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The direct association of the multiple PDZ domain containing proteins (MUPP-1) with the human c-Kit C-terminus is regulated by tyrosine kinase activity

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Abstract We have identified the multiple PDZ domain containing protein (MUPP-1 or MPDZ) as a novel binding partner of the human c-Kit. c-Kit binds specifically to the 10th PDZ domain of MUPP-1 via its C-terminal sequence. Furthermore, a kinase negative-mutant receptor interacted more strongly with MUPP-1 than the wild-type c-Kit. Strikingly, a constitutively activated c-Kit (D816V-Kit) did not bind to MUPP-1, although this oncogenic form retains the PDZ binding motif 'HDDV' at the C-terminal end. Deletion of V967 of c-Kit abolished binding to MUPP-1 and drastically reduced its tyrosine kinase activity, suggesting that the structure of the C-terminal tail of c-Kit influences its enzymatic activity.

Key words: Human c-Kit; PDZ domain; Oncogenic c-Kit; Tyrosine kinase; Multiple PDZ domain containing protein

1. Introduction

The c-kit protooncogene encodes the receptor for the stem cell factor (SCF). Interactions between c-Kit and its ligand induces differentiation, proliferation, survival, and/or secretion of mediators in several cell lineages including mast cells, melanocytes, hematopoietic stem cells, intraepithelial lymphocytes, interstitial cells of Cajal and germ cells [1].

The human c-Kit contains a cytoplasmic tyrosine kinase domain which is split into two subdomains, designated K1 and K2, by an insertion of approximately 76 amino acids, termed the kinase insert (KI) region. Furthermore, a segment of 36 amino acids, termed the juxtamembrane (JX) domain, separates the membrane spanning domain from the first tyrosine kinase (K1) domain. The C-terminal domain (CT) contains about 67 amino acids and proceeds the K2 domain. Interestingly, the amino acid sequence at the C-terminus is 'HDDV' which may provide a binding site for group II PDZ domains [2]. It has been demonstrated that the human c-kit$^+$ cell line (HMC-1), established from peripheral blood of a patient with mast-cell leukemia [3], or cells from mastocytosis with an associated hematologic disorder [4] exhibit point mutations in the cytoplasmic domain of c-Kit (D816V mutation), which promote ligand independent autophosphorylation of the mutant receptor [5]. Activation of the tyrosine kinase leads to autophosphorylation of the cytoplasmic domain of the receptor at multiple sites. The newly formed phosphotyrosine residues constitute binding sites for src homology (SH)2 domain-containing cytoplasmic proteins which may participate in the control of mitogenic pathways, cell differentiation, cell metabolism, or cell morphology.

In addition to SH2 domains, several interacting domains for the assembly of signaling complexes have been reported [6]. One of them, the PDZ domain, was first noted as repeated sequences in the brain-specific protein PSD-95, the *Drosophila* septate junction protein Disc-large, and the epithelial tight-junction protein ZO-1 [7,8]. PSD-95 interacts with cytoplasmic tail sequences of ion channel subunits, including the NR2 subunits of the NMDA receptor [9] and Shaker-type K^+ channel [10]. Recently, the C-terminus of AMPA receptors was shown to interact with one of the PDZ domains of GRIP [11]. Furthermore, the concept of receptor C-terminal interaction with PDZ domains also extends to G-protein coupled receptors [12,13], or members of the tyrosine kinase receptor family, including Eph [14,15] and ErbB-4 [16].

In this paper, we describe the cloning of a cDNA encoding a novel binding protein to c-Kit, the multiple PDZ domain containing protein (MUPP-1, or MPDZ) [13,17], by yeast two-hybrid screening using the cytoplasmic domain of c-Kit. This protein associated more strongly with the kinase negative c-Kit (K623M-c-Kit) than the wild-type (wt) c-Kit. Furthermore, an oncogenic c-Kit (D816V-c-Kit) did not bind to this protein, suggesting that the tertiary structure of the C-terminal end of c-Kit is important for its tyrosine kinase activity and that MUPP-1 may play a role for regulation of c-Kit tyrosine kinase activity.

2. Materials and methods

2.1. Plasmid constructions and yeast two-hybrid screening

For expression of the cytoplasmic domains of wt and mutant c-Kit as LexA-fusions, corresponding fusion genes were generated in pBTM116 and transformed into yeast strain L40 or YRN974 [18,19]. A single colony, selected for expression of the LexA–c-Kit fusion-protein, was tested for c-Kit autophosphorylation and used for transformation together with a VP16 cDNA library derived from a 10–11-day mouse embryo [19–21]. GST–c-Kit and GST–MUPP-1 fusion proteins were generated in the pGex system (Pharmacia, Freiburg, Germany).

2.2. Binding assay using the two-hybrid system

The qualitative and quantitative evaluations of various two-hybrid protein–protein interaction were described previously [18].

2.3. Cells and antibodies

TF-1 (kind gift from C. Stocking, Hamburg, Germany) and M-07e

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(from DSMZ) cells were grown in RPMI 1640 medium supplemented with 15% FCS and human recombinant granulocyte/macrophage colony stimulating factor (200 U/ml). Monoclonal antibody against phosphotyrosine (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY, USA), and polyclonal antibody against c-Kit was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Northern blotting

Northern blotting with MUPP-1-specific and actin-specific probes were performed as described [22]. Mouse multiple tissue Northern filters (MTN™) were purchased from Clontech Lab. Inc. (Palo Alto, CA, USA).

2.5. Immunoblotting

Immunoblotting was performed as published [18,23]. Bound radioactivity was quantified with a model BAS1500 bio-imaging analyzer (Fuji Photo Film Co., Kanagawa, Japan).

2.6. In vitro transcription/translation

In vitro transcription/translation was performed using TNT-coupled reticulocyte lysate systems (Promega, Madison, WI, USA).

2.7. GST binding assay

Purified GST fusion proteins were bound for 1 h at 4°C to glutathione (GT) agarose beads (10 μl slurry; Pharmacia) suspended in binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.01% Triton X-100, 1% trasylol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 400 μM sodium orthovanadate). Precharged beads were incubated overnight at 4°C with ^{35}S-labeled MUPP-1(PDZ8-13) as generated by in vitro transcription/translation in a total volume of 2 ml of binding buffer. Beads were washed five times with binding buffer and pellets were analyzed by SDS–PAGE.

3. Results and discussion

3.1. Isolation of MUPP-1 as binding protein of human c-Kit by yeast two-hybrid screening

We used a yeast two-hybrid screen to find specific binding proteins of the c-Kit tyrosine kinase. The cytoplasmic domain of c-Kit was expressed as a LexA-fusion protein. It should be noted that the LexA-domain induces dimerization of the fusion protein and thus causes activation of the tyrosine kinase [21]. We analyzed 200 clones and 90 of these encoded the SH2 domain of the p85 subunit of the PI-3′-kinase. Furthermore, 105 cDNA clones encoded the 10th PDZ domain of mouse MUPP-1 [13]. The Rat MUPP-1 cDNA was cloned previously from a brain cDNA library by yeast two-hybrid screening using serotonin receptor 2C as a bait [13]. To obtain the full length mouse cDNA we screened mouse spleen and brain libraries. Surprisingly, the MUPP-1 derived from the mouse brain cDNA library (Stratagene, La Jolla, CA, USA) lacked 114 amino acids (431–543) within the third to fourth PDZ domains, in comparison to the published sequence derived from mouse (AJ131869), rat (AJ001380) and human (AF093419). Furthermore, the MUPP-1 from the mouse spleen cDNA library (Stratagene) lacked 71 amino acids containing part of the seventh PDZ domain (Fig. 1A). Taken together, MUPP-1 transcripts may be expressed as multiple-spliced forms. We therefore next studied the tissue specific expression of the MUPP-1 gene using probes derived from

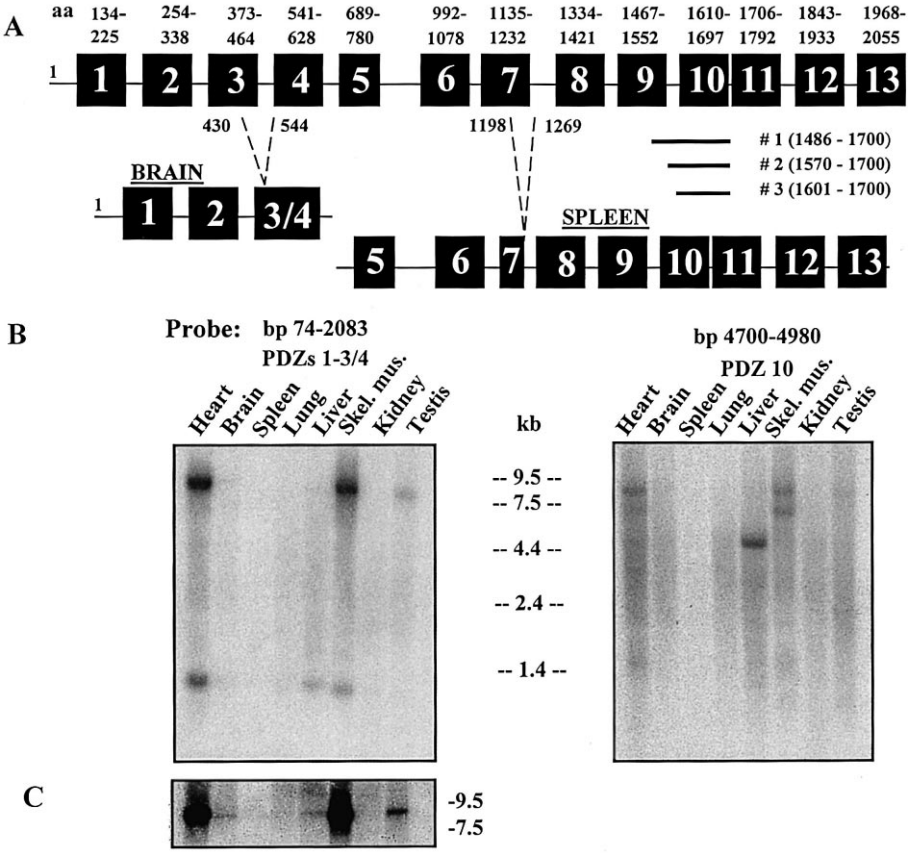

Fig. 1. Expression pattern of MUPP-1. A: Segmentation of the MUPP-1. Splice variants obtained from the spleen and brain cDNA libraries. 1–3: cDNA fragments detected by yeast two-hybrid screening of a murine embryo (11 days) cDNA library. B,C: Northern blot analyses of MUPP-1: ^{32}P-radiolabeled 5′ (74–2083) and 3′ (4700–4980) of MUPP-1 probes were hybridized to mouse MTN™ filter, Clontech Lab. Inc, Palo Alto, USA). B: 1 day exposure. C: 3 days exposure.

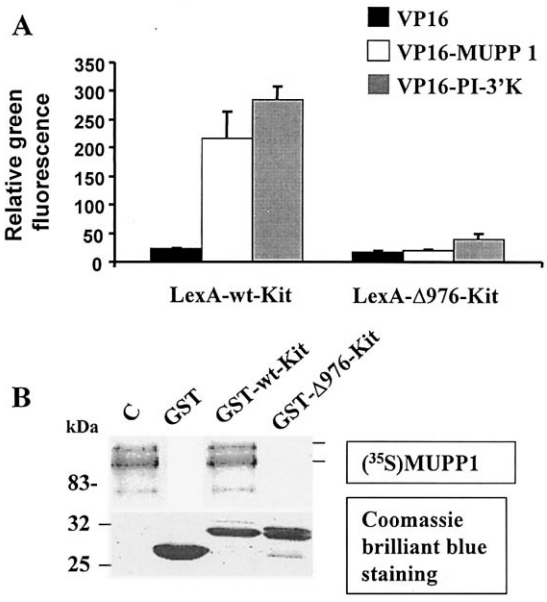

Fig. 2. Human c-Kit binds to MUPP-1 via the C-terminal end. A: Protein–protein interaction measured by the yeast two-hybrid analyses using the GFP gene as a reporter. Yeast strain YRN974 with an integrated LexA-Operator-GFP cassette was supplied for two-hybrid interaction analyses [18]. The flow cytometric quantification analyses of two-hybrid interactions of MUPP-1, and PI-3′-kinase (PI-3′K) with c-Kit and c-Kit mutants. pBTM116 carrying cDNAs which encode wt-c-Kit and the last V deletion mutant (Δ976-Kit) were transformed with either pVP16 vector alone (VP16), pVp16 carrying cDNAs encoding the 10th PDZ domain of MUPP-1 or the SH2 domains of the p85 subunit of PI-3′K. For each assay, standard deviations were calculated from four independent experiments. 10 000 cells of four independent transformants were analyzed for fluorescence intensity using a Becton Dickinson FACScan flow cytometer. B: GST fusion proteins containing the last 90 amino acids of the human c-Kit (c-Kit (877–976)) and the last V deletion mutant (Δ976-Kit) were incubated overnight at 4°C with ^{35}S-labeled MUPP-1, as generated by in vitro transcription/translation (Promega). Binding materials were analyzed by SDS–PAGE.

5′ (nucleotide number 74–2083) or 3′ (4700–4980) (numbers are according to the cDNA sequence published in Simpson et al. [17]). As shown in Fig. 1B, the expression level of MUPP-1 was rather weak, but the 8.5 kb MUPP-1 mRNA could be detected in all tissues after longer exposures (Fig. 1C). In short exposures, an 8.5 kb transcript was detected in tissues such as heart, skeletal muscle and testis using both 5′ and 3′ probes (Fig. 1B). In addition, we detected three additional transcripts of 7.5, 5.0 and 1 kb. Both the 7.5 kb and 5.0 kb mRNAs were only detectable using a 3′ probe. The 7.5 kb and 5.0 kb mRNAs were most predominant in skeletal muscle and liver, respectively. The 1.0 kb mRNA was only detectable, however, with the 5′ probe. At the moment we do not know whether these transcripts are derived from different genes or splice variants. In agreement with these data, Ullmer et al. [13] have also shown 8.5 and 5.0 kb mRNAs by Northern blot using a 3′ probe. Interestingly, another PDZ domain containing protein, PDZ-73, which contains three PDZ domains, has at least five different tissue-specific splice variants, encoding 73, 59, 54, 45, and 37 kDa proteins [24]. The biological significance of multiple transcripts of MUPP-1 remains to be studied.

3.2. Human c-Kit binds to MUPP-1 via the C-terminal end

In most of cases, PDZ domains interact with cytoplasmic tail sequences of proteins [9–15]. Analysis of the amino acid sequence of the C-terminus of human c-Kit revealed '-HDDV-COOH' the consensus sequence of the group II PDZ domain binding site [2]. To test whether the V976 of human c-Kit is required for this interaction, we generated a deletion mutant lacking V976 (Δ976-Kit). The cytoplasmic domain of the Δ976-Kit mutant was fused to LexA and the interaction with VP16-MUPP-1 by the yeast two-hybrid system was measured. To achieve better quantification of this interaction, we modified a currently available screening system [20] and generated a yeast strain, YRN974, in which the reporter gene, encoding the green fluorescent protein (GFP), was chromosomally integrated downstream of a LexA binding site [18,19,25]. Activation of GFP was quantified by flow cytometric analyses. As expected, the Δ976-Kit mutant failed to interact with MUPP-1 (Fig. 2B). To our surprise, however, this mutant lost its kinase activity in yeast (data not shown), and failed even to interact with the PI-3′-kinase (Fig. 2B). To further confirm the importance of the last V of c-Kit for this interaction, we generated GST fusion proteins containing the last 90 amino acids of the human wt-c-Kit (c-Kit (877–976)) and the Δ976 c-Kit (c-Kit(877–975)). As shown in Fig. 2C, GST–wt c-Kit interacted with in vitro ^{35}S-labeled last six PDZ domain, but GST–Δ976 c-Kit did not associate with MUPP-1.

3.3. The C-terminal end of c-Kit specifically binds to the 10th PDZ domain

Groups of PDZ domains have been classified by their recognition of unique C-terminal motifs [2]. Sequence 'HDDV' is

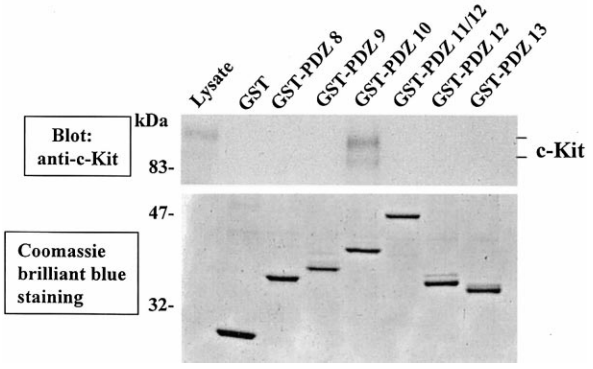

Fig. 3. 10th PDZ domain bound to the C-terminal end of c-Kit. GST, GST–PDZ8 (amino acid number 1334–1421), GST–PDZ9 (amino acid number 1467–1552), GST–PDZ10 (amino acid number 1570–1700), GST–PDZ11/12 (amino acid number 1706–1792), GST–PDZ12 (amino acid number 1843–1933), and GST–PDZ13 (amino acid number 1962–2054) were isolated from DH5α and were incubated overnight at 4°C with M-07e cell lysates. Beads were washed five times with binding buffer and pellets were analyzed by SDS–PAGE. For identification of c-Kit by immunoblotting, proteins were transferred onto Immobilon-P sheets (Millipore, Bedford, MA, USA) by a semi-dry blotting technique. Bound antibody was visualized by incubation of blots in 3 ml of 20 mM Tris–HCl, pH 7.6, containing 137 mM NaCl and 2 μCi of ^{125}I-labeled anti-rabbit immunoglobulin G (IgG, ICN). Bound radioactivity was quantified with a model BAS1500 bio-imaging analyzer (Fuji Photo Film Co., Kanagawa, Japan). Protein bands, stained with Coomassie brilliant blue, were quantified with a High Resolution Color Sharp JX-325 scanner.

recognized by the second group of PDZ domains, however, the 10th PDZ domain of MUPP-1 also binds to the low affinity receptor for the nerve growth factor, p75, (data not shown) whose C-terminal sequence is 'TSPV'. This motif, 'S/TxV', is recognized by the first group of PDZ domains. This fact raises the question of whether or not c-Kit binds specifically to a particular PDZ domain. Therefore, we tested the interaction of c-Kit with GST–PDZ8, GST–PDZ9, GST–PDZ10, GST–PDZ11/12, GST–PDZ12, and GST–PDZ13. As shown in Fig. 3, only GST–PDZ10 specifically bound to c-Kit.

3.4. Kinase negative c-Kit bound to MUPP-1 but not with oncogenic c-Kit

To see whether the binding of MUPP-1 and c-Kit correlates with tyrosine kinase activity, we generated two kit mutants: the kinase negative mutant (K623M) and the constitutively active oncogenic-form of c-Kit (c-KitD816V). As shown in Fig. 4, the kinase negative mutant bound more strongly than the wt c-Kit. In addition, the oncogenic mutant (D816V) failed to bind to MUPP-1. The oncogenic mutant, however, bound to the SH2 domain of PI-3′-kinase to a similar extent as the wt c-Kit and also associated with the SH2 domain of Grb10. In this assay system, Grb10 did not interact with the wt c-Kit (Fig. 3), indicating that the oncogenic c-KitD816V is more active than wt c-Kit in our yeast assay system. Indeed, phosphotyrosine specific immunoblot analyses revealed that oncogenic D816V mutant was phosphorylated about 20 fold more than wt c-Kit in yeast cells (data not shown). In agreement with these data, Yokouchi et al. [26] reported that oncogenic D816V mutant was highly active in yeast cells. Taken together, MUPP-1 may form a complex with c-Kit before stimulation with SCF, but is released from this complex after hyperactivation of the c-Kit tyrosine kinase.

We show here that c-Kit associates with the 10th PDZ domain of MUPP-1 via its C-terminal tail. This interaction

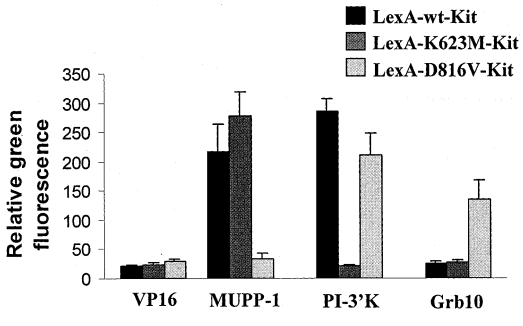

Fig. 4. Kinase negative c-Kit associates with MUPP-1 but not with oncogenic D816V-c-Kit. Protein–protein interaction measured by the yeast two-hybrid analyses using the GFP gene as a reporter. The flow cytometric quantification analyses of two-hybrid interactions of MUPP-1, PI-3′-kinase (PI-3′K) or Grb10 with c-Kit and c-Kit mutants. pBTM116 carrying cDNAs which encode wt-c-Kit, oncogenic c-Kit (D816V), and kinase negative mutant K623M-c-Kit were transformed with either pVP16 vector alone (VP16), pVp16 carrying cDNAs encoding the 10th PDZ domain of MUPP-1, or the SH2 domains of Grb10 or of the p85 subunit of PI-3′-kinase (PI-3′K). For each assay, standard deviations were calculated from four independent experiments. 10 000 cells of four independent transformants were analyzed for fluorescence intensity using a Becton Dickinson FACScan flow cytometer.

was shown by yeast two-hybrid assay and in vitro interaction study of c-Kit using GST–PDZ fusion proteins. Furthermore, we demonstrated that the binding of PDZ domain of MUPP-1 to the c-Kit is dependent on the catalytic activity of the receptor. The oncogenic D816V-c-Kit was not able to interact with MUPP-1, and wt c-Kit bound to MUPP-1 to a lesser extent than kinase negative c-Kit. This is the first evidence demonstrating that the C-terminal structure of oncogenic c-Kit is different from that of wt c-Kit. Interestingly, a single deletion of 'V' at the C-terminal end abolished the kinase activity, suggesting again that the structure of the C-terminal tail plays a key role in regulating c-Kit kinase activity. In this context, the viral oncogene product of Hardy-Zuckerman-4 strain of feline sarcoma virus, v-Kit, also has an altered C-terminal end [27]. Furthermore, this portion of the receptor acts as negative regulator of tyrosine kinase in many other receptor tyrosine kinases, including c-Fms and epidermal growth factor receptor [28–30]. For example, the replacement of a tyrosine residue at the C-terminal end of c-Fms with phenylalanine activates the Fms tyrosine kinase [28]. This tyrosine residue, however, is not phosphorylated, but rather the mutation modifies the tertiary structure of the CT of c-Fms.

It is generally accepted that PDZ domains play a role in protein clustering, so that signaling is triggered in a most efficient way. MUPP-1 may support signaling molecules expressed at very low levels for efficient participation of signaling cascades induced by SCF. Interestingly, MUPP-1 was isolated as a binding protein for the G protein-coupled receptor 5-HT2c [13]. MUPP-1 may be a connector between G-coupled receptor- and receptor tyrosine kinase-mediated signal transduction pathways.

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