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The Catenin p120^{ctn} Interacts with Kaiso, a Novel BTB/POZ Domain Zinc Finger Transcription Factor

JULIET M. DANIEL AND ALBERT B. REYNOLDS*

Department of Cell Biology, Vanderbilt University, Nashville, Tennessee 37232-2175

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p120^{ctn} is an Armadillo repeat domain protein with structural similarity to the cell adhesion cofactors β-catenin and plakoglobin. All three proteins interact directly with the cytoplasmic domain of the transmembrane cell adhesion molecule E-cadherin; β-catenin and plakoglobin bind a carboxy-terminal region in a mutually exclusive manner, while p120 binds the juxtamembrane region. Unlike β-catenin and plakoglobin, p120 does not interact with α-catenin, the tumor suppressor adenomatous polyposis coli (APC), or the transcription factor Lef-1, suggesting that it has unique binding partners and plays a distinct role in the cadherin-catenin complex. Using p120 as bait, we conducted a yeast two-hybrid screen and identified a novel transcription factor which we named Kaiso. Kaiso's deduced amino acid sequence revealed an amino-terminal BTB/POZ protein-protein interaction domain and three carboxy-terminal zinc fingers of the C_2H_2 DNA-binding type. Kaiso thus belongs to a rapidly growing family of POZ-ZF transcription factors that include the *Drosophila* developmental regulators Tramtrak and Bric à brac, and the human oncoproteins BCL-6 and PLZF, which are causally linked to non-Hodgkins' lymphoma and acute promyelocytic leukemia, respectively. Monoclonal antibodies to Kaiso were generated and used to immunolocalize the protein and confirm the specificity of the p120-Kaiso interaction in mammalian cells. Kaiso specifically coprecipitated with a variety of p120-specific monoclonal antibodies but not with antibodies to α- or β-catenin, E-cadherin, or APC. Like other POZ-ZF proteins, Kaiso localized to the nucleus and was associated with specific nuclear dots. Yeast two-hybrid interaction assays mapped the binding domains to Arm repeats 1 to 7 of p120 and the carboxy-terminal 200 amino acids of Kaiso. In addition, Kaiso homodimerized via its POZ domain but it did not heterodimerize with BCL-6, which heterodimerizes with PLZF. The involvement of POZ-ZF proteins in development and cancer makes Kaiso an interesting candidate for a downstream effector of cadherin and/or p120 signaling.

p120^{ctn} (hereafter referred to as p120) is the prototype for a conserved subfamily of Armadillo-related proteins that include ARVCF, p0071, δ-catenin/NPRAP, and plakophilins 1 and 2 (22–25, 42, 55, 59, 62, 67, 78) (reviewed in reference 60). Originally identified as a prominent substrate of the Src tyrosine kinase, p120 is also tyrosine phosphorylated in cells stimulated by epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor 1 (15, 31), implying a role in ligand-induced signaling and cell transformation. In addition, p120 localizes to sites of cell-cell contact and coprecipitates with multiprotein complexes containing E-cadherin and its cytoplasmic cofactors, the α-, β-, and γ-(plakoglobin) catenins (59, 66, 68). Like the prototypical catenins, β-catenin and plakoglobin, p120 binds directly to E-cadherin via its Armadillo repeat domain (9) and interacts with other members of the classical cadherin family (61). These observations strongly suggest a role for p120 in regulating cadherin function.

The importance of the Arm domain in protein-protein interactions is best illustrated by β-catenin which, via its Arm domain, forms mutually exclusive complexes with either E-cadherin, the tumor suppressor adenomatous polyposis coli (APC), or the transcription factor TCF/Lef-1 (T cell factor/lymphoid enhancing factor 1) (3, 28, 29, 45, 64, 69). Interestingly, β-catenin interacts with each of these proteins at different subcellular locations (E-cadherin–cell membrane, APC-cytosol, and Lef-1–nucleus), to perform unique functions in cell-cell adhesion and/or signaling. Recently, a p120-related Armadillo repeat protein, plakophilin 2, was localized to both cell junctions and the nucleus (42), indicating that this duality of function and subcellular localization may be applicable to other Armadillo family proteins.

p120 coprecipitates in E-cadherin complexes with either β-catenin or plakoglobin, indicating that it binds E-cadherin simultaneously at a site distinct from β-catenin and plakoglobin (9, 59). In most cell types, p120 exists as multiple isoforms (33, 44, 59) which probably compete for cadherin binding, in a manner akin to the competition between β-catenin and plakoglobin. The β-catenin and plakoglobin binding site maps to a carboxy-terminal region of the E-cadherin cytoplasmic domain (30, 46, 47, 52, 54), while the p120 binding site has been mapped to the juxtamembrane region (38, 70, 75). Deletion analysis of this juxtamembrane region has revealed crucial roles in regulating cadherin function (35, 53, 63, 75). For example, clustering of C-cadherin requires the juxtamembrane region (75). Moreover, in developing *Xenopus* embryos, cadherin mutants possessing the juxtamembrane region but lacking the catenin-binding domain display dominant-negative effects resulting in loss of cell adhesion (35). In tissue culture experiments analogous vascular endothelial cadherin mutants promote cell aggregation (48), and Chen et al. (5) reported a role for the juxtamembrane region in cell motility. Whether p120 directly mediates these effects is still unclear.

The differences between p120 and β-catenin imply unique roles for these proteins which are likely to be mediated, in part, through interaction with unique binding partners (9). To identify novel p120-specific interactions, we performed a yeast two-hybrid screen using p120 as bait. Here, we describe the cloning and characterization of a p120-interacting protein which we have named Kaiso. Kaiso specifically and efficiently interacts

* Corresponding author. Mailing address: Department of Cell Biology, Vanderbilt University, 1161 21st Ave. South, Nashville, TN 37232-2175. Phone: (615) 343-9533. Fax: (615) 343-4539. E-mail: al.reynolds@mcmail.vanderbilt.edu.

with p120 but not with β-catenin or other known components of the cadherin-catenin complex. Kaiso is a novel member of the rapidly growing BTB/POZ (Broad complex, Tramtrak, Bric à brac/Pox virus and zinc finger) family of zinc finger (ZF) transcription factors (hereafter referred to as POZ-ZF proteins) (reviewed in references 1 and 2). Like other POZ-ZF proteins, Kaiso homodimerizes via its POZ domain and localizes to the nucleus. Interestingly, all members of the POZ family so far identified are involved in development and/or cancer. For example, the human BCL-6 and promyelocytic leukemia ZF (PLZF) proteins are causally involved in non-Hodgkins' lymphoma and acute promyelocytic leukemia (APL), respectively, and they both form macromolecular complexes with nuclear transcriptional machinery regulators, such as histone deacetylase and the corepressors SMRT (silencing mediator of retinoid and thyroid receptor) or NCoR (nuclear corepressor) (10, 13, 19, 26, 40). The identification of Kaiso as a novel transcription factor and p120-binding partner, coupled with its ubiquitous expression in a wide range of tissues, makes Kaiso an interesting candidate for mediating p120 signaling effects in normal cells and in metastatic tumors.

MATERIALS AND METHODS

Cells and tissue culture. All cell lines were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, 2% L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Transfections and immunofluorescence. Cells were plated onto coverslips and transfected with Lipofectamine reagent (Gibco/BRL, Gaithersburg, Md.) or Superfect (Qiagen, Valencia, Calif.) according to the manufacturer's protocol. After 48-h incubation at 37°C, transfected cells were fixed for 7 min at −20°C in 100% methanol before blocking with 3% milk in phosphate-buffered saline (PBS) (pH 7.4). Primary antibody incubations were performed at room temperature (RT) for 30 min in 3% milk–PBS, at 0.5 to 1.0 μg/ml unless noted otherwise. Coverslips were then washed three times for 5 min each time with PBS (pH 7.4) before incubation at RT for 30 min with Cy3-conjugated donkey anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories, Westgrove, Pa.) in 3% milk–PBS, at 1:400 dilution. The coverslips were finally washed three times for 5 min each time with PBS (pH 7.4), mounted onto glass slides with Aqua Poly/Mount (Polysciences Inc., Warrington, Pa.), and examined with a Zeiss Axiophot fluorescent microscope.

Immunoprecipitation and Western blot analysis. Cells were lysed at 0°C and briefly sonicated in a buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM sodium vanadate, 0.1 trypsin inhibitor units of aprotinin, and 5 μg of leupeptin per ml. The proteins were immunoprecipitated from cell lysates, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37), and transferred to nitrocellulose membranes by using the Hoeffer semi-dry apparatus (Pharmacia, Piscataway, N.J.). Blots were briefly blocked at RT with 3% nonfat dried milk in Tris-buffered saline (TBS) (pH 7.4) and incubated overnight at 4°C with primary antibodies (p120 monoclonal antibodies [MAbs] at 1.0 μg/ml or Kaiso polyclonal antibodies at 1:1,000 dilution) in 3% milk–TBS. The membranes were then washed five times with TBS before incubation with the secondary antibody, peroxidase-conjugated donkey anti-mouse antibody (Jackson Immunoresearch Laboratories), in 3% milk–TBS for 2 h at RT. Blots were finally washed five times with TBS and then processed with the enhanced chemiluminescence immunodetection system (Amersham, Cleveland, Ohio) according to the manufacturer's protocols.

The generation and characterization of Kaiso MAbs (MAbs 6F8, 11D7, 12G2, and 12H9) are described elsewhere (9a). These antibodies were raised against purified six-histidine-tagged Kaiso antigens containing either amino acids 1 through 499 or amino acids 103 through 499 of Kaiso. Antibodies against p120 (MAbs 15D2, 8D11, 5A7, and 6H11) have been previously described (74). Rabbit polyclonal antibodies to β-catenin and α-catenin were purchased from Sigma (St. Louis, Mo.). Anti-E-cadherin and anti-BCL-6 MAbs were purchased from Transduction Laboratories (Lexington, Ky).

Yeast two-hybrid system. A full-length murine p120 cDNA was subcloned in frame into the pGBT9 DNA-binding domain vector (Clontech, Palo Alto, Calif.) and used to screen a murine liver activation domain (pGAD10) cDNA library (MATCHMAKER Two Hybrid System; Clontech). Approximately 3.3×10^6 transformants were screened according to the manufacturer's protocol. The DNA-binding domain and activation domain plasmids were segregated according to the manufacturer's protocol, and cDNAs from the activation domain segregants, encoding putative p120-interacting proteins, were isolated and retransformed with pGBT9-p120 into the HF7C yeast strain. Clones which did not transactivate with the DNA-binding vector alone but interacted with pGBT9-p120 were sequenced. The panel of pGBT9-p120 deletion mutants has been previously described (9). Kaiso deletion constructs were generated by PCR amplification of the desired fragments, which were then subcloned in frame into the pGAD424 activation domain vector or the pGBT9 DNA-binding domain vector. To test for protein-protein interactions, the appropriate pair of plasmids was cotransformed into the yeast strain HF7C by the lithium acetate-polyethylene glycol method according to the Clontech MATCHMAKER protocol. Transformants were grown and analyzed as previously described (9).

cDNA library screen. To obtain full-length Kaiso cDNAs, a total of 10^6 PFU from two λZapII (Stratagene, La Jolla, Calif.) cDNA libraries (spleen and lung) were screened according to the manufacturer's protocol by using the longest Kaiso clone (F-38) as a probe. Eighteen positive clones were put through a secondary screen, and six positive clones resulted. These positive phages were subjected to the in vivo excision protocol to obtain pBluescript phagemid for further analysis. Phagemid cDNAs were sequenced by the dideoxynucleotide chain termination method using the T7 Sequenase version 2.0 sequencing kit (Amersham).

Northern blot analysis. One hundred nanograms of cDNA of the longest Kaiso clone (F-38) was labelled with [^{32}P]dCTP (Dupont NEN, Boston, Ma.) and the Prime-it II random primer labelling kit (Stratagene), purified on a Sephadex column, and used to probe a murine multiple tissue Northern blot (Clontech) by using the ExpressHyb Hybridization Solution (Clontech) as outlined by the manufacturer. The blot was washed in a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, 40 min at RT, followed by a wash with 1× SSC–0.1% SDS, 20 min at 50°C, and finally a wash with 0.1× SSC–0.1% SDS for 20 min at 50°C. The blot was then exposed overnight to RX film (Fuji).

Plasmids and constructs. The pBluescript-S11/38 (pBS-S11/38) Kaiso construct was made as follows: nucleotides (nt) 1160 to 2690 were removed from pBS-S11 by digestion with *Nde*I and *Bam*HI and replaced with an *Nde*I-*Bgl*I fragment containing nt 290 to 1725 of pGAD-F-38. To remove the 5′ untranslated region (UTR) (~200 bp) from pBS-S11 and pBS-S11/38, the plasmids were digested with the *Sac*I restriction enzyme and ligated to *Sac*I-digested pBS to generate pBS-S11(Sac) and pBS-S11/38(Sac). These vectors were then used to generate eucaryotic expression vectors lacking the 5′ UTR by ligating blunt-ended *Sac*I fragments [from pBS-S11(Sac) and pBS-S11/38(Sac) respectively] into an *Eco*RV-digested and dephosphorylated pcDNA3 vector (Invitrogen, Carlsbad, Calif.).

pGBT9-BCL-6 (POZ domain only) and pActII-PLZF (full length) were kind gifts from Dominic Leprince and Arthur Zelent, respectively. Full-length Kaiso yeast two-hybrid constructs were made by ligating the *Eco*RI fragment from pcDNA3-S11/38(Sac) into *Eco*RI-digested and dephosphorylated pGBT9 or pGAD424. Additional Kaiso deletion constructs were generated by PCR amplification of the desired fragments by using Kaiso-specific oligonucleotides containing enzyme restriction sites (shown in lowercase letters in the primer sequences given below) for subsequent subcloning and ligation into *Eco*RI-*Bam*HI-digested pGBT9 or pGAD424. Kaiso-POZ (nt 217 to 663) was amplified by using the primers 5′-ccggaattcTCTTTGTGTGGGTCCCGG-3′ and 5′-acgggatcccTCAGCAGTACCATCCTG-3′, Kaiso-Zn (nt 1705 to 2019) was amplified by using the primers 5′-ccggaattcTTAATAGTAGATGGAAGG-3′ and 5′-acgggatcccTTGCATGGATGTAAGCG-3′, KaisoΔPOZ (nt 1594 to 2295) was amplified with the primers 5′-ccggaattcAAAGATGACCCTCATGGG-3′ and 5′-acgggatcccAAACTTTCTGATATTCC-3′, Kaiso-ZnΔC-term (nt 1597 to 2015) was amplified by using the primers 5′-ccggaattcAAAGATGACCCTCATGGG-3′ and 5′-acgggatcccTTGCATGGATGTAAGCG-3′ and Kasio-ZnΔN-term (nt 1707 to 2295) was amplified by using the primers 5′-ccggaattcTTAATAGTAGATGGAAGG-3′ and 5′-acgggatcccAAACTTTCTGATATTCC-3′. The KaisoΔPOZΔZn (nt 598 to 1782) was amplified with the primers 5′-gcgcgaattcGAGCTTGGTGTCCCACTG-3′ and 5′-gcgcctcgagTCTTCGCAAGCTTGTCAG-3′ and ligated into *Eco*RI-*Sal*I-digested pGBT9 or pGAD424.

GenBank accession number. The Kaiso cDNA sequence has been deposited in the GenBank/EMBL database under accession no. AF097416.

RESULTS

Isolation and identification of a p120-binding protein, Kaiso. To identify novel p120 binding partners, we screened a murine liver MATCHMAKER activation domain library (Clontech) by using a full-length p120 DNA-binding construct as bait (pGBT9-p120). Of 122 clones selected by growth on SD-His-Leu-Trp minimal medium agar plates, 63 clones were positive for activation of the *lacZ* gene (β-galactosidase assay). Five of these clones interacted specifically with p120 upon retransformation in yeast, and they turned out to be partial cDNAs of the same gene, which we named *kaiso*. The three shortest cDNAs, F-15, F-17, and F-32 (each 930 bp) were identical to each other and encoded a peptide of 220 amino acids with three ZF motifs of the C_2H_2 Kruppel-like DNA-binding type. The two larger cDNAs, F-9 and F-38, were like-

wise identical to each other but extended an additional 800 bp upstream of the two shorter clones to encode a 470-amino-acid peptide with no additional characteristic motifs.

To obtain full-length cDNAs, clone F-38 was used to screen lung and spleen λZapII cDNA libraries. The longest clone identified, S11, was 2.5 kb and extended an additional 700 bp upstream or 5′ of clone F-38 (Fig. 1A) to encode an additional sequence of 220 amino acids containing an initiation codon with a good Kozak sequence (36). In addition, its C terminus overlapped with and included a sequence identical to clone F-38 except that 15 bp upstream of the putative stop codon the S11 sequence diverged and encoded an additional sequence of 120 amino acids at its C terminus (Fig. 1A). This 3′ extreme S11-unique sequence appears to be a cloning artifact, but we have not ruled out the possibility that it may encode an alternative splice form. However, a human *kaiso* homolog recently appeared in the databases as an expressed sequence tag (EST) sequence (see below) and predicts an open reading frame (ORF) and stop codon identical to that of clone F-38 (Fig. 1C).

A BLAST search with the deduced amino acid sequence (Fig. 1B) revealed homology with a family of proteins containing a novel protein-protein interaction domain named the POZ (pox zinc finger) or BTB (Broad complex, Tramtrak, Bric à brac) domain (1, 2). This highly conserved, hydrophobic domain of approximately 120 amino acids is located at the extreme N terminus of these proteins, many of which also contain carboxy-terminal ZF motifs (Fig. 1A and B). Kaiso has the 6 invariant residues as well as 31 other highly conserved residues characteristic of POZ proteins (Fig. 2A). Kaiso's three carboxy-terminal C_2H_2 ZFs fit the characteristic motif [F/Y-X-C-X_2-C-X_{12}-H-$X_{3\text{-}4}$-H-X_5] (where C is cysteine, H is histidine, X is any amino acid, F is phenylalanine, and Y is tyrosine) considered diagnostic of transcription factors (16). In addition, the most-conserved residues between the second cysteine and the first histidine are highly basic, polar, or hydrophobic, implicating them as DNA-contacting residues (Fig. 2B).

Kaiso was most closely related to murine ZF-5, a protein identified during a screen for *myc*-promoter binding proteins (49), and human PLZF, a protein associated with translocation breakpoints in APL (6, 7). BLAST analysis with the *kaiso* nucleotide sequence subsequently identified a highly conserved human EST sequence (accession no. 002086), that mapped to the X-chromosome. This sequence localized to chromosome Xq23 and was 82% identical to *kaiso* at the nucleotide level, while its deduced amino acid sequence was 87% identical to Kaiso (Fig. 1C). The human EST predicts the same initiation codon as the *kaiso* S11 clone, thus supporting our assignment of the ORF. In addition, we have mapped murine *kaiso* to mouse chromosome X, indicating that the human EST and murine *kaiso* are synteneic (data not shown).

Kaiso coprecipitates with p120 in MDCK and HCT116 cells. To verify that Kaiso was a bona fide p120 binding partner, we performed coprecipitation studies to determine whether p120 and Kaiso interacted in vivo. Kaiso coprecipitated efficiently and specifically with several p120-specific MAbs (15D2 and 12F4) from MDCK and HCT116 cell lysates (Fig. 3, lanes 4 and 6). The p120 MAb 8D11, which reacts with canine (MDCK) but not human (HCT116) p120 (74), provided a stringent internal specificity control. This antibody selectively coprecipitated Kaiso from MDCK but not from HCT116 cell lysates (Fig. 3A, compare lanes 5 and 12), highlighting the requirement for p120 in the immunoprecipitate and verifying the specificity of the p120-Kaiso interaction. Although somewhat variable, the stoichiometry and specificity of coprecipitation appeared to be quite high; approximately 10 to 50% of endogenous Kaiso coprecipitated with p120 MAbs but not with the control antibody KT3 (Fig. 3A; compare directly immunoprecipitated Kaiso, shown in lanes 1 and 8, with p120-coimmunoprecipitated Kaiso, shown in lanes 4, 5, 6, 11, and 13) (Fig. 4; compare lanes 1 and 5). Because Kaiso is primarily nuclear, we compared p120 coprecipitates from sonicated and nonsonicated cell lysates; no significant differences were observed (data not shown). Hence, the subcellular location of the p120-Kaiso interaction remains unclear. Interestingly, MAbs 6H11 and 5A7, which were generated to an amino-terminal p120 epitope and recognize only the p120 type 1 isoforms, did not coprecipitate Kaiso from either cell line (Fig. 3A, lanes 2, 3, 9, and 10). This observation raises the possibility that Kaiso interacts only with the smaller p120 type 3 isoforms. Alternatively, the very low levels of p120 type 1 isoforms in these cells may preclude detection of an interaction with Kaiso. Figure 3B shows p120 Western blots of the same immunoprecipitates to indicate the amount of p120 precipitated by each p120-specific antibody. It also demonstrates the efficient immunoprecipita-

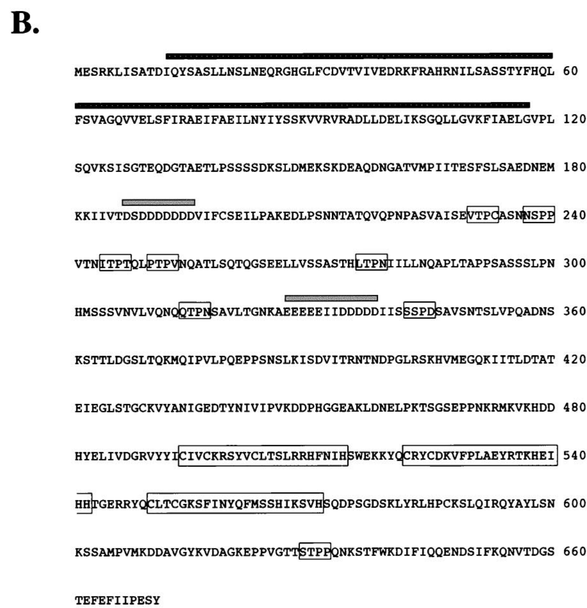

FIG. 1. (A) Schematic representation of Kaiso structure. The POZ domain and three C_2H_2 ZFs are indicated. The longest cDNA clone (S11) is shown aligned with clone F-38, which was isolated from the yeast two-hybrid screen. (B) Kaiso's deduced amino acid sequence. The POZ domain residues (amino acids 12 to 117) are highlighted by a hatched line above the sequence, while the residues of the three ZFs are boxed and shaded. Eight proline-dependent serine or threonine phosphorylation sites are boxed, and the two highly acidic regions are highlighted by a gray line above the sequence. (C) Kaiso alignment with its human homolog. Kaiso has 87% identity with the deduced amino acid sequence of a human EST which localizes to chromosome Xq23. Most amino acid differences are conservative changes and occur outside the highly conserved POZ and ZF domains. This human EST, coupled with comigration of overexpressed and endogenous Kaiso, confirms the designation of Kaiso's initiation and termination codons.

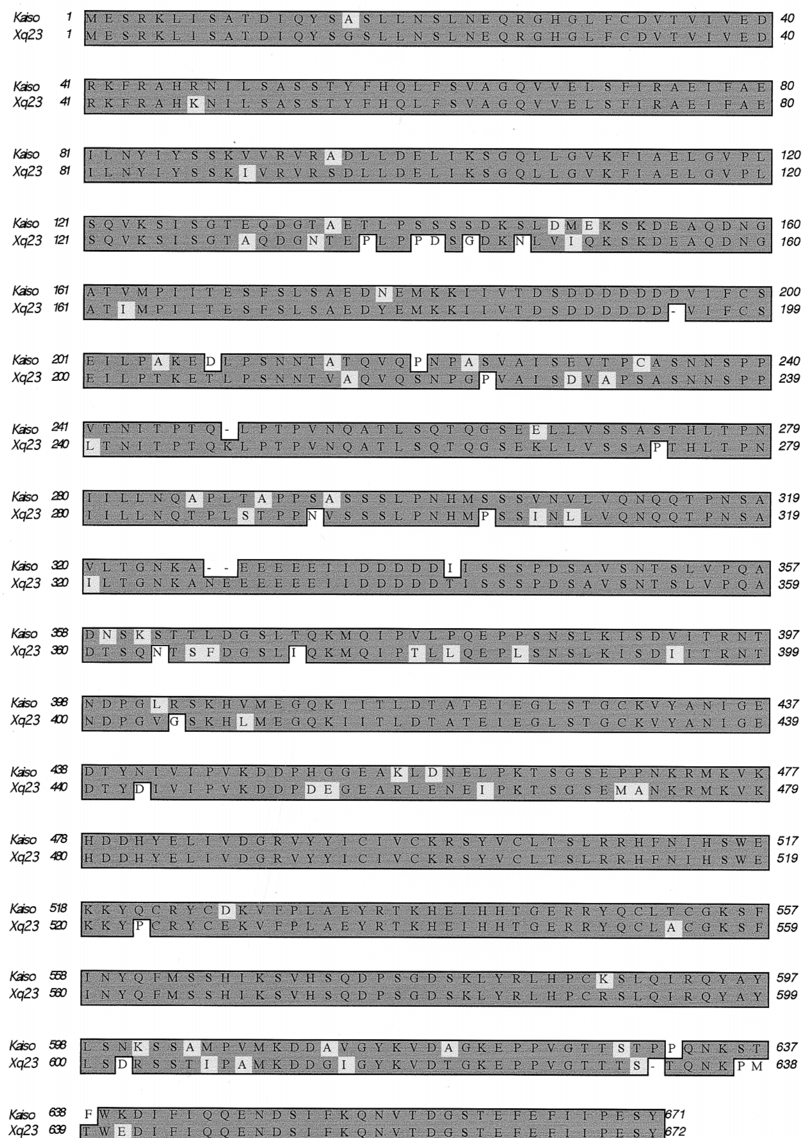

FIG. 1—*Continued.*

tion of p120 by MAb 8D11 from canine (MDCK) but not human (HCT116) cells. Attempts to get good reciprocal coimmunoprecipitates of p120 with Kaiso MAbs were unsuccessful. These MAbs were generated to an amino-terminal region of Kaiso which may be blocked sterically by association with p120. Therefore, any Kaiso immunoprecipitated by Kaiso-specific antibodies may for the most part not be associated with p120.

Kaiso does not interact with β-catenin, α-catenin, or E-cadherin. To further confirm the specificity of the Kaiso-p120 interaction, we immunoprecipitated individual components of the cadherin-catenin complex to determine if (i) Kaiso coprecipitates with other Arm repeat proteins (e.g., β-catenin) or if (ii) Kaiso could interact with cadherin complexes. In both MDCK and HCT116 cells, there was a strong and efficient coprecipitation of Kaiso by p120 only and not by any other component of the cadherin-catenin complex (Fig. 4). All of the antibodies used in these experiments efficiently immunoprecipitated their respective antigens (data not shown). The absence of Kaiso in E-cadherin immunoprecipitates suggests that Kaiso is not present in cadherin complexes and that p120 forms mutually exclusive complexes with either Kaiso or E-cadherin. This also suggests that Kaiso-p120 complexes localize to the cytosol and/or the nucleus.

Kaiso localizes to nuclear dots. Several POZ proteins (e.g., PLZF, BCL-6, and ZID) have been shown to localize to nuclear dots upon transient transfection of their full-length cDNAs (2, 12, 14). Immunofluorescence analysis of endogenous Kaiso revealed both diffuse and punctate nuclear staining which varied with cell type. For example, in MDCK cells, immunofluorescent staining with several Kaiso MAbs and polyclonal antibodies revealed a primarily diffuse nuclear localization (Fig. 5A), whereas in NIH 3T3 cells, nuclear dots were evident (Fig. 5C). Ectopically expressed wild-type Kaiso accumulated to high levels in the nucleus and was both dif-

A.

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Kaiso    IQYSASLLNSLNEQRGHGLFCDVTVIVEDRKFRAHRNILSASSTYFHQLFS---V
Xq23     IQYSGSLLNSLNEQRGHGLFCDVTVIVEDRKFRAHKNILSASSTYFHQLFS---V
PLZF     PSHPTGLLCKANQMRLAGTLCDVVIMVDSQEFHAHRTVLACTSKMFEILFHR---
Zn-5     DDHKTLFLKTLNEQRLEGEFCDIAIVVEDVKFRAHRCVLAACSTYFKKLFKKLEV

Consen   .......L.....QR..G.FCDV.V.VE...F.AHR.ILS..S.YF..LF.....

Kaiso    AG-QVVELSFIRAEIFAEILNYIYSSKVVRVRADLLDELIKSGQLLGVKFIAELG
Xq23     AG-QVVELSFIRAEIFAEILNYIYSSKIVRVRSDLLDELIKSGQLLGVKFIAELG
PLZF     NS-QHYTLDFLSPKTFQQILEYAYTATLQ-AKAEDLDDLLYAAEILEIEYLEEQC
Zn-5     DSSSVIEIDFLRSDIFEEVLNYMYTAKIS-VKKEDVNLMMSSGQILGIRFLDKLC

Consen   .....V.L.......F..IL.Y.Y..........L..LI..G..L.V..I....
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B.

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ZF-1     YICIVCKRSYVCLTSLRRHFNIH
ZF-2     YQCRYCDKVFPLAEYRTKHEIHH
ZF-3     YQCLTCGKSFINYQFMSSHIKSVH
Consen   Y.C..C...F.....L..H...H
```

FIG. 2. Functional Kaiso motifs. (A) POZ domain comparison. Alignment of amino acid sequences of POZ domains of the most closely related POZ family proteins, murine PLZF and ZF-5 (Zn-5), is shown. A consensus sequence (Consen) (2) is shown for comparison. Kaiso has all 37 highly conserved residues found in the majority of POZ proteins as well as the 6 invariant residues (underlined). (B) Alignment of Kaiso's three ZFs. Kaiso has three conserved ZFs of the C_2H_2 type that is commonly associated with DNA-binding transcription factors. All three fingers fit the consensus sequence [(F/Y)XCX$_2$CX$_{12}$HX$_{3-4}$H]. While the HX$_3$H (ZF-1 and ZF-2) spacing is more common than HX$_4$H (ZF-3), it is believed that HX$_4$H gives more flexibility for DNA binding.

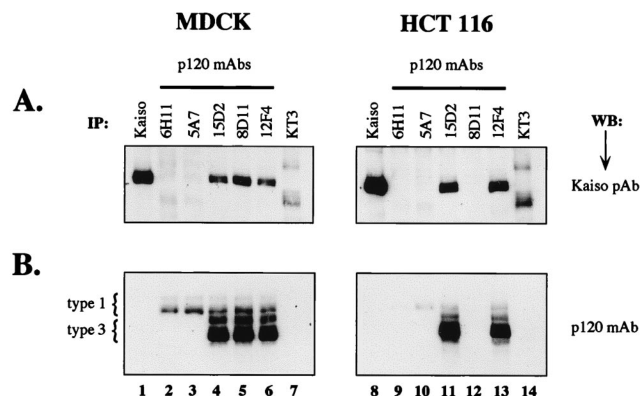

FIG. 3. p120 coprecipitates Kaiso from MDCK and HCT116 cells. Whole-cell lysates were immunoprecipitated with the indicated antibodies, separated by SDS-PAGE, transferred to nitrocellulose, and then Western blotted with Kaiso-specific polyclonal antibody (pAb). (A) Kaiso was coprecipitated by p120 MAbs 15D2 and 12F4 from both MDCK and HCT116 cells (lanes 4, 6, 11, and 13). The p120 MAb 8D11, which does not cross-react with human p120, coprecipitated Kaiso from canine MDCK cells but not from human HCT116 cells, highlighting the requirement for p120 in the immunoprecipitate (compare lanes 5 and 12). In addition, p120 MAbs 6H11 and 5A7, which recognize only the p120 type 1 isoforms, did not coprecipitate Kaiso, consistent with the poor expression of this splice form in epithelial cells. (B) The reciprocal Western blot (WB) indicates the levels of p120 immunoprecipitated by the p120 MAbs and illustrates the specificity of MAb 8D11 for canine (MDCK) but not human (HCT116) p120.

fusely distributed (Fig. 5B) and associated with punctate nuclear dot structures (data not shown). In a small fraction of transfected cells, Kaiso was clearly both cytosolic and nuclear (Fig. 5D). The cytosolic localization did not correlate directly with the level of Kaiso overexpression, suggesting that it is more likely due to cell cycle-related phenomena rather than to saturation of the nuclear capacity for overexpressed Kaiso.

Kaiso mRNA expression. High-stringency Northern blot analysis of a murine multiple tissue Northern blot performed by using a *kaiso* cDNA fragment (nt 870 to 2583) as a probe detected an approximately 5- to 6-kb message in all tissues, indicating ubiquitous expression of *kaiso* (Fig. 6A). The larger size of the message compared to the 2,100-bp ORF is reminiscent of other POZ-ZF proteins, which also have unusually large mRNAs compared to their ORF and cDNA length (2, 41, 51, 72). For example, the 2,019-bp coding sequence of PLZF is in stark contrast to the 7.5-kb mRNA detected by Northern blot analysis (7). These large messages may be explained by the fact that several POZ-ZF proteins have long 3′ UTR upstream of the poly(A) signal (AAUAAA) (73) (e.g., *Drosophila* Pipsqueak, Abrupt, and Tramtrak) (21, 27, 58, 72). The longest Kaiso clone had a 3′ UTR of only 300 bp and lacked a poly(A) signal (data not shown), suggesting that *kaiso* probably has additional 3′ UTR sequences.

Kaiso expression in different cell lines. To characterize Kaiso expression at the protein level, we analyzed a panel of cell lines by immunoprecipitation and Western blotting using Kaiso-specific MAbs. Kaiso was expressed and detected as a doublet of approximately 95 kDa in most cell lines examined (Fig. 6B), including colon (HCT116 and DLD-1), breast (MCF-7 and MDA-231), prostate (MLL, MC26, and AT2), and kidney (MDCK and COS) cell lines (Fig. 6B). Interestingly, Kaiso's apparent molecular mass (95 kDa) was larger than its predicted molecular mass (80 kDa) (Fig. 6B). We also observed that fibroblasts such as NIH 3T3 and CHO cells expressed a slightly larger, slower-migrating Kaiso doublet (~110 kDa) than their epithelial counterparts (Fig. 6B, lanes 1 and 4). This could be due to alternative splicing, a fibroblast-specific posttranslational modification, or species differences (rodent versus human). This phenomenon of a larger apparent molecular mass than predicted has also been reported for other POZ proteins, such as BCL-6, PLZF, Pipsqueak, Bach 1, and Bach 2 (14, 50, 51, 72), suggesting that POZ proteins may have some inherent protein folding conformation or modification which results in their aberrant migration on SDS-PAGE. Whether the Kaiso doublet detection and slow mobility reflect alternate splicing or posttranslational modification, such as

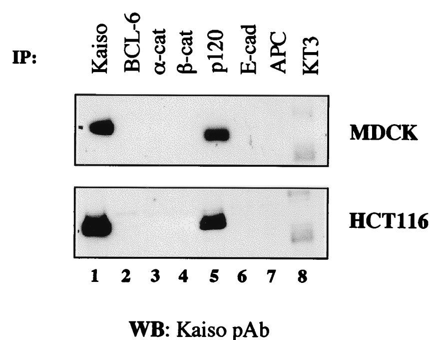

FIG. 4. Kaiso interacts specifically with p120. Whole-cell lysates from MDCK and HCT116 cells were immunoprecipitated with antibodies to different components of the cadherin-catenin complex (indicated across the top of the panel), separated by SDS-PAGE, and Western blotted with Kaiso-specific polyclonal antibody (pAb). Kaiso coprecipitated efficiently with p120 MAb (lane 5) but not with antibodies to α-catenin (α-cat), β-catenin (β-cat), E-cadherin (E-cad), APC, BCL-6, or the control antibody, KT3.

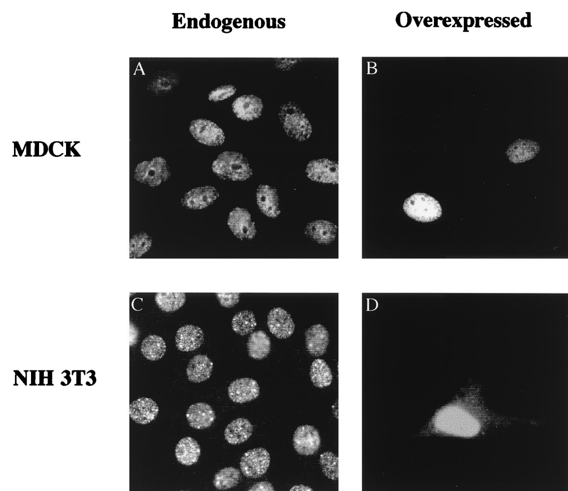

FIG. 5. Subcellular localization of Kaiso in MDCK and NIH 3T3 cells by immunofluorescence. Endogenous Kaiso was primarily concentrated in the nucleus (A and C) as detected by immunofluorescence with Kaiso polyclonal antibodies. The staining was mostly diffuse (panel A) but was also associated with small punctate dot structures (panel C). Overexpressed Kaiso was efficiently localized to the nucleus (B) as detected by the Kaiso-specific MAb 12H9. However, in some cells, nuclear and cytosolic Kaiso was detected (D).

serine, threonine, or tyrosine phosphorylation, remains to be determined. Onizuka et al. (50) have shown that, at least for BCL-6, phosphorylation accounts for aberrant migration on SDS-PAGE. Interestingly, the majority of POZ-ZF proteins have an unusually high number of potential proline-dependent serine and/or threonine phosphorylation sites (xSPx or xTPx) (71); for example, PLZF has 6 sites, Kaiso has 8, while BCL-6 and *Drosophila* Tramtrak have 14 and 16 sites, respectively.

Yeast two-hybrid mapping of the p120 and Kaiso interaction sites. To map the Kaiso-p120 interaction site, we performed a yeast two-hybrid interaction assay using two representative clones isolated from the original yeast two-hybrid screen, F-15 (short) and F-38 (long), and a panel of pGBT9-p120 deletion constructs (Fig. 7A). Both clones displayed similar patterns of interaction with the panel of p120 mutants and revealed that p120 Arm repeats 1 to 7 were necessary and sufficient for interaction with Kaiso (Fig. 7A). This region overlaps with the region of p120 required for binding the cell adhesion molecule E-cadherin (9), supporting our hypothesis that p120 binding to Kaiso and that to E-cadherin are mutually exclusive.

A panel of pGBT9-Kaiso deletion mutants was generated and used to map the Kaiso binding site. As expected, Kaiso's POZ domain did not interact with p120 (Fig. 7B, Kaiso-POZ). Kaiso constructs containing the ZF domain alone (Kaiso-Zn) or the ZF domain lacking either the proximate 5′ or 3′ sequence (Kaiso-Zn-C-term and KaisoΔC-term, respectively) did not interact with p120, indicating that the ZF domain by itself did not interact with p120 (Fig. 7B). We therefore postulate that the Kaiso binding site is a noncontiguous epitope that flanks the ZF domain. The assay with the KaisoΔPOZ construct was uninformative because this construct transactivated with the pGAD vector alone.

The POZ domain and protein-protein interactions. The POZ domain is characterized by 37 highly conserved residues, the majority of which are hydrophobic, with 6 of these being conserved in >85% of POZ proteins examined to date (1, 2). Kaiso has the characteristic 37 conserved residues, including the 6 nonvariable residues, and is clearly a member of the POZ-ZF transcription factor protein family (Fig. 2B). Four of these six residues (H, L, S, and F) form part of the most highly conserved alpha-helix, suggesting a structural or catalytic function. Using the yeast two-hybrid interaction assay, we demonstrated that, like other POZ proteins, Kaiso homodimerizes via its POZ domain. However, while BCL-6 and PLZF heterodimerize with each other via their POZ domains, Kaiso did not heterodimerize with BCL-6 (data not shown). Comparison of BCL-6 and PLZF POZ domain sequences did not reveal any unique or characteristic features that could explain why BCl-6 heterodimerizes with PLZF but not with Kaiso. Heterodimerization between Ttk and GAGA but not between Ttk and ZID has also been reported (2). The functional significance of heterodimerization remains to be determined. Heterodimerization compatibility of POZ-ZF proteins is probably dictated by subtle differences within the POZ domain itself or by the most-N-terminal amino acids, which are very divergent.

DISCUSSION

Accumulating evidence suggests that p120 and β-catenin have fundamentally distinct roles in cadherin-mediated cell adhesion and in signal transduction. The strongest evidence is the inability of p120 to bind α-catenin or APC protein and its

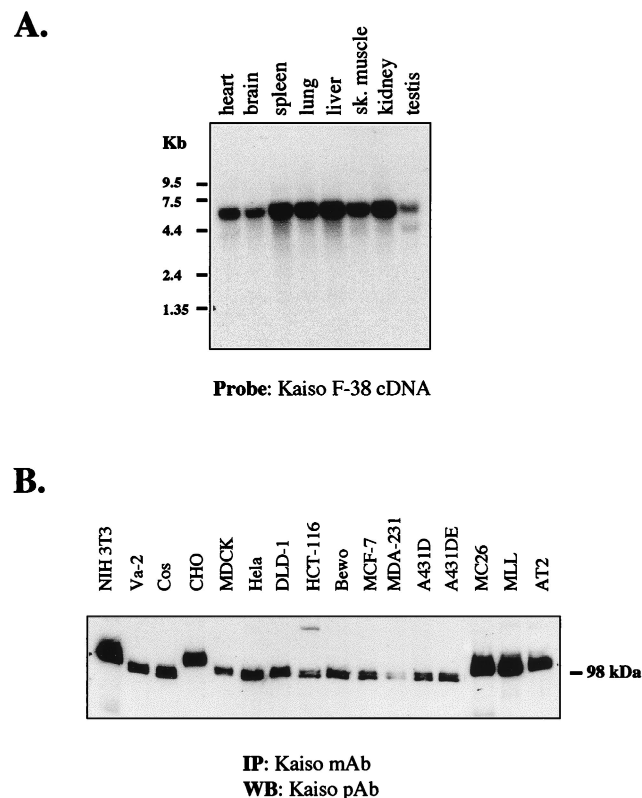

FIG. 6. Kaiso is ubiquitously expressed. (A) Northern blot analysis of Kaiso expression in tissues. A murine multiple tissue Northern blot (Clontech) was probed with a Kaiso cDNA probe (nt 870 to 2583) and washed under high stringency. A Kaiso mRNA of approximately 5 to 6 kb was ubiquitously expressed, and the lowest levels were found in the brain and testis. (B) Western blot analysis of Kaiso protein expression in various cell types. Whole-cell lysates of each cell line were normalized for equal protein amounts, immunoprecipitated with Kaiso MAb 6F8, and detected by Western blotting with a Kaiso polyclonal antibody (pAb). Cell lines are indicated at the top of the panel. Kaiso migrates as an ~95-kDa doublet in most cells, but interestingly rodent Kaiso (NIH 3T3, CHO, MLL, AT2, and MC26 cell lines) migrated as a slightly larger doublet (~110 kDa).

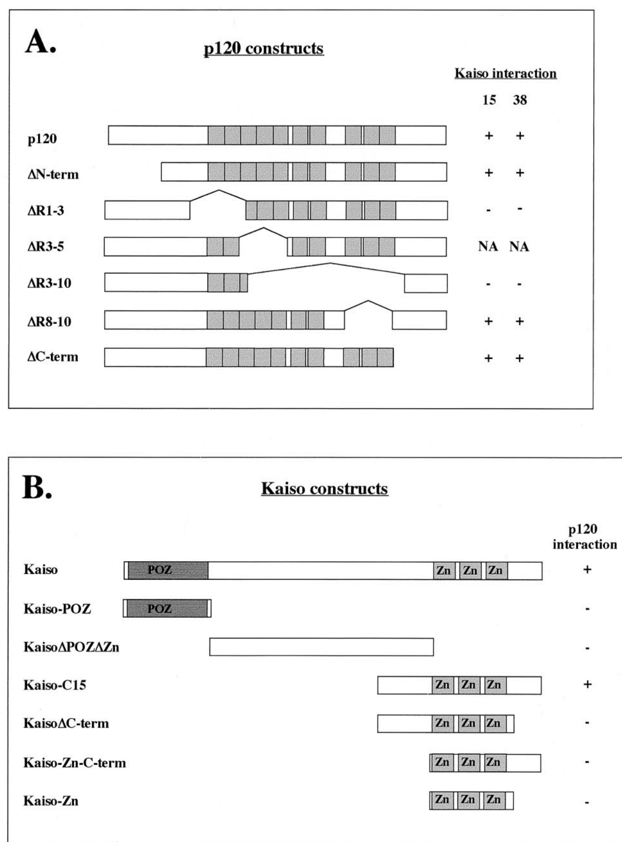

FIG. 7. Yeast two-hybrid interactions. Schematic representation of p120 and Kaiso mutant constructs. (A) The 10 Arm repeats which constitute p120's Arm domain are indicated by the hatched boxes. Clones F-15 (column 15) and F-38 (column 38) are representative short and long Kaiso clones isolated in the yeast two-hybrid screen. A summary of the binding interactions is shown on the right. NA, not addressed because of autoactivation. (B) Kaiso's POZ and ZF domains are indicated, and a summary of the binding results is shown on the right.

association with E-cadherin at a distinct juxtamembrane site. Unique roles of p120 are likely to be mediated by its own unique binding partners. To identify such proteins, we conducted a yeast two-hybrid screen using full-length p120 as bait. Surprisingly, the protein identified most frequently was a novel POZ-ZF transcription factor, which we have named Kaiso. Kaiso clearly belongs to the POZ-ZF transcription factor superfamily and may function downstream of p120 in a signaling pathway akin to the previously characterized β-catenin–Lef-1 interaction. Kaiso specifically interacts and coprecipitates with p120 but not with β-catenin or E-cadherin (Fig. 4). Since the interaction requires p120's Arm repeats 1 to 7, it is likely that p120 forms mutually exclusive complexes with E-cadherin at the membrane or with Kaiso in the cytoplasm and/or nucleus. In fact, multiple mutually exclusive interactions appear to be one of the hallmarks of Armadillo repeat proteins.

Several observations indicate that the p120-Kaiso interaction is highly specific. First, Kaiso coprecipitated efficiently with several p120-specific MAbs, thus ruling out the possibility of antibody cross-reactivity. Secondly, a good internal control for the specificity of the interaction was provided by the p120 MAb 8D11, which reacts with canine but not human p120. Using this antibody, Kaiso coprecipitated efficiently from canine (MDCK) but not human (HCT116) cell lysates, indicating an absolute requirement for p120 in the immunoprecipitate (Fig. 3). The relatively high stoichiometry and lack of association of Kaiso with E-cadherin or other Armadillo repeat proteins further emphasize the specificity of the p120-Kaiso interaction.

Interestingly, Kaiso was coprecipitated efficiently by p120 from several epithelial cell lines but inefficiently by p120 from fibroblasts (data not shown). Since fibroblasts express primarily the larger, p120 type 1 isoforms, it may be that the type 1 isoforms do not interact with Kaiso. Indeed, the p120 type 1-specific MAbs (6H11 and 5A7) did not coprecipitate Kaiso from MDCK or HCT116 cells, but this could be due to the low expression levels of type 1 isoforms in these cells. It is possible that the additional amino-terminal 101 amino acids in type 1 isoforms inhibit or block Kaiso binding by steric interference due to either modified p120 protein folding or recruitment of some other presently unknown p120-interacting protein. Ectopic expression of p120 type 3 isoforms in fibroblasts or, conversely, type 1 isoforms in epithelial cells might allow clarification of this issue. The apparent limitation of the p120-Kaiso interaction to p120 type 3 isoforms and the difference in precipitation from epithelial and fibroblast cell lines raise the intriguing possibility that the p120-Kaiso interaction, and hence function, is controlled or dictated in part by which p120 isoforms are expressed. For example, the failure of type 1 isoforms to bind Kaiso in fibroblasts could uncouple or downregulate the p120-Kaiso signal and specify epithelium-specific or fibroblast-specific events.

Under conditions of ectopic expression, it is clear that the majority of Kaiso is efficiently sequestered in the nucleus, although in a small subset of cells there was extensive cytosolic localization, which appeared to be cell cycle related. None of the POZ-ZF proteins characterized to date have a classical nuclear localization signal (NLS), raising the possibility that these proteins "piggyback" into the nucleus with other NLS-containing factors. Alternatively, POZ-ZF proteins may have a novel as yet unrecognized NLS. All p120 family members contain a conserved basic sequence between Arm repeats 6 and 7, but it is not clear whether these motifs can mediate nuclear localization. β-Catenin can translocate to the nucleus without an NLS, apparently through direct binding to the nuclear pore machinery (17). Moreover, nuclear transport of proteins with classical NLS motifs is mediated by a heterodimeric protein complex, both members of which contain Armadillo repeat domains (18). Based on these findings, it has been suggested that the capacity for nuclear docking and import may emerge as a general property of proteins containing Armadillo repeat domains (17). Thus, one potential role for p120 is to mediate the nuclear import or export of Kaiso and possibly other POZ-ZF proteins. Elucidation of the mechanism by which Kaiso and other POZ-ZF proteins localize to the nucleus should greatly enhance our understanding of the POZ-ZF protein family and the role of Kaiso as it relates to p120.

While many POZ-ZF proteins are transcriptional repressors (e.g., BCL-6 and PLZF), some are activators (e.g., Miz-1), while others (such as ZF-5, the POZ-ZF protein most similar to Kaiso) activate as well as repress natural promoters (4, 11, 32, 39, 51, 57, 65). Alanine-rich or negatively charged acidic regions have been found to correlate with transcriptional repression or transcriptional activation, respectively (reviewed in references 8 and 43). Miz-1 and ZF-5, POZ-ZF proteins with transcriptional activating abilities, have highly acidic regions upstream of their ZF motifs, suggesting that the two highly acidic regions in Kaiso (residues 187 to 195 and residues 327 to 338) could mediate transcriptional activation. On the other hand, recent studies have demonstrated that the POZ domains of BCL-6 and PLZF bind the corepressors mSin3A, SMRT,

NCoR, and histone deacetylase (10, 13, 19, 26, 40), raising the possibility that POZ domains may mediate transcriptional repression by recruiting histone deactylase complexes. Thus, whether Kaiso activates or represses transcription will have to be determined empirically; Kaiso's activity could differ from one cell type to the next.

While the majority of studies on POZ-ZF protein function have come from studies in *Drosophila* and reveal roles in development, most mammalian POZ-ZF proteins are linked to human cancer. For example, the human HIC-1 (hypermethylated in cancer) protein is underexpressed in a variety of human tumor cell lines and may act as a tumor suppressor (41). In contrast, BCL-6 and PLZF act as oncogenes. In non-Hodgkins' lymphoma, the chromosomal translocation 3q27 results in fusion of the *bcl-6* gene to heterologous promoters so that its expression is deregulated and results in proliferation of undifferentiated B cells (34, 50, 76, 77). PLZF translocations, on the other hand, are causally linked to APL and result in the reciprocal fusion of the PLZF POZ domain to the retinoic acid receptor alpha (RARα), apparently inducing constitutive repression of RARα activity (19). The ability of BCL-6 and PLZF to heterodimerize via their POZ domains raises the possibility of cross talk between the two proteins and adds another level of complexity to the pathogenesis of these diseases. Though not as thoroughly characterized, several other mammalian POZ-ZF proteins are also directly or indirectly linked to cancer. Miz-1, for example, binds the oncoprotein c-Myc, while ZF-5 presumably binds and regulates the c-*myc* promoter (49, 57). Together the data strongly implicate POZ-ZF proteins in development and cancer and imply a similar role for Kaiso.

While the role of Kaiso as a p120-specific binding partner is currently unknown, the specificity and stoichiometry of the interaction suggest a physiologically relevant function, possibly as a downstream effector of p120 signaling. The interaction is in many respects reminiscent of the recently identified β-catenin–Lef-1 association (3, 28). β-Catenin is normally absent from the nucleus of most cells, but in response to Wnt signaling, it accumulates to higher levels, enters the nucleus, and acts with Lef/TCF proteins to activate transcription (reviewed in references 20 and 56). It is not yet known whether similar events trigger nuclear translocation of p120, which would be necessary for p120 to directly affect transcription by Kaiso. Hence, one important issue is to determine the subcellular location of the p120-Kaiso interaction and events that effect the localization and functional interaction of these proteins. Plakophilin 2, which is 33% identical (57% similar) to p120 in the Arm domain, exists both in desmosomes and in nuclei (42), raising the possibility that plakophilins and other p120 family members might interact with Kaiso. In addition, it is important to define the transcriptional properties of Kaiso and to determine the effects of p120 mutation and misexpression. Identification of physiological Kaiso target genes will be important for determining how the p120-Kaiso interaction impacts on specific cellular events.

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