WD-Repeat FYVE Protein Interacts with the Akt Kinase and Promotes Adipogenesis

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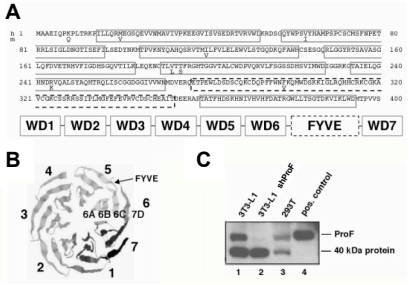
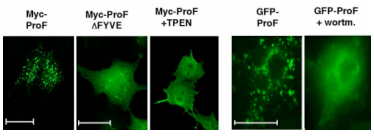
Introduction:

The protein kinase Akt is an important regulator of many processes, including metabolism, proliferation and differentiation. Recently, several studies have suggested a role of Akt in preadipocyte differentiation.
We have identified a protein, consisting of seven WD-repeats, presumably forming a β-propeller, and a FYVE domain, ProF, which interacts with the kinases Akt and protein kinase Cζ, PKCζ. The interaction between ProF and the kinases is increased upon hormonal stimulation of the cell [1]. We identified ProF as a positive regulator of preadipocyte differentiation. This is demonstrated by knockdown of ProF using RNA interference, which led to decreased adipogenesis, as shown by reduced lipid accumulation and impaired expression of the late differentiation markers PPARγ and C/EBPα, which indirectly led to decreased uptake of glucose into adipocytes, while overexpression of ProF caused the opposite.
We demonstrate ProF as positive regulator of preadipocyte differentiation and anticipate a role of ProF as potential mediator for the phosphorylation of Akt substrates by the kinase.

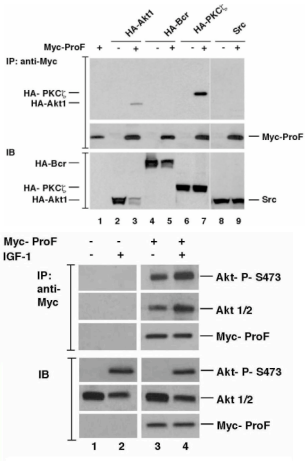
[1] Fritzius, T., Burkard, G., Haas, E., Heinrich, J., Schweneker, M., Bosse, M., Zimmermann, S., Frey, A.D., Caelers, A., Bachmann, A.S. and Moelling, K. (2006) A WD-FYVE protein binds to the kinases Akt and PKCzeta/lambda. Biochem J, **399**, 9-20.



Characterisation of ProF. (**A**) Deduced amino acid sequences of human (h) ProF and the murine (m) homologue, differing in eight indicated amino acids, are shown. The protein contains seven WD-repeats (WD 1–7), indicated as grey boxes, and one FYVE domain, indicated as black dashed box. (**B**) A model of the three-dimensional structure of ProF without the FYVE domain was generated by the 3D-PSSM program with the yeast protein Tup1 as template. The resulting ß-propeller consists of seven blades formed by the WD-repeats (1-7), whereby the D-strand of each WD-repeat is part of the previous blade. The FYVE domain is located between the ß-strands 6C and 7D (arrow). (**C**) ProF protein expression levels in different cell lines were analysed by Western blot antibody raised against the 15 C-terminal amino acids of ProF. 3T3-L1 shProF (lane 2) shows downregulation of the ProF protein in 3T3-L1 cells using a shRNA targeted against ProF. 293T + ProF (lane 4) indicates a lysate of HEK 293T cells overexpressing untagged ProF. In the case of 293T + ProF, only 1/20 of the protein content of lanes 1 – 3 was loaded to avoid excess signal strength by detection.

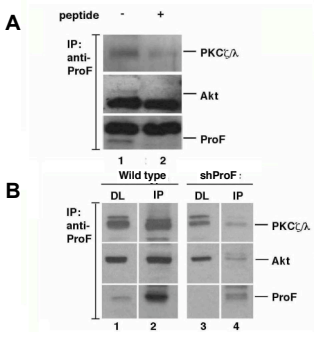


The FYVE domain of ProF is responsible for the vesicular localization of the protein. Immunofluorescence microscopy analysis of COS-7 cells transiently transfected with plasmid DNA encoding Myc-ProF, Myc-ProFΔFYVE, and GFP-ProF was performed. Cells were treated for 30 min with TPEN or wortmannin. Scale bars indicate 20 µm.

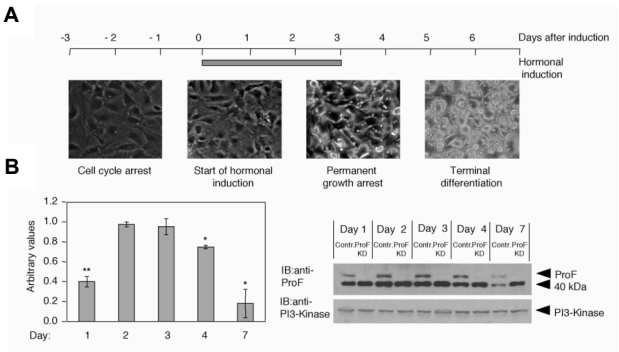


ProF interacts with Akt and PKCζ. HEK 293T cells were transiently transfected with HA-Akt1, HA-Bcr, HA-PKCζ, and Src in the presence or absence of Myc-ProF. Interaction of ProF with HA-Akt1, HA-Bcr, HA-PKCζ, and Src was analysed by IP with an antibody to Myc-tag followed by IB with antibodies against Src-, HA-, and Myc-epitopes (IP, top). Direct lysates are shown as expression controls (IB, bottom).

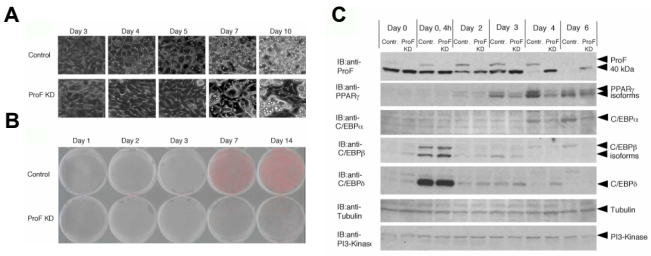
Interaction of ProF with Akt is increased upon hormonal stimulation of the cell (**A**) HEK 293T cells transiently expressing Myc-ProF were stimulated with IGF-1 and subjected to IP with an antibody against the Myc-epitope. Association of Myc-ProF with endogenous Akt1/2 was detected by an anti-Akt antibody and phosphorylation of Akt by an antibody to phosphorylated Ser 473 (P- S473), indicating increased coimmunoprecipitation of Akt after stimulation by IB analysis. Expression and immunoprecipitation of Myc-ProF was verified by IB of direct lysates and IP with an antibody against the Myc epitope. Samples were loaded on one gel, separating lines were included for clarity. Similar results were found for PKCζ.



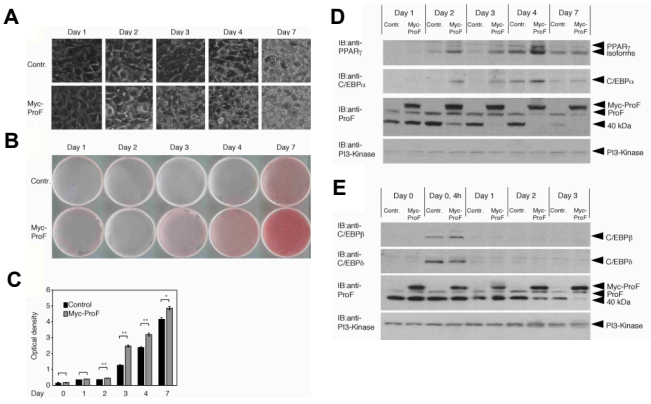
ProF interacts with endogenous Akt and PKCζ. (**A**) Mouse brain extract was treated with anti-ProF antibody in the absence (-) or presence (+) of an excess of ProF peptide to compete for the antibody followed by IB. Lane 2 shows competition of the ProF protein by addition of an excess of competing peptide. (**B**) Undifferentiated 3T3-L1 cells, which were untransduced (lane 1,2) or transduced with an shRNA targeted against ProF to downregulate ProF expression (lane 3,4), were subjected to IP in lanes 2 and 4. IP was performed with the peptide antibody against ProF, which was crosslinked to Sepharose beads. Association of endogenous ProF with endogenous kinases was detected in untransduced control cells (lane 2), and in cells with shRNA-mediated downregulation of ProF (lane 4). Lane 1 shows the direct lysates of control cells, lane 3 shows the direct lysates of shProF- transduced cells. Lysates were loaded on one gel, separating lines were included for clarity.



ProF expression is unregulated during early adipogenesis. (**A**) The general morphology of 3T3-L1 cells is shown during cell cycle arrest (day –3), start of hormonal induction (day 0), permanent growth arrest (day 3 to 4) and terminal differentiation (day 7) as observed by light microscopy (from left to right). Day 0 to 3 in the time line corresponds to the days of addition of the hormonal induction medium (indicated as a gray bar). (**B**) The expression of the ProF protein at days 1, 2, 3, 4, and 7 after induction of preadipocyte differentiation was measured by densitometric scanning of ProF protein bands from immunoblots. One representative blot shows the expression of ProF during preadipocyte differentiation in control cells (Contr.) and cells with knock-down of ProF (ProF KD).



Knockdown of ProF impairs preadipocyte differentiation. (**A**) Morphology of 3T3-L1 preadipocytes, transduced with a control shRNA against firefly luciferase (Control) or a shRNA targeted against ProF mRNA for ProF knockdown (ProF KD) was observed by light microscopy during the indicated days of preadipocyte differentiation. (**B**) Oil Red O staining of control cells (top) and ProF knockdown cells (bottom) at days 1, 2, 3, 7, and 14 of preadipocyte differentiation, showing fat development. (**C**) 3T3-L1 control cells (Contr.) or cells with ProF knockdown (ProF KD) were lysed at the indicated days after addition of hormonal induction medium; (Day 0, 4h) denotes lysis of cells after 4 h at day 0. Time-course analysis of expression of preadipocyte differentiation markers was performed by Western blot with antibodies against ProF, PPARγ, C/EBPα, C/EBPβ, C/EBPδ, tubulin, and PI3-Kinase (from top to bottom). Expression of tubulin and PI3-Kinase were assessed as loading control.



Overexpression of ProF increases preadipocyte differentiation. (**A**) Morphology of 3T3-L1 cells, transduced with an empty control vector (Contr.) or a vector expressing Myc-tagged ProF (Myc-ProF) was observed by light microscopy at the indicated time points of preadipocyte differentiation. (**B**) Oil Red O staining of control cells (Contr.) or Myc-ProF expressing cells (Myc-ProF) at days 1, 2, 3, 4, and 7 of preadipocyte differentiation, showing fat development. (**C**) Triglyceride content of control cells or Myc-ProF expressing cells (Myc-ProF) was measured spectrometrically at days 0, 1, 2, 3, 4, and 7 after induction of preadipocyte differentiation (**D** + **E**): 3T3-L1 cells, transduced with an empty control vector (Contr.) or a vector expressing Myc-tagged ProF (Myc-ProF) were lysed at the indicated days after addition of hormonal induction medium; (Day 0, 4h) denotes lysis of cells after 4 h at day 0. Time-course analysis of expression of preadipocyte differentiation markers was performed by Western blot with antibodies against PPARγ , C/EBPα, C/EBPβ, C/EBPδ, ProF, and PI3-Kinase. Expression of PI3-Kinase was assessed as loading control.