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TITLE: Characterization of the PKB/AKT Pathway in the Role of Tumorigenesis and Transformation of MCF10A Human Mammary Epithelial Cells

PRINCIPAL INVESTIGATOR: Scott L. Ackler

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057

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6. AUTHOR(S)
Scott L. Ackler

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University Medical Center
Washington, DC 20057

E-Mail: acklers@gusun.georgetown.edu

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13. ABSTRACT (Maximum 200 Words)
The Akt homology domain (AH) of Akt has been identified as a potential dimerization site within the kinase, and potentially could serve as a selective inhibitor of these kinases. We fused the AH domains of Akt1 (AH1) and Akt2 (AH2) to EGFP and performed expression studies with these constructs. Overexpression of both in PTEN deficient U87 cells sensitized these cells to apoptosis from camptothecin treatment, while neither was able to do this in PTEN+ MCF-7 cells. AH2 was able to block overexpressed Akt1 and endogenous Akts inhibition of AFX as measured by an AFX reporter assay, but not when Akt2 was overexpressed. AFX disinhibition in U87 and MCF-7 cells patterned their effects in the cytotoxicity assay. Finally, we were able to demonstrate that AH2 bound to Akt1 and Akt2, but AH1 only bound to Akt1 using co-immunoprecipitation assays; both bound to PDK1. Binding of AH domains to Akt decreased phosphorylation within the activation loop of Akts. We hypothesize that the AH2 domain binds to the AH domain within Akts, and this blocks access either to 3'OH-phosphoinositides or PDK1, and thereby inhibits Akt activation. We further hypothesize that PTEN deficient cells are more susceptible to Akt inhibition PTEN expressing cells.

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KEY ACCOMPLISHMENTS:

- As of the prior report, we generated the Akt homology domain of mouse Akt2 (AH2) by PCR and cloned this fragment into pEGFP-C1. Expression of EGFP-AH2 protein was verified by western blotting. Comparison of the sequences of AH1 and AH2 is shown (Fig 1).

- EGFP-AH1 and -AH2 domains were overexpressed in PTEN deficient U87 glioblastoma cells and MCF-7 breast carcinoma cells with wild type PTEN. Cells were then treated with varying doses of camptothecin, and apoptosis quantified by counting pyknotic nuclei following Hoechst staining. The PTEN deficient U87 cells were sensitized to camptothecin, with a log IC50 shift of 0.5 units (Fig 2). MCF-7 cells, however, showed higher variability in there response to camptothecin, and neither AH domain was capable of significantly altering the response of these cells to camptothecin.

- A luciferase system was used to examine downstream markers of Akt activity in transfected 293T cells. AFX wild type (AFXwt) or a triple mutant of AFX which cannot be regulated (AFXmut) were combined with a Forkhead response element (FKHRE) driven luciferase construct. AFXmut dramatically increased luciferase production in 293T cells, while AFXwt increases were more moderate (Fig 3A). Overexpression of Akt1 or Akt2 lowered AFXwt transactivation of the FKHRE (Fig 3B,C). Addition of AH2 significantly increased AFXwt transactivation activity in the presence of endogenous Akts (Fig 3D) and Akt1 (Fig 3B), but not

Akt2 (Fig 3C). AH1 had little effect in this assay. Increases in luciferase by AH2 were not as high as AFXmut, suggesting that other pathways are involved in regulating AFX that are not inhibited by AH2.

- Using this same assay system, AFXmut was modestly able to increase luciferase activity in both U87 and MCF-7 (Fig 4A,C), while AFXwt had little effect. Addition of AH2 increased luciferase activity to near AFXmut levels in U87, while AH1 had little effect (Fig 4B). The effect of both AH domains in MCF-7 was highly variable and inconsistent, which was in agreement with the camptothecin data (Fig 4D).

- Co-immunoprecipitations were performed to demonstrate direct interaction between Akts and AH domains in 293T cells. Akt1 or Akt2, or empty vector, were co-expressed in 293T cells with either pEGFP, -AH1 or -AH2. Akt was precipitated using a pan-Akt antibody recognizing the C-terminus of Akt, and precipitated proteins were analyzed by Western blotting. Interaction with Akt1 and Akt2 was seen with AH2, while AH1 was only co-precipitated with Akt1. There was no evidence of EGFP alone precipitating with Akts (Fig 5). Furthermore, phosphorylation of Akt at Thr308 (or Thr309) was significantly reduced by GFP-AH2, and to a lesser extent GFP-AH1. Phosphorylation of Ser473 was either unchanged or slightly enhanced (Fig 6).

- In similar co-immunoprecipitation experiments, myc-tagged PDK1 was co-expressed with either pEGFP, -AH1 or -AH2. Both AH domains demonstrated interaction with PDK1, while EGFP did not (Fig 7). Inhibition of Akts was not due to a competitive inhibition of phosphoinositides by the ensconced PH domains, or inhibition of PDK1, since phosphorylation of p70S6 kinase (downstream of PI-3 kinase/PDK1 but not of Akt) was not altered (data not shown).

- We hypothesize that the EGFP-AH proteins bind directly to the PH domain of Akts. This binding inhibits the activation of Akt by preventing the phosphorylation of the activation loop of Akt, presumably by sterically hindering access to either PDK1 or to membrane 3'OH-phosphoinositides. The effect of overexpressing AH domains to sensitize cancer cells appears to be PTEN specific; future experiments are planned with PTEN null and wild type cell lines to test this hypothesis. This method of targeting Akt appears to be specific for Akts, and may provide an adequate method for selectively targeting this pathway in cancer therapy.

REPORTABLE OUTCOMES

- Ackler S, Kristipati ST and Glazer RI. **Examination of AKT Dimerization and its Viability for Drug Development**. Abstract, Era of Hope 2003.

- Expected to earn Doctorate of Philosophy in Pharmacology, Fall 2003 from Georgetown University, Department of Pharmacology.

- Received position of Senior Research Pharmacologist, Cancer Therapeutics Group, Abbott Labs, Chicago, IL. Currently working at Abbott Labs while completing writing for Thesis Defense.

APPENDIX

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       1
AH1:   MSDVA IVKEG WLHKR GEYIK TWRPR YFLLK NDGTF IGYKE
AH2:   MNEVS VIKEG WLHKR GEYIK TWRPR YFLLK SDGSF IGYKE
        -  -                                 -
      41
AH1:   RPQDV DQREA PLNNF SVAQC QLMKT ERPRP NTFII RCLQW
AH2:   RPEAP DQTLP PLNNF SVAEC QLMKT ERPRP NTFVI RCLQW
         ---   ---          -
      81
AH1:   TTVIE RTFHV ETPEE REEWT TAIQT VADGL KKQEE EEMDF
AH2:   TTVIE RTFHV DSPDE REEWM RAIQM VANSL KQRGP GEDAM
                             - -   -   --     -- - ---
     121
AH1:   RS--G SPSDN SGAEE MEVSL AKPKH RVTMN
AH2:   DYKCG SPSDS STSEM MEVAV NKARA KVTMN
       --        -  -- -    -  - - -
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Fig. 1. Comparison of AH1 and AH2 sequences. The PH domain of each is in bold, while the DiD domain is in normal script. Significant amino acid sequence differences are underlined below.

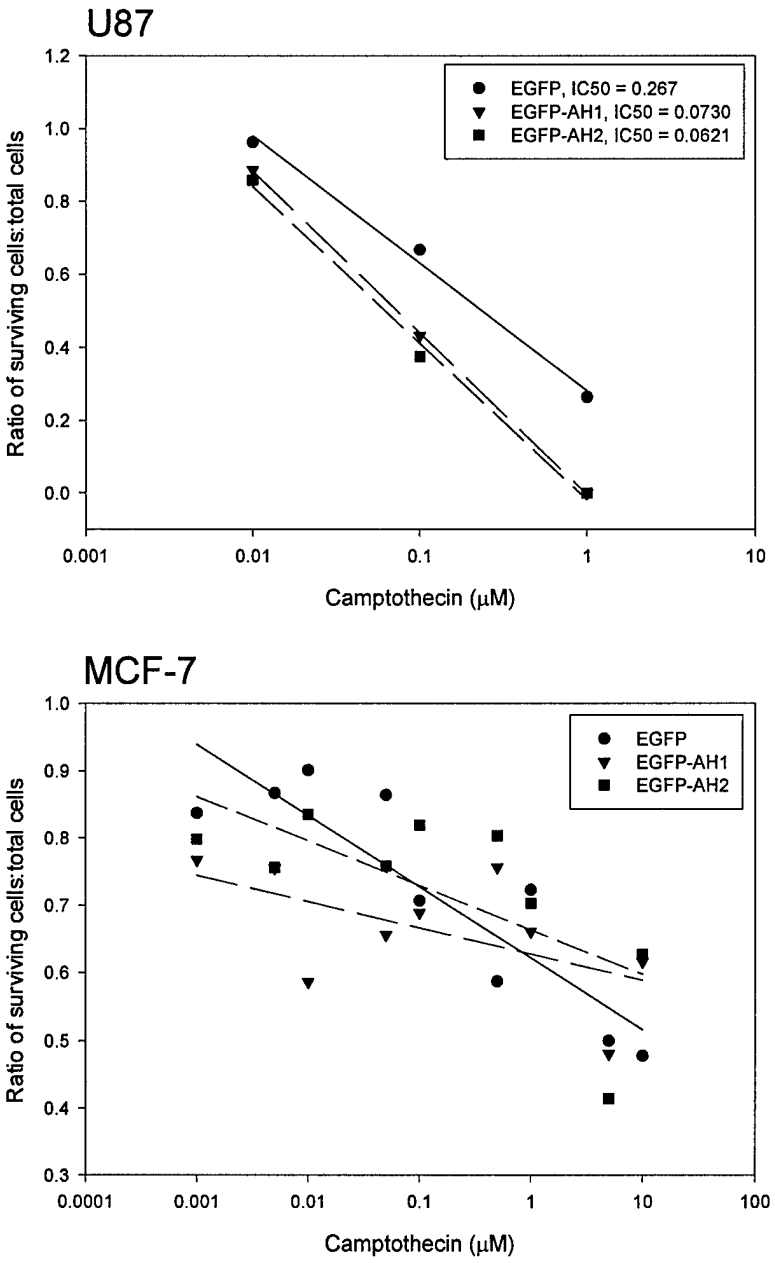

Fig. 2. Sensitization of human cancer cells to camptothecin treatment by overexpression of AH1 or AH2. U87 or MCF-7 overexpressing either GFP-AH1 or -AH2 were treated with varying doses of camptothecin for 48 h or 16 h, respectively. Apoptosis was determined by Hoechst staining of greater than 500 cells per dose.

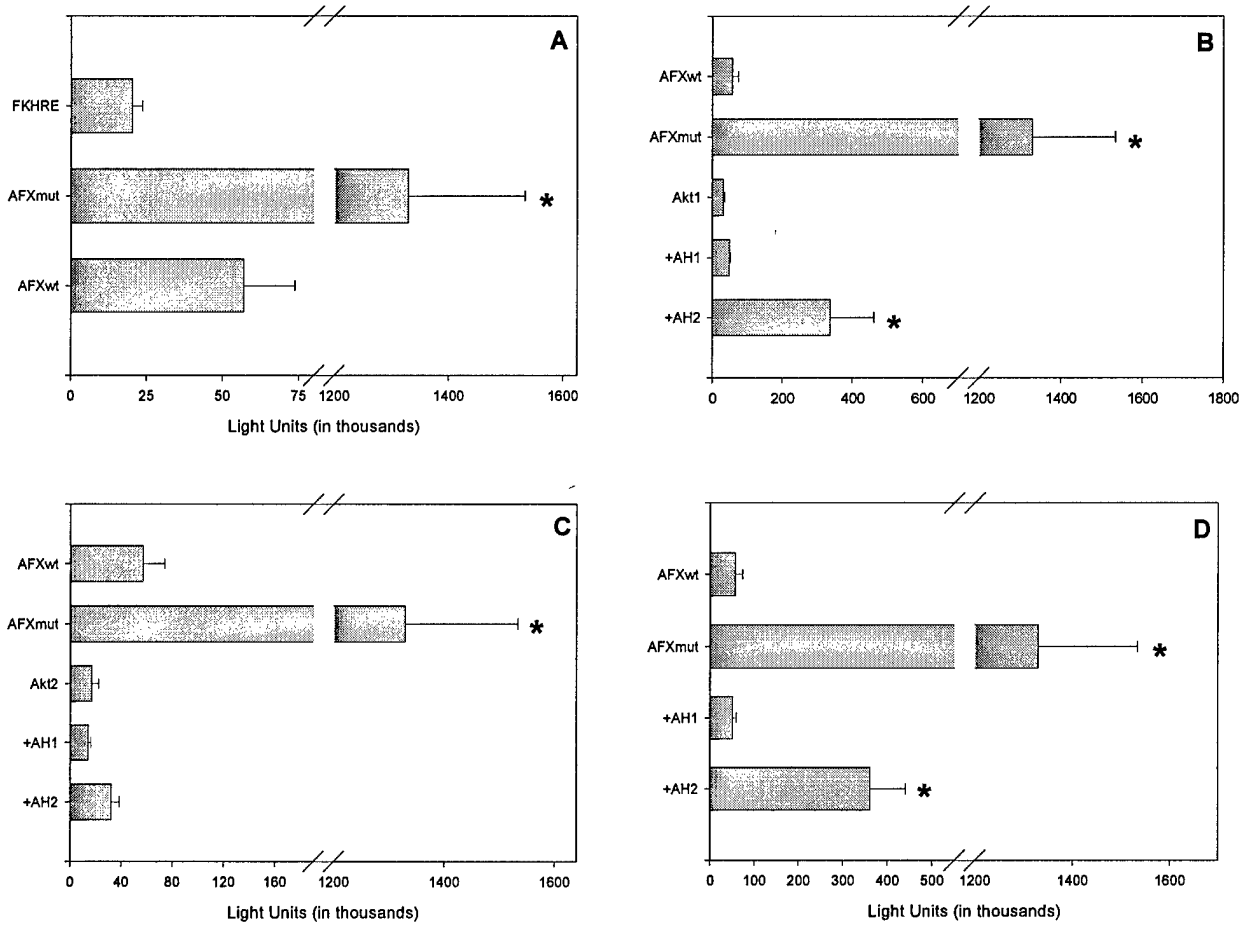

Fig 3. Disinhibition of AFX transactivation activity by AH2 overexpression in 293T cells. A) Effect of overexpression of AFX wt or constitutively active AFX (AFXmut) on Forkhead responsive luciferase activity. B) Alteration of AFX wt transactivation by overexpression of Akt1 in the presence or absence of GFP-AH1 or -AH2. C) Alteration of AFX wt transactivation by overexpression of Akt2 in the presence or absence of GFP-AH1 or -AH2. D) Alteration of AFX wt transactivation by overexpression of GFP-AH1 or -AH2 in native 293T cells. *, $p < 0.05$.

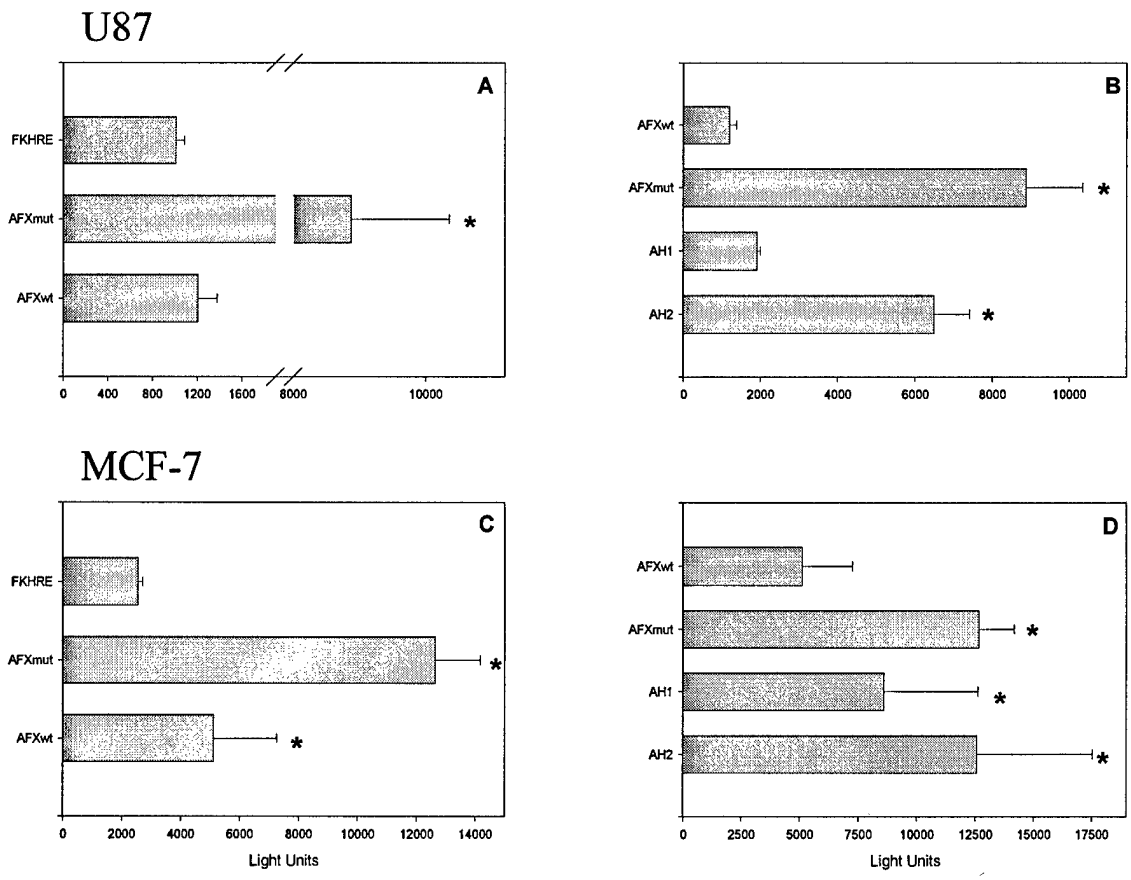

Fig 4. AFX transactivation activity in U87 and MCF-7 cells in the presence and absence of GFP-AH1 or -AH2. A,C) Effect of overexpression of AFX wt and AFXmut on Forkhead responsive luciferase activity in U87 and MCF-7, respectively. B,D) Alteration of AFX wt transactivation by overexpression of GFP-AH1 or -AH2 in U87 and MCF-7, respectively.

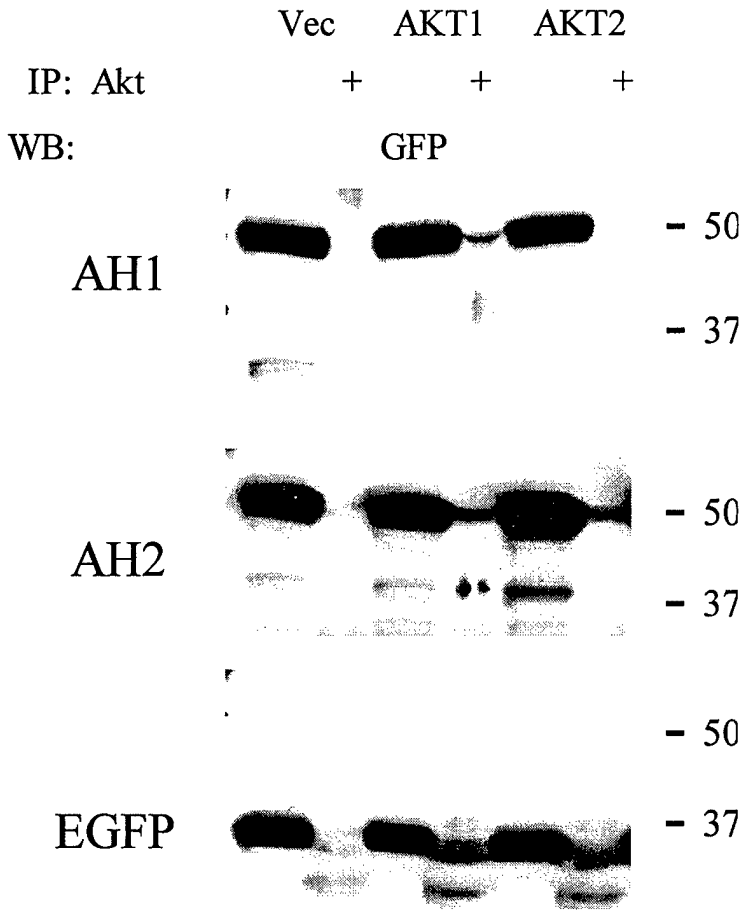

Fig 5. Co-immunoprecipitation of GFP-AH1 or GFP-AH2 with Akt1 or Akt2 in 293T cells. 293T co-transfected with either Akt or vector and either AH or vector and precipitation performed with a pan-Akt antibody.

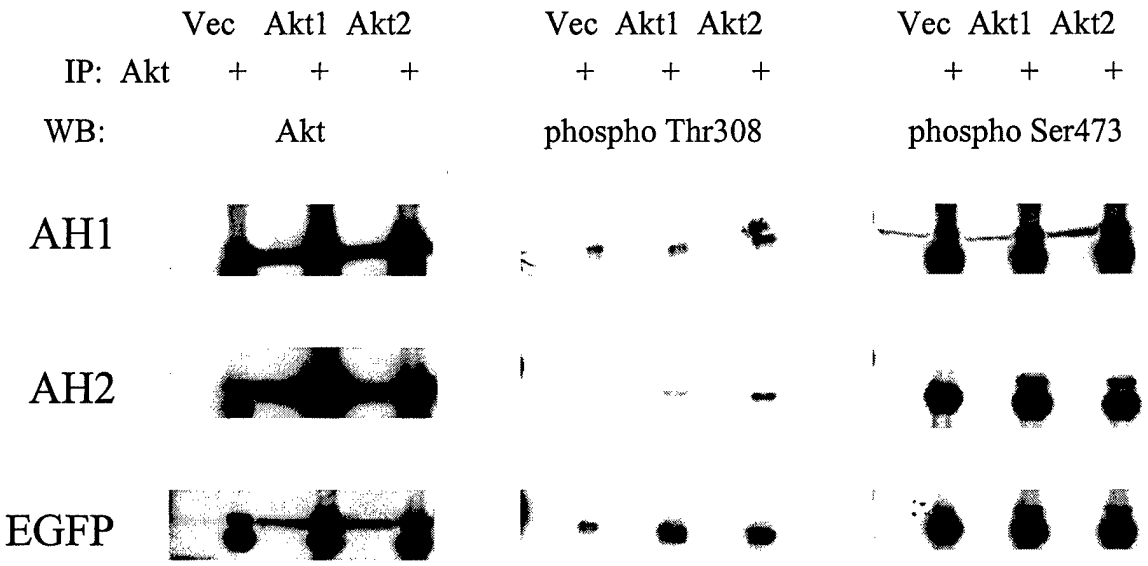

Fig 6. Co-immunoprecipitation experiment as described for Fig 6. Membranes stripped and reprobed for Akt, phosphoThr308 Akt and phosphoSer473 Akt. In the case of Akt and phosphoSer473 Akt, the upper band represents Akt, while the lower band is the heavy chain of the immunoprecipitating antibody

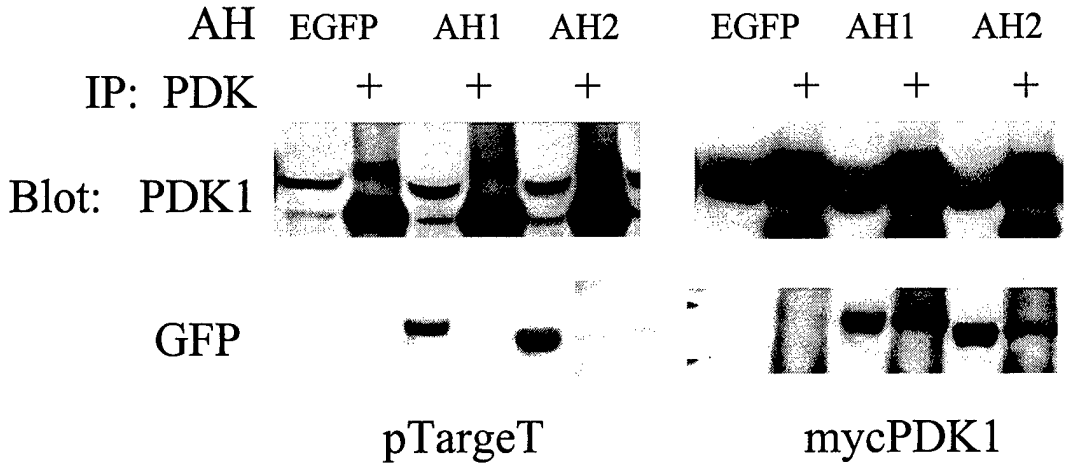

Fig 7. Co-immunoprecipitations performed as in Figs 6-7, using an anti-PDK1 antibody. Membranes probed for PDK1 and EGFP.