKs.

Methods

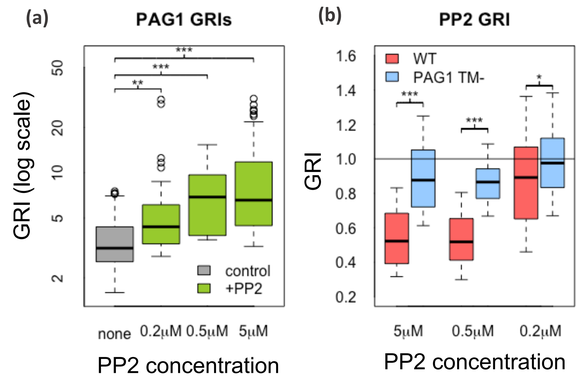
Proliferation assays:
Five MTT assays were carried out with cells grown for either 2 or 3 days in different concentrations of PP2 (an SFK inhibitor). Growth rate indices (GRIs) were calculated for each assay individually with the formula $GRI = 2^{R'/R} - 1$, where R' and R represent the number of doublings per day for experiment and control conditions respectively.

Exosome Isolation by ultracentrifugation:
Conditioned media was collected from cells after incubation for 24 hours. Two spins were done to remove cells, and larger cell fragments: one at 2,000 x g for 20 minutes, and a second at 5,000 x g for 60 minutes. Exosomes were pelleted from the supernatant by spinning at 40,000rpm for 90 minutes, then dissolved in Urea buffer containing SDS. Digested exosomes were analyzed by Western blotting.

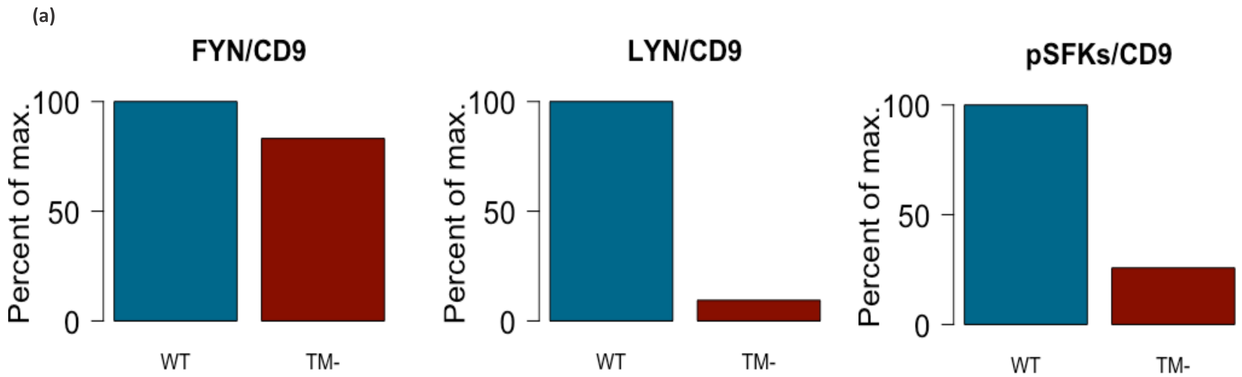
Cloning and sequencing of LAN6 cells:
LAN6 cells were previously transfected with CRISPR plasmids targeting exon 5 of the PAG1 gene. Transfected cells were then cloned by limiting dilution. The targeted region of exon 5 was amplified using PCR prior to sequencing.

Proliferation of PAG1^{TM-} cells

Increased growth rate in PAG1^{TM-} cells and decreased sensitivity to PP2. PP2 was used at three different concentrations to inhibit SFKs. Growth rates were calculated as the number of doublings per day and compared by calculating the growth rate index (GRI), a normalized ratio (see methods for formula). Boxplots show the interquartile range of GRIs comparing PAG1^{TM-} cells to WT cells (**a**) or of PP2-treated to control cells (**b**) for each concentration of PP2. *P<0.05, **P<0.005, ***P<0.0005. Student's t test was also calculated for pooled growth rates of WT vs. PAG1^{TM-} cells. All P-values were less than 0.05.

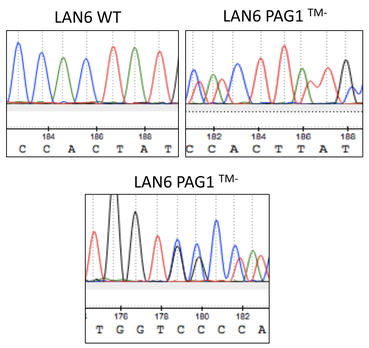


SFKs in exosomes of SH-SY5Y cells



Western blot results of exosomes: Exosomes were isolated from WT and PAG1^{TM-} cells and probed for FYN, LYN, pSFK and the exosomal marker CD9 (also known as Tetraspanin-29) in a Western blot. To determine the relative amount of SFKs expressed in exosomes, the SFK signal was divided by the CD9 signal for each sample. The SFK to CD9 ratio is shown as a percentage of the maximum between WT and PAG1^{TM-} exosomes (a). Images of the experiment are shown in (b).

LAN6 PAG1^{TM-} cells



Allele 1- T insertion
Wild-type:
126 GGAAAAGAAGCCGCGACAGCATAGTGGGGACCATGAGAACCTGATGAACGTG 177
--E--K--K--P--R--Q--H--S--G--D--H--E--N--L--M--N--V-
Clone 14:
126 GGAAAAGAAGCCGCGACAGCATA**A**GTGGGGACCA**TGA** 170
--E--K--K--P--R--Q--H--K--W--G--P-**stop**

Allele 2- Frameshift deletion
Wild-type:
126 GGAAAAGAAGCCGCGACAGCATAGTGGGGACCATGA 161
Clone 14:
126 GGAAAAGAAGCCCCA**TGA** 155
-E--K--K--P--P-**stop**

PAG1^{TM-} LAN6 mutants: . LAN6 cells, another neuroblastoma cell-line, have a slower growing and less differentiated phenotype compared to SH-SY5Y cells. The same PAG1^{TM-} mutation was attempted in LAN6 cells. Several clones were isolated and sequenced. Clones 14 and 32 were identified as heterozygous mutants, with mutations in both copies of exon 5. Sequencing results for clone 14 are shown above. Unfortunately, we have been unable to verify a mutant phenotype due to difficulty detecting the protein.

Results

- PAG1^{TM-} cells proliferate faster than WT cells.
- Proliferation in PAG1^{TM-} cells is less sensitive to SFK inhibition by PP2 than proliferation in WT cells.
- PAG1^{TM-} mutation caused decreased levels of total pSFKs and LYN incorporated into exosomes, but had minimal effect on FYN in SH-SY5Y cells.

Conclusions

- PAG1^{TM-} mutation increases proliferation in SH-SY5Y cells.
- Increased proliferation in PAG1^{TM-} cells is not affected by concentrations of SFK inhibitors that inhibit proliferation in WT cells.
- Membrane-bound PAG1 is required for incorporation of pSFKs and LYN into exosomes.

Questions for future directions

- Are SFKs effectively inhibited by PP2 in PAG1^{TM-} cells?
- Are increased levels of pSFKs important for proliferation in PAG1^{TM-} cells?
- Does PAG1^{TM-} bind SFKs in the cytosol and interfere with targeting of SFKs?
- Does PAG1^{TM-} prevent pSFKs from reaching MVBs, resulting in diminished release of pSFKs in exosomes?
- Alternatively, are pSFKS in MVBs recycled back to the cytosol or degraded in lysosomes?
- Is PAG1 exported into exosomes along with SFKs in WT cells?
- Is PAG1 required to escort SFKs into exosomes?