BIOLOGIJA. 2005. Nr. 1. P. 24–26
© Lietuvos mokslø akademija, 2005
© Lietuvos mokslø akademijos leidykla, 2005

Interaction between protein kinase D and components of SUMO conjugation enzyme complex

V. Tunaitis,
M. Ger,
M. Stoškus,
M. Valius

Institute of Biochemistry,
Department of Development Biology,
Mokslininkø 12, LT-08662
Vilnius, Lithuania

E-mail: virgis.tunaitis@bchi.lt;

Protein kinase D (PKD) is a serine/threonine protein kinase which is implicated in signaling mechanisms controlling cell proliferation, apoptosis, tumor metastases and immune response. In the previous study we have isolated a clone which could interact with PKD in the yeast two-hybrid screen. Sequence analysis has shown that the clone encodes the C-terminal part of small ubiquitin-related modifier (SUMO) activating enzyme Uba2 which together with another activator protein, Aos1, is involved in covalent modification of the target protein by SUMO peptide. Here we show that intracellular Uba2 co-immunoprecipitates with PKD and recombinant GST-Uba2 fusion protein associates with cellular catalitically active PKD. In addition, the recombinant GST-PKD insert fusion protein recruits endogenous Uba2. Data suggest that PKD, by interacting with Uba2, might play a role in the regulation of protein covalent modification with SUMO.

Key words: protein kinase D, SUMO modification complex

INTRODUCTION

Protein kinase D (PKD) is a serine/threonine protein kinase that plays an important role in the regulation of a variety of intracellular signaling processes. PKD is involved in diverse cellular functions such as differentiation, growth control, tumor promotion and cell death. Typically, PKD activity is regulated by lipid second messenger diacyglycerol (DAG) produced after activation of PLCβ-γ by various receptors, Gβ-γ subunits in the Golgi or after cleavage by caspases [1].

Several signaling pathways are affected by PKD activation. For instance, activation of PKD leads to activation of the Raf-MEK-ERK pathway, inhibition of JNK activation and subsequently enhances NF-kB transcription factor activity [2, 3]. These pathways may interplay, with protein covalent modification, with SUMO peptide, which is recognized as a new important protein regulatory mechanism. SUMO modification is important in nuclear protein targeting of some proteins, formation of the so-called subnuclear structures of nuclear bodies, regulations of transcription activities or DNA binding abilities of transcription factors, and control of protein stability [4, 5]. Protein modification by SUMO pathway consists of three different types of enzymes: E1-activating enzymes (Aos1/Uba2), E2-conjugating enzyme (Ubc9), and E3-ligases (PIAS1, Siz1, Siz2) [6–8].

Our previous study using a yeast two hybrid screening system revealed several new PKD-interacting cDNA clones [9]. In the current study, we have identified a new interaction of protein kinase D with Uba2, one of the components of the SUMO conjugation complex. A combination of GST pulldown and co-immunoprecipitation experiments presents evidence of an interaction of PKD and Uba2 proteins in human A431 cells. These data suggest that PKD might be involved in the SUMO-depending regulation of different cellular processes.

MATERIALS AND METHODS

Construction of expression vectors. The pGEX-14 plasmid was obtained by subcloning the C-terminal region of Uba2 from the *cDNA* library (465–640 a.a.) into the pGEX-6P-1 vector. To generate the pGEX-Zn.fn and pGEX-Zn.fn.insert expression vectors, a PKD zinc finger (encoding 144–326 a.a.) and zinc finger insert (encoding 194–276 a.a.) were amplified by PCR and cloned into pGEX-6P-1. The retrovirus expression vector pLXSHD-Myc-PKD was obtained by ligation of c-Myc tag adapter to C-end of PKD in pBluescript and subcloning of a new generated Myc-PKD into pLXSHD vector.

Expression and purification of GST fusion proteins. The plasmids pGEX-14, pGEX-Zn.fn, and pGEX-Zn.fn.insert expression vectors were introduced into the competent *E. coli* strain BL21, Expression of GST fusion proteins in growing cells was induced with 0.8 mM of IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. Purification was performed according to the manufacturer's instruction (Amersham-Pharmacia, USA).

Generation of antiserum. Rabbit antiserum was produced using GST fusion proteins expressed in and purified from *E. coli* as antigen. For production of Uba2-specific antiserum, rabbits were immunized with GST fusion protein encoding 465–640 a.a. of Uba2. To produce anti-PKD antiserum, rabbits we immunized with GST-Zn. fn fusion protein (encoding 144–326 a.a. of PKD).

Cell culture. A431 cells overexpressing Myc-PKD were established by infection with virus expression vectors encoding cDNA of Myc-PKD (A-Myc-PKD) or the empty vector pLXSHD (A-HD) using a retrovirus-mediated transfection-infection protocol selected in 16 mM of L-Histidinol (SIGMA, USA). The established cell lines were cultured in DMEM medium containing 10% (vv) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in 5% CO_2 atmosphere.

Preparation of cell lysates and immunoprecipitation. Culture of A431 containing about 3.0 × 10^6 cells per dish was synchronized in DMEM medium for 24 h and treated with 100 nM PMA (phorbol 12-meristate 13-acetate) (SIGMA, USA), or left without treatment. After 30 min cells were washed three times with PBS and lysed in EB lysis buffer (10 mM TrisHCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 20 mg/ml Aprotinin, 1 mM PMSF, 2 mM Na_3VO_4). The lysate was cleared by centrifugation at 20000g for 15 min. 1.5 ml of lysate was incubated for 1.5 h at 4 °C with 20 μl of the specific rabbit antiserum. Immunocomplexes were precipitated with 20 μl of protein A-Sepharose (Amersham-Pharmacia, USA) for 1 h, washed 4 times with EB lysis buffer, resuspended in SDS-PAG electrophoresis sample buffer and analyzed by protein immunoblot using protein-specific antiserum or c-Myc tag specific antibodies (SantaCruz, USA).

GST-pull down assay. To demonstrate the interaction between Uba2 and PKD, approximately 20 μg of purified from bacteria GST, GST-14, or GST-Zn.fn.insert fusion proteins were coupled with 15 μl of glutathione-Sepharose beads and incubated with corresponding cell lysates. Glutathione sepharose-bound proteins were washed 4 times with EB lysis buffer, resuspended in 50 μl of sample buffer, fractionated by SDS-PAG electrophoresis, and analyzed by protein immunoblotting.

RESULTS AND DISCUSSION

Our previous study of PKD Zn finger insert-interacting *cDNA* clones obtained from yeast two-hybrid screening revealed three potential PKD-binding proteins. DNA sequence analysis of one of these clones showed an extensive homology with C-terminus of human Uba2.

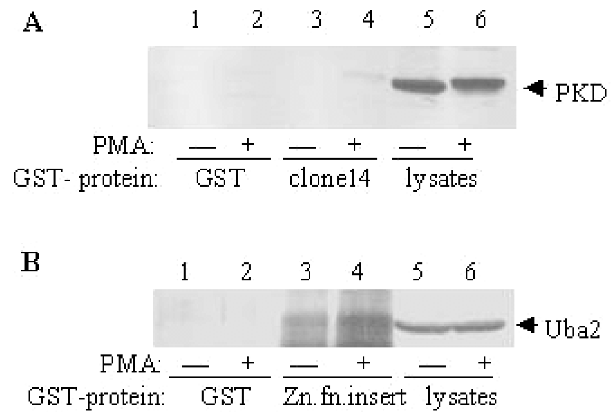

Fig. 1. Interaction of full length PKD and Uba2 with GST fusion proteins in pulldown experiments. A-Myc-PKD cells were untreated or treated with PMA, and total lysates were subjected to pulldown assays. Proteins that interacted with GST fusions were detected by immunoblot analysis using monoclonal C-Myc antibody (A) or anti-Uba2 antiserum (B)

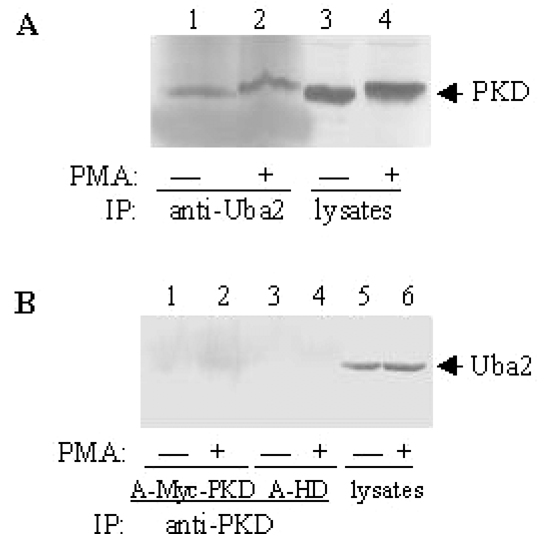

Fig. 2. Interaction conformation by co-immunoprecipitatios. A-Myc-PKD and A-HD cells were untreated or treated with PMA and total lysates were subjected to immunoprecipitation analysis using Uba2-specific or PKD antiserum. Immunocomplexes were subjected to immunoblot analysis using monoclonal C-Myc antibody (A) or anti-Uba2 antiserum (B). A-HD cells without PKD overexpression were used as negative control

To identify stable complex formation between PKD and Uba2, we applied a GST fusion protein pulldown assay. We produced bacterial GST fusion proteins Uba2 (465–640 a.a.) and PKD (194–276 a.a.), coupled them to glutathione-Sepharose and incubated with cell lysate. Immunoblot analysis showed that in the A-Myc-PKD cells overexpressing Myc tagged PKD, PMA treatment induced GST-Uba2 interaction with PKD, as well as endogenously expressed Uba2 interaction with GST-Zn-fingers insert (Fig. 1).

To obtain evidence for molecular complex formation in the cell environment, we co-immunoprecipitated endogenous Uba2 from A-Myc-PKD cells with PKD-specific antiserum (Fig. 2). We found that after PMA stimulation, PKD translocated into the nucleus where it could interact with the nuclear protein Uba2, suggesting that activation of PKD might be essential for this interaction (data not shown).

In summary, our data show that in the human cell lines PKD and Uba2 interact. The insert portion between two Zn fingers of PKD molecule is responsible for association with the C-terminus part of Uba2.

ACKNOWLEDGEMENTS

This work was supported by Lithuanian Science and Studies Foundation (Grants K-005 and G-046).

Received 27 January 2004
Accepted 15 November 2004

References

1. Van Lint J, Rykx A, Maeda Y, Vantus T at al. Trends in Cell Biology 2002; 12(4): 193–200.
2. Van Lint J, Ni Y, Valius M, Merlevede W, Vandenheede JR. J Biol Chem. 1998; 273: 7038–43.
3. Johannes F-J, Horn J, Link G, Haas E, Siemenski K, Wajant H, Pfizenmaier K. Eur J Biochem 1998; 257: 47–54.
4. Yang S-H, Jaffray E, Hay RT, Sharrocks AD. Molecular Cell 2003; 12: 63–74.
5. Desterro JMP, Rodriguez MS, Kemp GD, Hay RT. J Biol Chem 1999; 274(15): 10618–24.
6. Kim KI, Baek SH, Chung CH. J Cell Physiol 2002; 191: 257–68.
7. Rodriguez MS, Dargemont C, Hay RT. J Biol Chem 2001; 276(16): 12654–9.
8. Johnson ES, Swienhorst I, Dohmen RJ, Blobel G. EMBO 1997; 16(18): 5509–19.
9. TunaitisV, Van Lint J, Valius M. Biologija 2000; Nr. 2 (priedas): 153–6.

V. Tunaitis, M. Ger, M. Stoškus, M. Valius

SÀVEIKA TARP PROTEINKINAZËS D IR SUMO MODIFIKACINIO KOMPLEKSO BALTYMØ

Santrauka

Serino / treonino proteinkinazë D (PKD) yra átraukta á làstelës veiklà reguliuojanèius procesus, tokius kaip làstelës proliferacija, apoptozë, vëþio metastazës, imuninis atsakas. Naudojant mieliø dvihibridinæ sistemà rastas naujas su PKD sàveikaujantis baltymas Uba2, kuris kartu su kitu aktyvatoriumi Aos1 áeina á baltymø modifikavimo SUMO peptidu kompleksà. Ðiame darbe mes biocheminiais metodais nustatëme, kad Uba2 ir PKD sàveikauja làstelëje. Remdamiesi literatûra ir naujausiais duomenimis galime teigti, kad ði sàveika gali turëti átakos baltymø SUMO modifikacinei sistemai.