p67 isoform of mouse disabled 2 protein acts as a transcriptional activator during the differentiation of F9 cells

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The mouse disabled 2 (*mDab2*) gene is a mouse homologue of the *Drosophila* disabled gene and is alternatively spliced to form two isoforms, p96 and p67. Although p96 has been known to regulate the Ras–Sos G-protein signal transduction pathway by interacting with Grb2, little is known about the biological function of p67. Recent studies have shown that the expression of *mDab2* is markedly up-regulated during the retinoic acid (RA)-induced differentiation of F9 cells, suggesting another role for mDab2 in cell differentiation [Cho, Lee and Park (1999) Mol. Cells **9,** 179–184). In the present study, we first elucidated the biological function of p67 isoform of mDab2 and identified its binding partner. Unlike p96, p67 largely resides in RA-treated F9 cell nuclei. In this system, p67 interacts with mouse androgen-receptor interacting protein 3, termed the mDab2 interacting protein, which acts as a transcriptional co-regulator. By using a fusion protein with a heterologous DNA-binding domain (GAL4), we showed that p67 had an intrinsic transcriptional activation function. These results suggest that mDab2 p67 may function as a transcriptional co-factor for certain complexes of transcriptional regulatory elements involved in the RA-induced differentiation of F9 cells.

Key words: mDab2 interacting protein, proline-rich domain, retinoic acid, transactivation.

INTRODUCTION

mDab2 belongs to the disabled (*dab*) gene family and represents two major alternatively spliced isoforms, p96 and p67 [1]. Recently, it was found that mouse disabled 2 (mDab2), especially p67, is highly expressed in retinoic acid (RA)-treated F9 cells but not in untreated cells. Murine F9 embryonal carcinoma cells can be induced to differentiate into non-malignant cells resembling either parietal or visceral endoderm, facilitating the study of the formation of extra-embryonic endoderm [2,3]. In addition to being up-regulated during F9 cell differentiation, p67 is highly expressed in embryos 7.5 days post-coitus, but p96 was not expressed in embryos 6.5 and 14.5 days post-coitus. Also, the two isoforms of mDab2 are differentially expressed in a tissue-specific manner; p96 is expressed in nerve and macrophage cell lines [1] and p67 is highly expressed in kidney and rat ventral prostate [4,5]. These data suggest that each isoform of mDab2 may have a different function.

The structure of mDab2 includes a conserved phosphotyrosine-interacting domain (PID) and a distinct proline-rich domain [6]. The N-terminal PID shares significant similarity with the members of the Dab family. However, unlike other PIDs, the mDab2 PID does not bind to tyrosine-phosphorylated peptide ligands or phospholipids [7,8]. The C-terminal proline-rich domain of mDab2 is quite distinct and bears very little similarity to other Dab proteins [8]. It contains approx. 20% proline residues within the 200 amino acids of the C-terminal region, which resembles the proline-rich activation domains of AP-2 and CTF/NF1 [9,10].

The function of mDab2 is not clear at present, although several studies argue for a role in signal transduction. Xu et al. [6] have shown that p96 interacts with the adaptor protein Grb2, and may negatively regulate cell growth by down-regulation of Ras activation by sequestering Grb2 from the Sos–Grb2 complex. However, p67 does not interact with Grb2 and no other proteins that interact with the p67 isoform have been identified.

In the present study another function of mDab2 is described. Surprisingly, in the nucleus of RA-treated F9 cells, p67 binds through its PID to the mDab2-interacting protein (mDIP), a mouse homologue of the rat androgen receptor-interacting protein 3 (ARIP3). ARIP3 is a transcriptional co-regulator of the androgen receptor and belongs to a novel family of nuclear proteins [11]. Furthermore, mDab2 p67 harbours a strong, autonomous transactivation function when fused with the GAL4 DNA binding domain.

EXPERIMENTAL

Materials

All oligonucleotides were synthesized by Gibco–BRL Life Technology (Grand Island, NY, U.S.A.). The Dye Terminator cycle sequencing kit was obtained from Perkin–Elmer Applied Biosystems (Norwalk, CT, U.S.A.).

Cell cultures

F9, NIH3T3, and COS-1 cells were obtained from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-bovine serum at 37 °C in a 5% CO_2 incubator. For induction of differentiation into visceral endoderm cells, F9 cells were treated with 1×10^{-7} M all-*trans*-RA for 4 days in aggregation culture on bacterial Petri dishes.

Abbreviations used: ARIP3, androgen receptor-interacting protein 3; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; HA, haemagglutinin; mDab2, mouse disabled 2; mDIP, mDab2-interacting protein; PID, phosphotyrosine-interacting domain; RA, retinoic acid.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under accession number AF201391.

Antibodies

A rabbit polyclonal antibody to mDab2 was prepared by immunizing rabbits with a glutathione S-transferase (GST)–mDab2 fusion protein, corresponding to residues 1–700. Anti-mDIP polyclonal serum was prepared by immunizing rabbits with a GST–mDIP fusion, corresponding to residues 1–572. The anti-mDab2 monoclonal antibody, anti-ERK2 monoclonal antibody, anti-haemagglutinin (HA) monoclonal antibody and anti-Myc polyclonal rabbit antibody were obtained from Signal Transduction Laboratories (Lexington, KY, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Subcellular fractionation

RA-treated and untreated F9 cells (3×10^6) were collected by centrifugation and resuspended in buffer [20 mM Tris/HCl (pH 7.5)/10 mM $CaCl_2$]. After homogenization in a Dounce homogenizer, the homogenate was centrifuged at 1000 ***g*** for 5 min. The supernatant was removed and the procedure was repeated twice to remove any non-nuclear membranes; the resulting pellet was the nuclear fraction and the supernatant the cytosolic fraction. Proteins from each fraction were separated by SDS/PAGE (8 % gel) and analysed by immunoblotting, using specific antibodies and ECL detection according to the manufacturer's instructions (Santa Cruz Biotechnology).

Yeast two-hybrid screen

The mDab2 cDNA fragment, corresponding to residues 1–229, was cloned into pLexA (Clontech) to generate a bait plasmid. The AD fusion library in pB42AD (Clontech) was constructed using the cDNAs made from RA-treated F9 cells as described previously [12,13]. This library was screened for mDab2 interacting proteins according to the manufacturer's instruction (Clontech). Forty positive clones were isolated from 3×10^6 primary transformants. All of these clones were partial-length cDNAs from the same gene, which we named mDIP.

Isolation of full-length mDIP cDNA

Poly(A)$^+$ RNA was isolated from F9 cells treated with RA for 4 days. A F9 cDNA library was constructed in a Uni-ZAP XR vector (Stratagene, La Jolla, CA, U.S.A.) using a ZAP-cDNA synthesis kit according to the manufacturer's instructions (Stratagene). The full-length mDIP cDNA was obtained by screening the library with the dioxygenin-labelled mDIP cDNA fragment isolated from the yeast two-hybrid screen (Roche Diagnostics GmbH, Mannheim, Germany). cDNA was subcloned into T7 Blue (Novagen, Madison, WI, U.S.A.) vector and its sequences were determined by direct cycle sequencing (Amplitaq; Perkin–Elmer).

Co-immunoprecipitation analysis

RA-treated F9 cells (1×10^6) were lysed, on ice, in 1 ml of TX-IPB [10 mM Hepes (pH 7.5), 0.1 M NaCl, 1 % (v/v) Triton X-100, 2 mM EDTA, 0.1 % (w/v) 2-mercaptoethanol, 50 mM NaF, 0.2 mM Na_3VO_4, 2 mM PMSF containing 20 μg/ml aprotinin, 1 μg/ml pepstatin and 10 μg/ml leupeptin], incubated for 10 min on ice and centrifuged at 20000 ***g*** for 30 min at 4 °C. Lysates were cleared with Sepharose CL-4B (Sigma) and incubated with specific antibodies or pre-immune serum for 2 h at 4 °C. Protein A–Sepharose beads were added and the mixtures were incubated for a further 1 h. After three washes with TX-IPB buffer, the samples were processed for immunoblot analysis.

Generation of GST fusion proteins and the construction of mDab2 and mDIP expression plasmids

pGST–mDab2N (N-terminal fragment; residues 1–229), pGST–mDab2C (C-terminal fragment; residues 487–720) and pGST–p67 (residues 1–837) were constructed by cloning PCR-generated mDab2 cDNA fragments into pGEX2T vectors (Amersham Pharmacia Biotech). DNA constructs for GST fusion proteins of mDIPN (1–460), mDIPC (460–572) and the full-length mDIP (1–572) were prepared by PCR using the mDIP cDNA as template. All constructs were verified by sequencing. The expression and purification of the fusion proteins from *Escherichia coli* were performed as described previously [14]. To obtain HA-tagged proteins, the full-length p67 and mDIP cDNA were respectively subcloned into pHM6 (Roche Diagnostics GmbH). The pHM6–mDIP (Δ460–572) was constructed by cloning the PCR-generated cDNA fragment corresponding to residues 1–459 into pHM6.

GST pull-down experiments

pHM6–mDab2 and pHM6–mDIP were each expressed in COS-1 cells by transfection and cell lysates were prepared. Pre-cleared lysates (500 μg/ml) were incubated with 50 μl of glutathione-agarose and various GST-fusion proteins (approx. 10 μg of each fusion protein) for 4 h at 4 °C. The glutathione beads were washed rapidly three times in TX-IPB buffer for 5 min, bound proteins were separated by SDS/PAGE (8 % gel), and analysed by immunoblotting using anti-HA monoclonal antibodies and ECL detection.

GAL4–mDab2 fusion constructs

pM–mDab2 p67, an expression plasmid containing a fusion of p67 with the GAL4 DNA-binding domain, was constructed by cloning the p67 cDNA into the *Eco*RI and *Bam*HI sites of pM (Clontech). pM–mDab2 p96, pM–mDab2N (residues 1–229), and pM–mDab2C (residues 487–720) were constructed by cloning appropriate PCR-generated cDNA inserts into pM. pG5CAT (chloramphenicol acetyltransferase), driven by a GAL4-response element in front of the E1b TATA sequence (Clontech), was used as the reporter plasmid. Correction of all constructs was verified by sequencing.

Transcription assays

F9 cells were plated at a density of 10^6 cells per 100-mm tissue-culture dish 24 h prior to transfection. The cells were co-transfected with 4 μg of GAL4-fusion expression plasmids, 5 μg of reporter pG5CAT and 3 μg of an internal reference plasmid (pRSV–β-galactosidase) by the calcium phosphate method, as described previously [15]. Approx. 36 h after transfection, the cells were harvested and CAT assays were performed according to the protocol of Gorman et al. [16]. CAT activities were normalized to β-galactosidase activity, in order to correct for differences in transfection efficiency. In all experiments, the values given represent the means $\pm$ S.E.M of at least five independent experiments.

RESULTS

p67 isoform of mDab2 localize to the nucleus of RA-treated F9 cells

The subcellular localization of mDab2 in RA-treated F9 cells was investigated. Cells were fractionated into cytoplasmic and

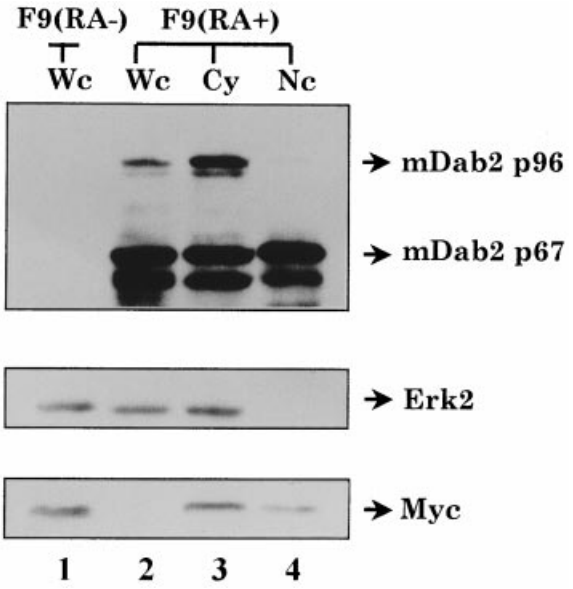

Figure 1 Subcellular localization of mDab2

RA-treated [F9(RA+)] and untreated [F9(RA−)] F9 whole cell extracts (Wc) were separated into cytoplasmic (Cy) and nuclear (Nc) fractions. Proteins were separated by SDS/PAGE, blotted and probed with mDab2 monoclonal antibodies. To validate the fractionation procedure, immunoblotting was performed using antibodies to Erk2 and Myc (lower panels).

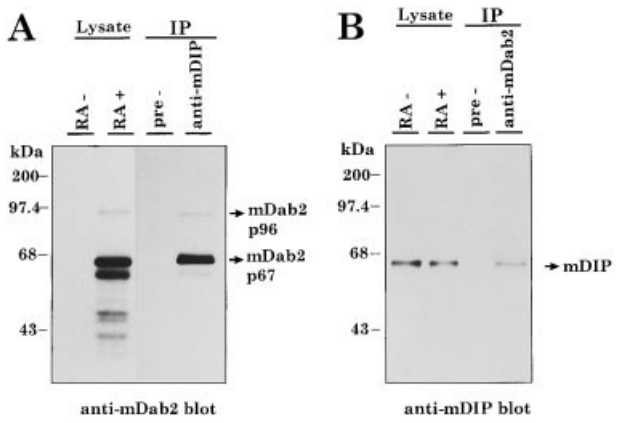

Figure 2 Interaction of mDab2 with mDIP in RA-treated F9 cells

Lysates from F9 cells, grown in the absence (−) or presence (+) of RA for 4 days, were immunoprecipitated (IP) using antibodies to (**A**) mDab2, (**B**) mDIP or irrelevant pre-immune serum (pre-). The cell lysates and immunocomplexes were fractionated by SDS/PAGE (8% gels) for analysis by immunoblotting. The positions of molecular-mass standards (kDa) are shown on the left of each panel.

nuclear fractions and equal amounts of protein from each fraction were analysed by immunoblotting using an anti-mDab2 monoclonal antibody. Only in RA-treated F9 cells was mDab2 detected (Figure 1). mDab2 p96 was detected at a low level mostly in the cytoplasm (lane 3), whereas, although p67 was detected both in the cytoplasm and nucleus at high levels and in similar amounts, compared with p96, it was exclusively localized in the nucleus (lanes 3 and 4). In each type of mDab2 protein, two bands were observed. Since mDab2 is reported to be a phosphorylated protein [1,5], we speculated that the upper band is the phosphorylated mDab2 and lower band is the unphosphorylated one. To validate the fractionation procedure, the blot was stripped and reprobed with antibodies against the cytoplasmic Erk2 protein and the nuclear Myc protein (Figure 1, lower panels). The nuclear localization of mDab2 was further confirmed by immunofluorescence. mDab2 was also detected both in the cytoplasm and in the nucleus (results not shown), consistent with the above results. The presence of mDab2 p67 in the nucleus suggested that it might function as a nuclear protein.

mDab2 interacts with mDIP in RA-treated F9 cells

We employed a yeast two-hybrid system to identify proteins that interact with mDab2. As the bait protein we used the N-terminal PID, corresponding to residues 1–229, fused with the LexA DNA-binding domain to screen a RA-treated F9 cell cDNA library constructed in the pB42AD plasmid. Since high expression levels of mDab2 had been observed in F9 cells treated with RA for 4 days, the cDNA library made from this source was used. After two rounds of screening, we obtained 40 positive clones. DNA-sequence analyses revealed that all of these clones were derived from the same gene. The complete cDNA sequence of the mDIP isolated from the RA-treated F9 cell cDNA library contained an open reading frame of 572 amino acids with a calculated molecular mass of 63.3 kDa (GenBank accession number AF201391). Sequence-similarity searches revealed that the mDIP had extensive sequence identity with a recently cloned protein termed rat ARIP3, which interacts with the androgen receptor [10]. Both cDNA and amino-acid sequences of mDIP were 97% identical with those of the rat ARIP3, suggesting that the mDIP is a mouse ARIP3.

To examine whether mDab2 and mDIP formed a complex in F9 cells, a co-immunoprecipitation experiment was performed. mDIP was constantly expressed at low level during the differentiation of F9 cells, whereas mDab2 was expressed only in RA-treated F9 cells. Cell lysates, prepared from RA-treated F9 cells, were immunoprecipitated with antibodies to mDIP, and mDab2 was detected by immunoblotting (Figure 2A). A large amount of the p67 isoform of mDab2 was co-immunoprecipitated with mDIP, relative to mDab2 p96, but neither was detected in the pre-immune serum. Only the upper band of mDab2 was immunoprecipitated by the anti-mDIP antibody. This implies that the phosphorylated form of mDab2 mainly interacts with mDIP. At this point, however, it is not evident why mDab2 is phosphorylated and why only phosphorylated mDab2 interacts with mDIP; further experiments are required to resolve this anomaly. mDIP was also detected in anti-mDab2 immunoprecipitates (Figure 2B). These results indicated that mDab2 and mDIP formed a stable complex in RA-treated F9 cells.

Mapping of p67 isoform of mDab2 and mDIP interaction domains

To determine which region of the mDab2 p67 protein was bound to the mDIP, a series of mutant GST–p67 proteins was tested for their ability to interact with full-length mDIP (Figure 3A). HA-tagged mDIP, expressed in transfected COS-1 cells, strongly interacted with full-length p67 (lane 2) and mDab2N (lane 3), but not with mDab2C (lane 4) or with GST alone (lane 5). The N-terminal region of p67 was sufficient for binding to mDIP. The binding of p67 to mDIP *in vitro* was further examined using expressed GST-fusion proteins containing full-length mDIP and various truncation mutants. The full-length GST–mDIP and HA-tagged p67 were also associated in transfected COS-1 cells (Figure 3B, lane 2), and deletion of the mDIP residues 460–572 caused a marked diminution in this interaction (lane 3). All of

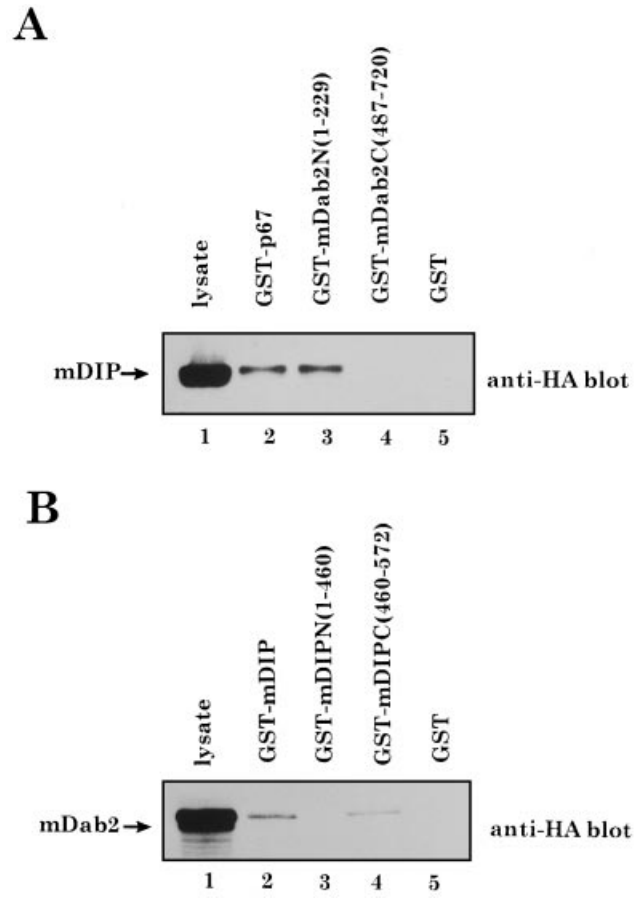

Figure 3 N-terminal region (1–229) of mDab2 p67 interacts with the C-terminal region (460–572) of mDIP

(**A**) HA-tagged mDIP was expressed in COS-1 cells by transfection. Similar amounts of cell lysates (lane 1) were incubated with GST-p67 (lane 2), GST-mDab2N (lane 3), GST-mDab2C (lane 4) or with GST alone (lane 5). Bound proteins were separated by SDS/PAGE (8% gel) followed by immunoblotting with anti-HA antibody. (**B**) Interaction of mDab2 p67 with GST-mDIP (lane 2), GST-mDIPN (lane 3) or GST-mDIPC (lane 4). Cell lysate from COS-1 cells transfected with the HA-tagged mDab2 expression plasmid (lane 1) is shown for comparison.

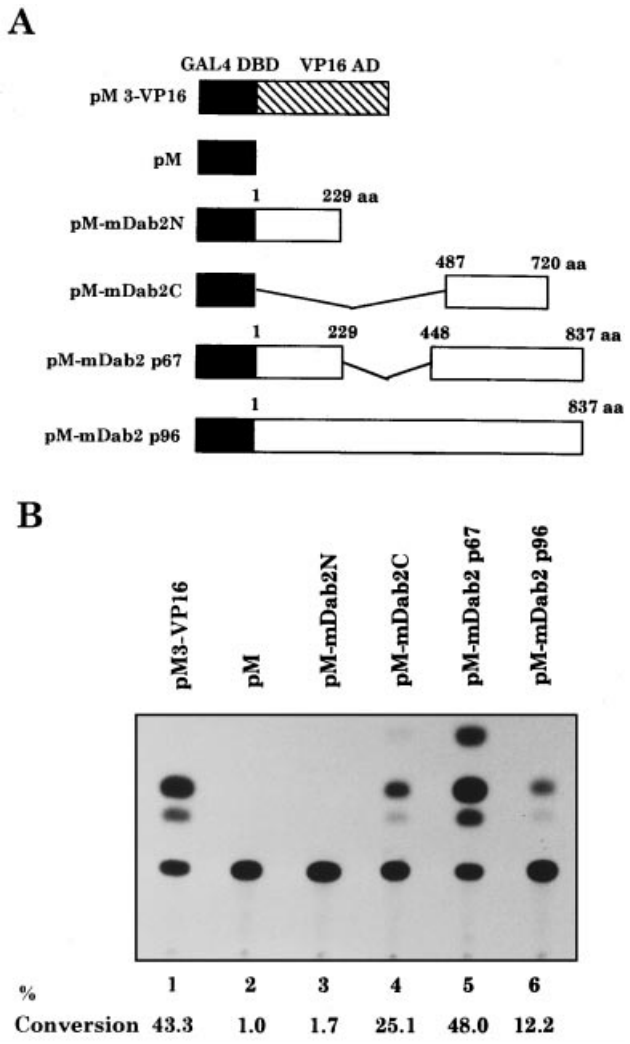

Figure 4 Transcriptional activation by mDab2 p67 in mammalian cells

(**A**) Full-length p96, p67, mDab2N and mDab2C (open bars) were fused to the DNA-binding domain of GAL4 (black bars). pM3-VP16, encoding the fusion of the GAL4 DNA-binding domain with the VP16 activation domain (hatched bar), and pM, encoding only a GAL4 DNA-binding domain, were used as a positive and negative control respectively. (**B**) RA-untreated F9 cells were transfected with the CAT reporter construct together with various GAL4 fusion expression plasmids. CAT assays were performed, with extract quantities normalized to β-galactosidase activity. Percentage conversion of chloramphenicol to its acetylated forms is shown below the panel.

these results demonstrate that residues 1–229 of p67 and residues 460–572 of mDIP are critical for p67–mDIP interaction.

Transcriptional activation by the p67 isoform of mDab2

Our experimental data have shown that the p67 isoform of mDab2 is localized to the nucleus of RA-treated F9 cells and it interacts with a mouse ARIP3, which is known as a transcriptional co-regulator. Also, we observed that p67 can act as a transactivator when fused to a LexA DNA-binding domain when expressed in *Saccharomyces cerevisiae* EGY48 (results not shown). These data suggest that the cellular function of mDab2 might be related to transcriptional regulation. To test this possibility, full-length mDab2 p96 and mDab2 p67, and mDab2N and mDab2C fragments were fused to the DNA-binding domain of the GAL4 protein (Figure 4A) and each fusion construct was co-transfected with a GAL4-responsive CAT reporter plasmid into RA-untreated F9 or NIH3T3 cells devoid of endogenous mDab2. The expression and integrity of the various GAL4 fusion proteins were assayed by immunoblotting extracts from the transfected cells (results not shown). pM3-VP16 (Clontech), encoding fusion of the GAL4 DNA-binding domain with the VP16 activation domain, and pM, encoding only a GAL4 DNA-binding domain, were used as positive and negative controls respectively. When compared with the negative control, pM–mDab2 p67 and pM–mDab2C activated transcription by 48- and 25-fold respectively, whereas transcriptional activation by pM–mDab2N was not detectable (Figure 4B). When various amounts (1, 4, 8 or 16 μg) of pM–mDab2 p67 were transfected into F9 cells, CAT activity was increased in a concentration-dependent manner (Table 1). These results indicate that the p67 isoform of mDab2 can stimulate transcription and that the C-terminal region has minimal information for transcriptional activation.

Since mDIP is a transcriptional co-regulator, it is likely that the interaction between mDIP and mDab2 p67 may affect the

Table 1 Transcriptional activity of mDab2 p67

The indicated amounts of the expression plasmid were transfected into F9 cells with pM–mDab2 p67 and the reporter plasmid pG5CAT. CAT activity was normalized with respect to the activity of β-galactosidase. Data are expressed as the percentage of CAT activity relative to the value of cells transfected with 16 μg of pM and are means ± S.E.M of five independent experiments.

Amount of pM–mDab2 p67 plasmid (μg)	Relative CAT activity (%)
0	1.2 ± 1.1
1.0	21.3 ± 1.4
4.0	39.3 ± 2.7
8.0	58.5 ± 3.0
16.0	66.9 ± 3.7

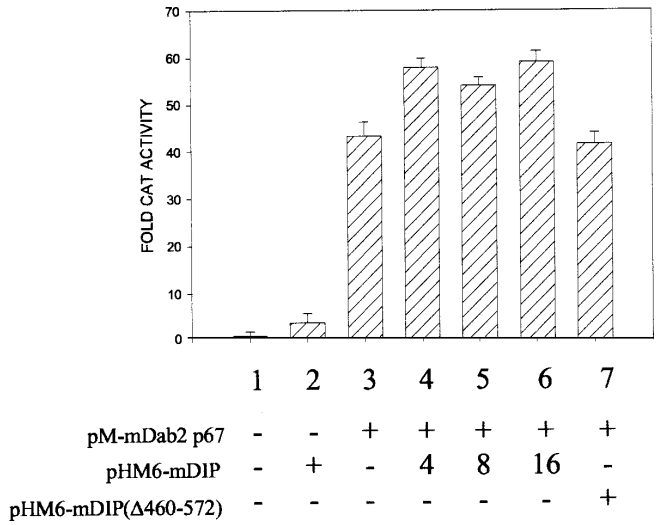

Figure 5 mDIP influences the transactivating activity of mDab2 p67

RA-untreated F9 cells transfected with 5 μg of pG5CAT, 4 μg of pM–mDab2 67, 3 μg of pRSV-β-galactosidase and increasing amounts (in μg) of pHM6-mDIP, or with 4 μg of pHM6-mDIPΔ460–572. The total amount of DNA (28 μg) was kept constant by adding empty pM as needed. The two mDIP forms were expressed at similar levels, as shown by immunoblot analysis using anti-HA antibody. CAT activity was calculated as the percentage of chloramphenicol converted into the acetylated forms, determined by liquid scintillation counting of extracts of the acetylated forms after TLC. Data are expressed as fold CAT activity related to that of pM, which have been given a value of 1.0.

transactivating function of p67. To examine the potential regulation of p67 activity by mDIP, we co-transfected pM–mDab2 p67 with mDIP expression plasmids (Figure 5). mDIP alone had no transcriptional activity (lane 2), but it slightly enhanced the transactivating activity of p67 (lane 4). However, its enhancing effect was very low and the increasing amounts of co-expressed mDIP did not influence the transactivating activity of p67. Deletion of mDIP Δ460–572 abolished this enhancing property (lane 7), implying that direct interaction between p67 and mDIP is indeed required for mDIP to influence p67 function. Similar results were obtained in experiments where NIH3T3 cells were used (results not shown).

DISCUSSION

mDab2 and DOC-2/DAB2, which is the human homologue of mDab2, are serine phosphorylated and believed to be potent negative regulators of carcinoma cell growth [4,17]. The p96 isoform of mDab2 is down-regulated in breast and ovarian carcinoma cell lines, where it prevents Grb2 from binding to Sos [4,6]. DOC-2/DAB2 is up-regulated in rat ventral prostate after castration, suggesting that it can be regulated directly or indirectly by the androgen receptor [5]; however, it does not interact with Grb2 [17]. Rather, it inhibits cell growth through inhibiting AP-1 activity, although it is not known how DOC-2 inhibits AP-1 activity in the nucleus [17]. Although it has been speculated that mDab2 p96 is a candidate tumour suppressor in several cancer cell lines, the function and regulation of the p67 isoform are still unknown. In the present work, the transcriptional activity of p67 has been clarified and the functional domains of transcriptional activation and mDIP binding have been mapped.

As a first step toward elucidating the biological function of mDab2, we determined its cellular localization. So far, Dab proteins were believed to be cytoplasmic proteins and, therefore, their functions have been confined to the cytosol. Surprisingly, however, p67 was found to be located not only in the cytoplasm but also in the nucleus of RA-treated F9 cells, where it interacted with mDIP, which acts as a transcriptional co-regulator. mDab2 p67 binds via the N-terminal PID to the C-terminal region of mDIP. mDab2 PID is believed to have a distinct property, since it binds to neither tyrosine-phosphorylated peptide ligands nor phospholipid bilayers [7,8]. Sequence-similarity searches revealed that mDIP is a mouse ARIP3. During the development of the prostate, ARIP3 binds to the androgen receptor as a co-regulator and thereby influences androgen-receptor-dependent transcription [11]. In addition to ARIP3, its homologues interact with tissue-type specific transcription factors: PIASxα, a human homologue of ARIP3, interacts with Stat1 in haematopoietic cells [18], and MiZ-1, an alternatively spliced form of ARIP3, interacts with Msx2 in the process of skull development [19]. It is noteworthy that all of these proteins, which possess at least 96 % amino-acid sequence identity, interact with totally different transcription factors in a cell-type-specific manner. Our present data and those of these reports suggested to us the possibility that mDab2 could be another transcription factor specific to F9 cell differentiation.

This possibility was further supported by the results showing that mDab2 p67, fused to the heterologous DNA-binding domain, stimulated transcription of the reporter gene without the presence of any other transactivation domain. Furthermore, our deletion analyses revealed that a transcriptionally active domain is contained within the C-terminal region of mDab2 p67. The C-terminal region of mDab2 p67 consists of almost 20 % proline residues, which may contribute to the transcriptional activation function of mDab2. A number of other known, or suspected, site-specific transcription factors contain similar proline-rich domains. First, all of the known members of the CTF/NF-1 family of proteins contain a proline-rich region, although the overall sequence of this C-terminal domain shows extensive differences in amino acid sequence [20–22]. In addition to the CTF family, a number of proteins involved in the developmental control of gene expression in *Drosophila*, including the fushi tarazu and Kruppel proteins, contain 50–100 amino acid regions of high proline content [23,24], similar to that of mDab2. Steroid receptors for progesterone and oestrogen also contain proline-rich sequences in regions of the molecule that participate in their transcriptional activity [25,26]. According to the current model, the proline-rich domain folds into a unique structure consisting, for instance, of omega loops [27], which form protein–protein contact with the transcription machinery and then function as a transactivation domain.

pM–mDab2 p96 was less active than pM–mDab2 p67 for transcriptional activation. These results, and the observation

that RA-induced expression level of p96 is very low in F9 cells (Figure 2A), suggest that p96 may play an ancillary role to p67 or a different role, such as down-regulation of Ras.

Although the function of mDab2 has not yet been fully clarified, it is obvious that mDab2 p67 can behave as an intrinsic transcriptional activator. The protein is supposed to mediate its transactivating activity through both the PID and the proline-rich region. In our experiment, it is clearly revealed that the proline-rich region is responsible for the transactivation domain; however, the molecular function of PID is not fully understood. The primary structure of mDab2 p67 does not contain any consensus DNA-binding motif. We hypothesize that mDIP binds to the PID of mDab2 and may facilitate the recruitment of mDab2 to a sequence-specific DNA-binding transcription factor. In the present study, we found that mDIP did not significantly enhance the transcriptional activity of mDab2 p67. This may be because p67 was fused to the GAL4 DNA-binding domain. Further experiments are needed to define precisely the molecular mechanism by which mDIP affects the transactivating activity of mDab2 p67. We hope to identify also the sequence-specific DNA binding partner of mDab2 p67 in RA-treated F9 cells. Recently, we have isolated a protein that possesses well-conserved sequences related to the homeodomain. Maybe the characterization of this protein will provide more convincing evidence for the function of mDab2 p67.

In conclusion, our experiments have resulted in several important findings: (i) the detection of mDab2 p67 in the nucleus of RA-treated F9 cells; (ii) the identification of mDab2 p67 binding partner, mDIP, which acts as a transcriptional coregulator; and (iii) the recognition that mDab2 p67 functions as a transcriptional activator. Presently, mDab2$^{-/-}$ F9 cells, in which the expression of mDab2 is eliminated by targeted disruption of the gene, are being developed in our laboratory. It will be of interest to examine the discrepancies in the biochemical and physiological properties of the mDab2$^{-/-}$ F9 and wild-type F9 cells during RA-induced differentiation.

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