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Zinc Finger Protein Overexpressed in Colon Carcinoma Interacts With the Telomeric Protein hRap1

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Abstract The OZF (ZNF146) protein is a 33 kDa Kruppel protein, composed solely of 10 zinc finger motifs. It is overexpressed in the majority of pancreatic cancers and in more than 80% of colorectal cancers. We found an interaction between OZF and the telomeric hRap1 protein with a yeast two-hybrid screen. hRap1 (TERF2IP) is an ortholog of the yeast telomeric protein, scRap1 originally identified as a regulator of telomere length. In HeLa cells, it interacts with TRF2, a telomere repeat binding factor whose inactivation causes a dysregulation of telomere length and structure. Immunoprecipitation with anti-hRap1 antibodies in conditions that allow the purification of proteins associated with hRap1, demonstrated that OZF binds to hRap1 in HeLa cells. Using deletion mutants, we mapped the interacting domain of each protein. The three zinc fingers at the C-terminus of OZF interact with a region of hRap1 located downstream of the coil domain. It involves a stretch of at least 25 amino acids at the C-terminus of hRap1 that interact with TRF2. This suggests that OZF overexpression in tumours may alter the balance between hRap1 and other telomeric proteins and therefore that OZF function may be linked to telomere regulation. J. Cell. Biochem. 95: 763–768, 2005.

Key words: zinc finger protein; telomere; yeast two-hybrid assay; colon cancer

The OZF protein is a nuclear zinc finger protein of the Kruppel family. Unlike other Kruppel proteins, OZF is devoid of a transactivation domain and consists of 10 zinc finger motifs, preceded by 10 amino acids [Le Chalony et al., 1994; Ferbus et al., 1996]. Amplification and overexpression of OZF was reported in pancreatic cancers [Ferbus et al., 1999a]. Recently, it was also found to be overexpressed at an early stage in more than 80% of colorectal cancers [Ferbus et al., 2003] and to be a target of myc protein [Fernandez et al., 2003]. Despite the absence of a transactivation domain, protein interaction may be important for the unknown OZF function and may involve the zinc finger motifs [Lee et al., 1993; Perkins et al., 1994; Merika and Orkin, 1995]. Using the two-hybrid system, we screened for factors that interact with OZF and may give us a clue to explain OZF overexpression in human tumours. Half of the sequenced clones coded for UBC9, a SUMO-1 conjugating enzyme (Antoine et al., in press) and 7% to hRap1, a telomeric protein. Telomere length is controlled in part by cis-acting negative regulators that limit telomere extension. In budding yeast, the major telomere length regulator scRap1 binds to telomeric DNA and acts to inhibit telomere elongation in cis. In contrast human Rap1 appears to have lost its DNA binding activity and associates with telomeres primarily through its association with TRF2 [Li et al., 2000]. TRF2 was originally characterized as a telomere repeat binding factor and inactivation of its function causes a dramatic dysregulation of telomere length and structure [van Steensel et al., 1998; Smogorzewska et al., 2000]. Loss of TRF2 resulted in

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chromosome fusions and other chromosome aberrations associated with aberrant telomeres.

MATERIALS AND METHODS

LexA Two Hybrid Screen and Plasmid Construction

Hybrid screen, yeast strains, his growth and β-galactosidase assay have been described (Antoine et al., in press). Four OZF deletion mutants were subcloned into pLEX10 plasmid using PCR amplification and internal primers. All mutants contained the ten first amino acids of OZF and the five, six, and seven N-terminal zinc fingers in pLEX10-OZF1-5 (aa: 1–150), pLEX10-OZF1-6 (aa:1–178), and pLEX10-OZF1-7 (aa: 1–206), respectively. pLEX10-OZF6-10 (aa: 151–293) contains also the first 10 amino acids. Numbering refers to originally OZF sequence [Le Chalony et al., 1994]. A partial 1 kb cDNA I.M.A.G.E hRap1 clone and a clone isolated from the two-hybrid screen were used to construct the 1.2 kb hRap1 ORF. Inserted into the pGAD1318 vector, it led to the synthesis of a fusion protein between the GAL4 activation domain and hRap1 protein. Four deletion mutants were constructed using PCR amplification and internal primers. M1 (aa: 1–234), M2 (aa: 292–399), M3 (aa: 234–399), and M4 (aa: 266–399). All contructions were sequenced before transfection into L40 yeast.

Cell Culture and Transfection

HeLa cells (3×10^6) were plated onto petri dishes (100 mm) and cultured with DMEM supplemented with 10% fetal calf serum. Transfection was performed 24 h later using an effectene transfection kit (QIAGEN) with 2 μg of each plasmid.

Plasmids and Cell Extracts

Full length and deleted OZF ORFs were cloned into the hemaglutinin-tagged pRK5 vector (pRK5-HA). All mutants contained the HA-tagged in phase with amino acids 2–10 of OZF and amino acids 2–150 (pRK5-OZF-1-5), 151–293 (pRK5-OZF-6-10), 173–293 (pRK5-OZF-7-10), and 207–293 (pRK5-OZF-8-10), respectively. The hRap1 ORF was inserted into the pRK5myc2 expression vector. Transfected cells were lysed after 24 h for 15 min at 0°C with 250 μl per dish of modified RIPA buffer without SDS (25 mM Tris HCl pH 7.2, 125 mM NaCl, 1% NP40, 0.5% DOC, 1 mM EDTA, in the presence of protease inhibitors). After centrifugation pellets were lysed in 200 μl per dish of SDS sample buffer for SDS–PAGE to obtain nuclear extracts. They were boiled, sonicated, and quantified on SDS–PAGE.

Antibodies and Immunoprecipitation

Antibodies were produced in rabbit against a recombinant GST-hRap1 protein. Hyperimmune serum was passed over two immunoabsorbant columns consisting of GST and GST-hRap1coupled to Sepharose 4B (Pharmacia). Expression of hRap1 and OZF constructs were checked by Western analysis in crude nuclear extract. Nuclear extract corresponding to 22×10^6 transfected cells were diluted into 0.4 ml PBS and incubated with affinity purified rabbit anti-hRap1 covalently linked to protein G-Sepharose beads using the Seize-X-beds protein G immunoprecipitation kit (Pierce). Absorption in the PBS buffer (0.14M NaCl, 0.008M Na_2PO4, 0.002M K_2PO4, 0.01M KCl, pH 7.4), and washing (3X with PBS) were performed in conditions that preserved association of hRap1 with other proteins. Proteins were eluted with 0.14M NaCl and 100 mM glycine, pH 2.5 and neutralized with 2M Tris-HCl pH 9.5. Aliquots of the anti-hRap1 immunoprecipitate (IP-hRap1), preimmune serum immunoprecipitate (IP-control), and nuclear extracts were analyzed by SDS–polyacrylamide gel electrophoresis on 10%–14% gel under reducing conditions. Proteins were transferred onto polyvinylidene difluoride membrane (Immobilon-P Millipore). After blocking, blots were probed for 2 h with primary antibodies (monoclonal anti-Myc 1:10,000 and anti-HA 1:1,000) then with peroxidase conjugated donkey anti-rabbit IgG and rabbit anti-mouse diluted 2,000 and 1,000-fold, respectively. Peroxidase activity was detected by chemiluminescence with Super Signal pico (Pierce) and visualized by charge-couple device (CCD) imaging system (Fuji). Both hRap1 myc-tagged and OZF HA-tagged proteins were expressed in transfected HeLa cells.

RESULTS AND DISCUSSION

hRap1 Was Identified as a Partner of OZF in a Yeast Two-Hybrid Screen

In order to identify proteins that interact with OZF, a yeast two-hybrid screen was performed. A bait expression vector was constructed by

fusing the pLEX-10-encoded LexA-DNA binding protein to OZF protein and used to transform a yeast strain containing the HIS3 and LacZ LexA-inducible reporter genes. pLEX10-OZF yeast cells were used to screen a HeLa cDNA library cloned into pACT2 vector. Approximately 650 clones grew on his selection plates from 6×10^6 yeast transformants. Most of them were positive in the β-galactosidase assay. Forty clones were randomly chosen and their inserts were sequenced. Twenty-one encoded the cDNA of UBC9 (Antoine et al., in press) and four encoded partial hRap1 sequence in frame with the pACT2-GAL4 activation domain. Among 46/560 clones that did not encode UBC9, 8 contained hRap1 sequence. To demonstrate the binding of hRap1 to OZF, yeasts containing the full length OZF ORF in the pLEX10 vector were transformed with a pACT2 plasmid expressing hRap1 cDNA. Significant growth indicating an interaction between hRap1 and OZF was observed on plates containing his selection medium and were positive in the β-galactosidase assay (data not shown). In contrast, no growth was detected in cells transfected with the empty pLEX10 vector or the pLEX10 expressing lamin cDNA.

Mapping of the Interacting Domains of OZF and hRap1 in Yeast

We first mapped the region of hRap1 that interacts with OZF. Human Rap1 has three domains in common with yeast Rap1p: (1) a central Myb motif with specific sequence features also present in the yeast Rap1p R1 Myb domain [Li et al., 2000]; (2) a BRCT domain at the N-terminus; (3) a conserved motif (the RCT domain) in their C-terminus. In addition, a coil domain between myb and RCT regions has been identified in the hRap1 protein [Li et al., 2000]. We constructed several mutants (M1–M4) carrying one or several of these domains (Fig. 1a). M1 carries both BRCT and Myb domains absent from the clone isolated during the screening of the library and did not present a significant interaction (Fig. 1a). No interaction was observed with the M2 construct that carries the RCT C-terminal region. In contrast M3 and M4 constructs interacted with OZF. The coil region present in M3 and absent in M4 appeared not to be essential for efficient interaction. We therefore concluded that a short region downstream of the coil region (amino acid 267–292) is necessary for the interaction with OZF (Fig. 1a). The

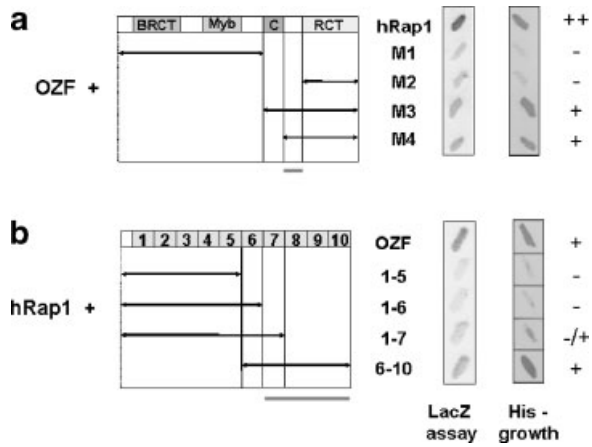

Fig. 1. Two-hybrid mapping of hRap1–OZF interacting domains. L40 yeast was co-transformed with plasmids encoding a fusion protein between the GAL4 activation domain and full-length or hRap1 deleted constructs and plasmids encoding a fusion protein between LexA-DNA binding domain and full-length or deleted OZF constructs. All yeast clones grew on SD Leu/Trp medium, confirming that both plasmids were transformed. Growth on SD Leu/Trp/His medium results from the protein–protein interaction activating the *HIS3* prototrophic reporter (not shown). Histidine growth and β-galactosidase activity in the yeast two-hybrid LacZ assay are presented on the right of the schema. ++ indicates strong interaction, + normal interaction, ± weak interaction, and – no interaction. BRCT, Myb, C, and RCT indicate the domains identified in hRap1 (see text). **a:** Mapping of the region of hRap1, between amino acid 267 and 292, interacting with OZF. **b**: Mapping of the region OZF interacting with hRap1. The OZF constructs used in this experiment contain zinc fingers 1–5, 1–6, 1–7, and 6–10. The zinc fingers are numbered from 1 to 10 and bars show the region of interaction in hRap1 and OZF.

interaction of hRap1 with the mutants appeared weaker as compared to that of the native OZF protein and is likely to result from conformational modifications.

To map the region of OZF interacting with hRap1, the pGAD1318 vector carrying the full-length hRap1 cDNA was co-transfected in yeast with OZF or with truncated constructs carrying a number indicating the borders of the OZF region inserted in pLEX10 (Fig. 1b): that is OZF1-5 carries zinc fingers 1–5. LacZ activity and his growth were observed with OZF and a truncated construct containing the last five zinc fingers (OZF6-10). A weak coloration appeared with OZF1-7. No activity was observed with the other truncated OZF constructs, indicating that the sixth finger of OZF does not participate in the interaction with hRap1. We concluded that the region of OZF interacting with OZF involves the four zinc fingers at the C-terminus. This region carries a single amino acid substitution

between human, mouse, and cow. Its high conservation among mammals suggests that it bears an essential function [Ferbus et al., 1999b].

OZF Co-Immunoprecipitate With hRap1 in HeLa Cell Extracts

Subsequent immunoprecipitation of hRap1 complex and Western analysis were used to confirm in human cultured cells, the interaction observed in yeast between OZF and hRap1. The monoclonal antibodies against OZF previously characterized failed to immunoprecipitate the native protein. Therefore, we raised a polyclonal antibody against a GST tagged-hRap1 recombinant protein. Affinity-purified rabbit anti-hRap1 serum detected the recombinant and the cellular hRap1protein in Western blotting as a single band of 60 kDa and no cross reactivity with other cellular proteins was observed (Fig. 2a,b and data not shown). Moreover, in the presence of competing recombinant hRap1, the binding was inhibited. To check that the protein migrating at 60 kDa corresponds to the theoretical 44 kDa hRap1, we transfected HeLa cells with a myc-tagged hRap1 cDNA. A protein of identical mobility was detected with anti-hRap1 antibody and a monoclonal antibody directed against the myc-tag (Fig. 2). To immunoprecipitate the hRap1-OZF complex, we used a method based on antibody immobilization that eliminates the antibody heavy and light chains contamination interfering with hRap1 detection. Nuclear extracts were incubated with protein G-sepharose beads coupled with purified anti-hRap1 antibody. The purified proteins, eluted from the beads, were analyzed by Western blotting using anti-hRap1 and anti-HA antibodies, and compared to crude nuclear extracts or an immunoprecipitate using a non-specific antibody. HA-antibody was used in place of OZF monoclonal antibody, which is directed against an epitope located at the junction between the first 10 amino acids and the first zinc finger. This epitope is absent in the deletion mutants except in OZF1-5 [Ferbus et al., 1999b]. In cells co-transfected with myc-hRap1 and HA-OZF, both antibodies detected two proteins of 60 and 33 kDa (Fig. 2). Their size is in agreement with the expected size of hRap1 and OZF, respectively, indicating that both constructs are expressed in transfected HeLa cells. Western blot analysis of proteins released from the beads showed that OZF co-immunoprecipitated with hRap1. Immunoprecipitation performed using a preimmune serum failed to immunoprecipitate hRap1. The transfection of expression vectors may induce high level of expressed proteins, creating non-specific interactions. To demonstrate a physical interaction in vivo between OZF and hRap1, we examined whether endogenous proteins would co-purify by immunoprecipitation in HeLa cells. Figure 2b shows that OZF co-eluted with hRap1 from protein G-sepharose beads coupled with purified anti-hRap1 antibody and confirm the endogenous interaction between these two proteins. This association is specific since no OZF was recovered from an affinity column conjugated with a preimmune serum. These results demonstrate a physical interaction between hRap1 and OZF in human cells.

The yeast two hybrid assay showed that the region encompassing zinc finger 7–10 is involved in the interaction with hRap1 (Fig. 1). To confirm in vivo the interaction observed in yeast, we co-transfected myc-tagged hRap1 with HA-tagged OZF truncated constructs into HeLa cells. After immunoprecipitation with anti-hRap1, overproduction of myc-hRap1 and the constructs containing zinc finger 1–5 failed to show any interaction with the 17 kDa OZF-truncated protein (Fig. 2a). Interaction was observed when HeLa cells were transfected with OZF6-10 and OZF7-10. The observed association between hRap1 and OZF8-10, limited the domain of interaction to the three zinc fingers located at the C-terminus. Therefore, the domain of OZF interacting with hRap1 mainly involves a region of at most 90 amino acids located at its C-terminus.

The results from the yeast two-hybrid assay and affinity chromatography with HA-tagged OZF constructs showed that hRap1 interacts in HeLa cells. This interaction involves the region between the coil and the RCT regions (amino acid 267–292) and the C-terminus of OZF. Immunofluorescence was performed to search for hRap1 and OZF co-localization in resting cells and during mitosis. Preliminary results show a nuclear double staining in resting cells but it represents a fraction of hRap1 and OZF signal (data not shown). This suggests that only a fraction of OZF and hRap1 are associated together.

We have identified hRap1 as a protein interacting with OZF, a protein overexpressed in pancreatic and colonic carcinomas and at an

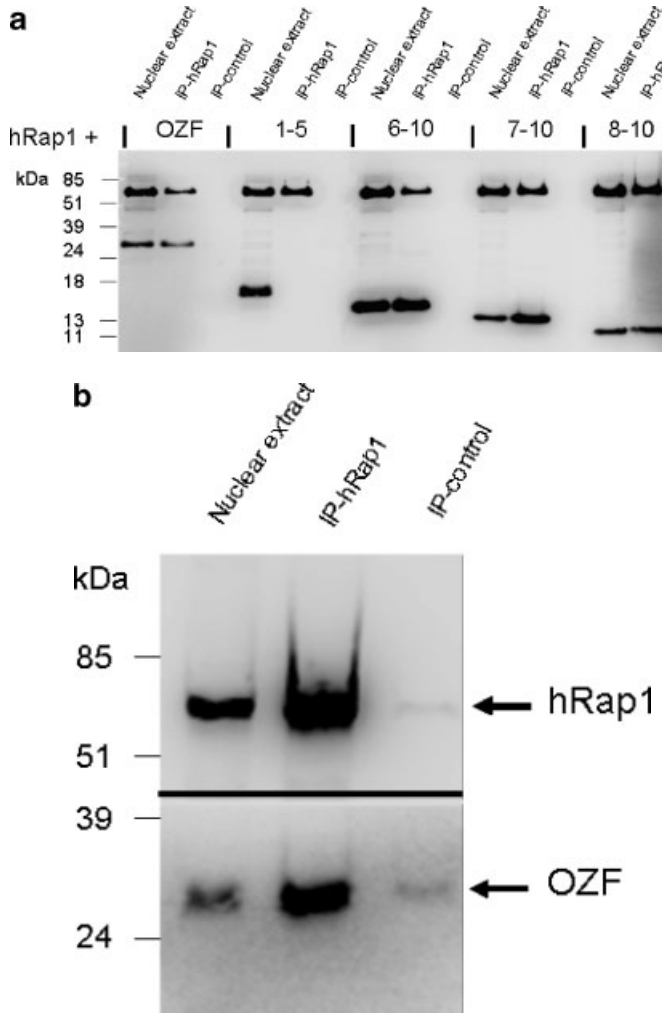

Fig. 2. Interaction of hRap1 and OZF in human HeLa cells. **a**: The three last fingers of OZF interact with hRap1. HeLa cells (3×10^6) were transfected with 2 μg of pRK5 myc-tagged-hRap1 and 2 μg of each four OZF deletion constructs in the pRK5 HA-tagged vector. After 24 h, cells were lysed and incubated with affinity purified rabbit anti-hRap1 covalently linked to Protein G-Sepharose beads. Co-elution of hRap-1 with full-length OZF, OZF6-10, OZF7-10, and OZF8-10 indicates interaction of these constructs with hRap-1. No interaction was observed between hRap1 and the construct encoding the five first zinc finger (OZF1-5). Interaction between hRap1 and OZF occurs in vivo and involves zinc finger 8–10. **b:** Endogenous hRap1 and OZF proteins interact in HeLa cells. Nuclear extract corresponding to 60×10^6 untransfected HeLa cells was subjected to immunoprecipitation in the conditions described in (a) for exogenously expressed hRap1. Detection of each protein was performed on aliquots of the same eluat run in parallel in the same gel. After transfer, the membrane was split and each half membrane probed separately with affinity purified hRap1 rabbit polyclonal antibody (1:2,000) and anti-OZF monoclonal antibody (1:500). Immunoprecipitation of hRap1 (IP-hRap1) led to the purification of hRap-1 together with OZF. No hRap-1 complex was immunoprecipitated with pre-immune serum (IP-control). Blots are representative of three separate experiments. Size of the markers is indicated on the left.

early stage of colorectal carcinogenesis. hRap1 has been shown to be directly involved in the control of telomere length [Li and de Lange, 2003; O'Connor et al., 2004]. Telomere maintenance is essential for cell immortality and in tumour cells, it is achieved by de novo synthesis of telomere repeats at the terminus by the enzyme telomerase [Kim et al., 1994] or by a recombination-based mechanism known as alternative lengthening of telomeres (ALT) [Lundblad and Blackburn, 1993; Bryan et al., 1995; Li et al., 2000]. We delineated the binding domain of TRF2 in the region of hRap1 downstream of the coil domain between amino acids 267 and 372. Thus, the region between 267 and 292 binds both OZF and TRF2. It is therefore tempting to speculate that increased expression of OZF may alter the balance between hRap1

and TRF2, leading to some telomere dysregulation. Direct demonstration of telomere dysfunction was addressed by transfection of constitutive or inducible vectors expressing OZF. No cell line overexpressing OZF could be established using several methods of transfection and recipient cells. We also failed to recover cell lines expressing anti-sense OZF. This suggests that levels of OZF expression are tightly regulated. A slight modification is likely to alter the balance with other cell partners leading to a selective growth disadvantage. Despite the absence of this direct proof, our data suggest for the first time a link between OZF overexpression in tumours and telomere regulation.

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