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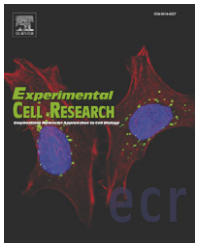

Research Article

Ankyrin repeat domain 28 (ANKRD28), a novel binding partner of DOCK180, promotes cell migration by regulating focal adhesion formation

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ABSTRACT

DOCK180 is a guanine exchange factor of Rac1 originally identified as a protein bound to an SH3 domain of the Crk adaptor protein. DOCK180 induces tyrosine phosphorylation of p130^{Cas}, and recruits the Crk-p130^{Cas} complex to focal adhesions. To understand the role of DOCK180 in cell adhesion and migration, we searched for DOCK180-binding proteins with a nano-LC/MS/MS system, and identified ANKRD28, a protein that contains twenty-six ankyrin domain repeats. Knockdown of ANKRD28 by RNA interference reduced the velocity of migration of HeLa cells, suggesting that this protein plays a physiologic role in the DOCK180-Rac1 signaling pathway. Furthermore, knockdown of ANKRD28 was found to alter the distribution of focal adhesion proteins such as Crk, paxillin, and p130^{Cas}. On the other hand, expression of ANKRD28, p130^{Cas}, Crk, and DOCK180 induced hyper-phosphorylation of p130^{Cas}, and impaired detachment of the cell membrane during migration. Consequently, cells expressing ANKRD28 exhibited multiple long cellular processes. ANKRD28 associated with DOCK180 in an SH3-dependent manner and competed with ELMO, another protein bound to the SH3 domain of DOCK180. In striking contrast to ANKRD28, overexpression of ELMO induced extensive lamellipodial protrusion around the entire circumference. These data suggest that ANKRD28 specifies the localization and the activity of the DOCK180-Rac1 pathway.

Introduction

Cell migration is an important event during early development, inflammatory responses to infection, and wound healing, and an important pathological event during tumor invasion and metastasis. The family of Rho GTPases regulates this process through remodeling of the actin cytoskeletons, and by generating focal complexes and focal adhesions [1].

DOCK180 was originally identified as one of two major binding proteins of the adaptor protein Crk [2]. Later, genetic and

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biochemical studies revealed that DOCK180 functions as a guanine-nucleotide exchange factor (GEF) for a small GTPase Rac [3,4]. Orthologs of DOCK180 in *C. elegans* and *D. melanogaster* have been identified as Ced-5 (cell death abnormal 5) and Mbc (Myoblast city), respectively, and together with DOCK180 itself comprise an evolutionarily conserved protein group called the CDM (*C*ED-5, *D*OCK180, *M*BC) family [5]. In addition, studies in *C. elegans* identified orthologs of Crk (Ced-2) and Rac (Ced-10) to show that the Crk-DOCK180-Rac signaling pathway is evolutionally conserved [6].

The localization and function of DOCK180 are regulated through interaction with binding partners. DOCK180 contains N-terminal SH3 and C-terminal proline-rich domains, which are required for ELMO and CrkII interaction, respectively [7,8]. Subsequent studies identified two other regions, designated DHR1 and DHR2 (or CZH1 and CZH2/DOCKER, respectively) [2,9], both of which showed high sequence homology among the 11 human DOCK180 superfamily proteins. Numerous recent findings have demonstrated that DHR2 functions as a GEF for Rho-family proteins [8,10–12]. *In vitro* and *in vivo* data indicated that phosphatidylinositol 3,4,5-trisphosphate (PIP_3) binds to and recruits DOCK180 through DHR1 at the site of lamellipodia in response to platelet-derived growth factor [13]. Coexpression of CrkII and its binding protein $p130^{Cas}$ induces the accumulation of CrkII, $p130^{Cas}$, and DOCK180 at the focal adhesions in NIH3T3 cells [14], and the enhancement of Rac GEF activity of DOCK180 toward Rac1 [3]. Ced-12 in *C. elegans* has been identified as a required gene for engulfment of apoptotic cells [7]. Its mammalian homolog ELMO binds to DOCK180 directly and cooperates with CrkII and DOCK180 to promote cell shape changes through enhancement of DOCK180-induced Rac1 activation [15]. It has been suggested that the SH3 domain of DOCK180 binds directly to the DHR2 of DOCK180 to inhibit the activity of the protein in the steady state [16], and that these binding proteins and lipids induce the conformational changes of DOCK180 upon stimulation.

Here we identify ANKRD28 as a novel binding partner of DOCK180, and show a potential role of ANKRD28 in regulating focal adhesion for cell migration, of which mechanism is different from that of the other binding partner ELMO.

Materials and methods

Plasmids

cDNAs encoding the full-length ANKRD28 (KIAA0379) were obtained from Kazusa DNA Research Institute. To construct expression vectors, cDNA were amplified by PCR with the primer set GTCGACATGGCGTTCCTCAAACTCCGT (forward) and GCGGCCGCTCAGTAGGTCTCAGAATCGGA (reverse) and subcloned into pDrive vectors (QIAGEN, Hilden, Germany). After confirming the nucleotide sequences, the cDNA was digested with SalI and NotI and cloned into expression vectors to obtain pCAP-FLAG-ANKRD28 and pCAGGS-EGFP-ANKRD28. pCXN2-FLAG-DOCK180 and its deletion mutants (PS and dSH3) pCAGGS-EGFP-DOCK180, pSSRp130^{Cas}, and pCAGGS-Myc-CrkII were described previously [14,17,18]. pCAGGS-DOCK180-1-357 and pCNX2-DOCK180-72-520 encode amonoacid residues 1–357 and 72–520 of DOCK180, respectively. pCXN2-FLAG DOCK180-ISP is a mutant in which three contiguous residues at positions 1487 to 89, Ile-Ser-Pro, were replaced by Ala-Ala-Ala, as described previously [15] with accidental mutation by PCR at amino acid 1456 (Asn to Asp). The plasmids pEBB-mELMO1/2-wt-FLAG and pEBB-mELMO1/2-wt-GFP were gifts from Dr. K.S. Ravichandran [7].

For vector-based knockdown, the short hairpin RNA (shRNA) sequences targeting human DOCK180 (DK1-#4: 5′-GTTTCTTCAG-GACACGTTG-3′; DK1-#7: 5′-GTACGGAGATATGAGGAGA-3′), human ANKRD28 (ANK-#2: 5′-GTACCTTCTAGATCTTGGA-3′; ANK-#5: 5′-GGTGCTGCTGAGATGTTAA-3′), and firefly luciferase (Luc) (5′-GATTATGTCCGGTTATGTA-3′) were cloned into pSuper.retro.puro vector (OligoEngine, Seattle, WA) or pSuper-DsRed2, in which the puromycin-resistant sequence of pSuper.retro.puro was replaced by that for DsRed. For RNAi rescue experiments, shRNA (ANK-#5 and DK1-#4)-resistant cDNAs were created by introducing seven silent point mutations in the target sequences (GAGCGGCGGAAATGTTGATAGATA and TTCCTGCAGGATACCTTA-GACGCT), generating pCAGGS-EGFP-ANKRD28^{R} and $DOCK^{R}$, respectively [19]. For retroviral infection, Moloney murine leukemia virus (MuLV)-based retroviral vector plasmids, pMSCVpac-3HA-ANKRD28, -ELMO2, pCX4bsr-EGFP-Cas, and pCX4bsr-mCherry-paxillin, were constructed [20].

Antibodies

The polyclonal antibodies against DOCK180 and GFP were developed in our laboratory and described previously [2,21].The antibody against ANKRD28 was raised in a rabbit using a human ANKRD28 C-terminus peptide (corresponding to amino acids (aa) 1026–1040 CSFNNIGGEQEYLYT) (Covalab, Lyon, France). Other antibodies used in this study were purchased from the following suppliers: Rat anti-HA antibody (Roche, Basel, Switzerland); mouse anti-c-Raf-1 antibody, mouse anti-$p130^{Cas}$ antibody, mouse anti-phosphotyrosine (pTyr) antibody, mouse anti-Crk antibody, and mouse anti-Rac1 antibody (BD Biosciences, San Jose, CA); Mouse anti-FLAG monoclonal antibody (Sigma-Aldrich, St. Louis, MO); Mouse anti-α-tubulin antibody (EMD Chemical Inc., San Diego, CA); goat anti-ELMO2 antibody (IMGENEX, San Diego, CA); and Goat Alexa Fluor-conjugated anti-mouse and rabbit IgG (Invitrogen, Paisley, United Kingdom).

Cell culture, transfection, and retroviral infection

HeLa, HEK 293T, and BOSC23 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 1 μg/ml of penicillin-streptomycin and 10% fetal bovine serum. 293F cells were maintained at 37 °C in FreeStyle 293 expression medium (Invitrogen), and transfected with various plasmids using 293 fectin reagent (Invitrogen) according to the manufacturer's instructions. In some experiments, a MuLV-based retroviral system was utilized to obtain cells expressing the proteins of interest [20]. Briefly, the murine ecotropic retrovirus receptor EcoVR was first introduced by using viruses, which were produced from BOSC23 cells by transfection of pCX4hyg-EcoVR together with the plasmids pGP (the packaging plasmid) and pVSV-G (the envelope) [20,22]. After selection by hygromycin, the cells expressing EcoVR were infected with retrovirus for various tagged proteins. cDNA of the protein of interest was cloned into pMSCVpac- or pCX4bsr-based vectors, which are resistant to puromycin and blasticidin, respectively, and