Id2 Is a Target of the β-Catenin/T Cell Factor Pathway in Colon Carcinoma*

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Activation of β-catenin/TCF transcription as a result of mutations in the adenomatous polyposis coli (APC) and/or β-catenin genes occurs in the majority of colon tumors. An increasing number of genes, including c-myc and cyclin D1, have been implicated as targets of this pathway. We now report that the dominant negative helix-loop-helix regulator Id2 is also a target of the β-catenin/TCF transcription pathway in colon adenocarcinoma. Investigation of the mechanism for the overexpression of Id2 in colon carcinoma cells demonstrated that the Id2 promoter is activated, and the Id2 protein is up-regulated by β-catenin. Conversely, reducing free β-catenin blocked this induction of promoter activity. We have also used an electrophoretic mobility shift assay and supershift to identify a motif in the Id2 promoter that binds to TCF4 protein. Site-directed mutagenesis of this motif abolished promoter reporter activity. Both transfection of Id2 into SW480 cells and induction of Id2 in HT29 colon cells was found to increase anchorage-independent survival of these cells. Growing evidence associates disruption to Id2 expression with tumorigenesis, and our findings suggest that this dysregulation of Id2 expression is due to the activation of the β-catenin/TCF pathway.

Mutations in the APC and β-catenin genes are common in human colon cancer (1, 2). These mutations lead to the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus. In the nucleus, β-catenin associates with T cell factor (TCF)1 leading to the formation of a bipartite transcription factor that activates transcription of target genes such as c-myc and cyclin D1 (for a review, see Ref. 2).

The helix-loop-helix (HLH) family of transcription factors has been implicated in transcriptional regulation, oncogenesis, cell type determination, and differentiation (3). The HLH structure that distinguishes this class of molecules mediates protein dimerization within the HLH family (4). Many members of the HLH family contain a domain of basic amino acids, aminoterminal to the HLH motif, which mediates DNA binding following dimerization. A HLH subfamily has been identified, known as the Id (inhibitors of differentiation/DNA binding) proteins, that lacks this basic region and possesses the ability to act as dominant negative molecules (5). One member of the Id subfamily, Id2, regulates proliferation and differentiation. Thus, deregulated Id2 expression has the potential for malignant transformation. In support of this hypothesis, overexpression of Id2 has been demonstrated in human pancreatic cancer (6). Preliminary observations suggesting a correlation between β-catenin and Id2 expression in colonic polyps led us to explore the possibility that Id2 is a target of β-catenin/TCF-mediated transcription.

Experimental Procedures

Tissue Samples—Samples of tumor, polypl, and normal tissue were obtained from patients undergoing Surgery at Western Hospital, Victoria, Australia. The collection of tissues was approved by the relevant hospital ethics committee.

Cell Lines—The colon carcinoma cell lines used in this study have been described previously (7). Colon carcinoma cell lines and the adenovirus immortalized human kidney cell line (HEK-293) were grown as monolayers in RPMI 1640 medium containing 10% (v/v) bovine calf serum (CSL Ltd., Parkville, Victoria, Australia) at 37 °C in a fully humidified atmosphere containing 5% CO2 in air.

Immunohistochemistry—Immunohistochemistry was performed on serial paraffin sections as described previously (8) according to a commercially adapted method (DAKO Corp., Carpinteria, CA) using new fuchsin chromogen as substrate. Sections of 5 μm thickness were incubated with either rabbit anti-Id2 polyclonal antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) or an equivalent dilution of non-immune rabbit serum. Id1, Id3, Id4, and E2A rabbit polyclonal antibodies (Santa Cruz Biotechnology) were used to control for nonspecific binding. Specific Id2 immunoreactivity was also demonstrated by competition with an Id2-specific blocking peptide (Santa Cruz Biotechnology). Anti-β-catenin monoclonal antibody (Transduction Laboratories, Los Angeles, CA) was used at 1:100 on sections treated by boiling in 0.1 M sodium citrate (pH 6.0) for 20 min to unmask antigen prior to blocking.

In Situ Hybridization—To confirm the expression of Id2, we performed in situ hybridization essentially as described by Brand et al. (9). Briefly, digoxigenin-labeled RNA was generated using T7 polymerase (40 units, Promega Corp., Madison, WI) and restriction endonuclease-linearized pCDNA3.1-Id2 sense and antisense plasmids as templates. Sections were hybridized at 50 °C overnight. Bound probe was detected using an anti-digoxigenin monoclonal antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals GmbH), and the color reaction was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate. Sections were counterstained with acid fuchsin for 2 min at room temperature.

Immunoblot Analysis—Cell lysates (50 μg/lane) were resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) and incubated with anti-Id2 rabbit polyclonal antiseraum (1:500, Santa Cruz Biotechnology).
Biotechnology) for 18 h at 4 °C followed by swine anti-rabbit polyclonal antibody conjugated to horseradish peroxidase. Blots were washed in TBS-T, and bound antibody was detected with an enhanced chemiluminescence kit (ECL kit, Amersham Pharmacia Biotech). The membranes were then stripped by incubation in 62.5 mM Tris-HCl buffer, pH 6.7, containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol at 50 °C for 30 min. Membranes were subsequently blocked and immunoblotted with a pan actin monoclonal antibody (ICN Biomedicals Inc., Aurora, OH) or anti-V5 monoclonal antibody (Invitrogen, Groningen, The Netherlands).

Expression Plasmids and Id2 Sense and Antisense Inducible Cell Lines—Full-length sense and antisense Id2 constructs were cloned into the pcDNA3.1 expression vector (Invitrogen). V5-tagged Id2 constructs were generated using the pcDNA3.1/V5 vector (Invitrogen) with a reverse primer without the Id2 stop codon (nucleotides 479–498; GC-CACACAGTGGCTTTGGCTGT). The Myc-tagged ΔN89 β-catenin (lacking the N9 N-terminal amino acids) expression vector has been described previously (10). The human expression vectors E-cadherin cytoplasmic domain and E-cadherin cytoplasmic domain Δ (that lacks the β-catenin interaction domain) have also been described elsewhere (11). To create the Id2 Tet-inducible HT29 cell line, Id2 cDNA was subcloned into the green fluorescent protein (GFP)-expressing plasmid pBl-EGFP (CLON-TECH Laboratories, Palo Alto, CA). Sense and antisense constructs were co-transfected with pTet-On/IRESPuro (a gift of Dr. M. O’Connell, Peter MacCallum Cancer Institute, Melbourne, Australia). Transfected cells were grown in puromycin (2 μg/ml) sorted against GFP without induction by fluorescence-activated cell sorting initially and then by sorting following addition of doxycycline (100 ng/ml). In Vitro Promoter Assays—Cells were plated at 4 × 10^5 cells/well 24 h prior to transfection. One microgram of each plasmid was transfected with 3 μl of FuGENE 6 (Roche Diagnostics) according to the instructions of the manufacturer. Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (12). The −934 to +30 Id2 promoter-CAT reporter (13) activity was compared with pCAT-basic (Promega Corp.). Site-directed mutagenesis of the −834 to +30 Id2 plasmid was performed by using the “QuickChange” site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the instructions of the manufacturer. In all cases the addition of empty vector pcDNA3.1 was used to ensure equivalent DNA levels in all transfections.

Gel Retardation Assay—Id2 promoter binding of in vitro synthesized protein (TNT, Promega Corp.) or nuclear lysates were assessed by electrophoretic mobility shift assays following the methods described previously (14). The oligonucleotide sequence corresponding to bases −109 to −85 within the 5′ Id2 flanking region (GATCCGCTCGCTTGGATAAGCC) and containing a putative TCF binding site (underlined) was used. Oligonucleotides modified by the addition of a T to create a consensus TCF4 site (underlined) (GATCCGCTCGCTTGGATAAGCC) or a C to disrupt the putative TCF binding site (GATCCGCTCGCTTGGATAAGGG) were used as controls. Confirmation of in vitro synthesized TCF4 protein was determined by incorporation of [35S]Sulfonine (Amersham Pharmacia Biotech) and standard SDS-PAGE. Binding reactions (2 μg of nuclear extract protein) were incubated for 20 min at room temperature before addition of 0.25 μg of either anti-TCF4 monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY) or an irrelevant control monoclonal antibody and then incubated for a further 20 min. The samples were subsequently subjected to nondenaturing PAGE at 4 °C. The gels were examined using Molecular Dynamics PhosphorImager SI and analyzed using ImageQuant software (Amersham Pharmacia Biotech).

RESULTS

We have used immunohistochemistry to examine the expression of Id2 in human adenomatous polyps. Increased levels of Id2 expression were observed in the majority of adenomas examined (49/59) (Fig. 1A). No staining was detected in normal colon mucosa (data not shown). The expression of Id2 in polyps was confirmed in selected cases by in situ hybridization (Fig. 1B). Where Id2 expression was raised, the pattern of expression was heterogeneous with areas of high expression and areas without staining (Fig. 1C). We also detected heterogeneous β-catenin expression in 51/59 adenoma cases (Fig. 1C). In 48/59 adenoma cases there was a striking similarity in the pattern of β-catenin and Id2 expression with staining confined to specific crypts (Fig. 1, A and C). Of the 11 cases with no Id2 staining, 8 also failed to stain with β-catenin, and 3 had only low β-catenin expression.

We have also used the TOPFLASH/FOPFLASH-TCF reporter system (15) to demonstrate a close correlation between β-catenin/TCF-mediated transcriptional activity and the expression of Id2 in these colon cancer cell lines (Table I). Cell lines exhibiting TOPFLASH activity (for example SW480 and LIM1215) (Table I) were found to express high levels of Id2 by Northern (Fig. 2) and Western analysis (data not shown). Conversely cell lines that did not activate the TOPFLASH reporter (for example LIM2405 and LIM2412) expressed only low levels of Id2. The TOPFLASH/FOPFLASH activity and Id2 expression were consistent with the APC and β-catenin mutation status of the cell lines (Table I).

To confirm the relationship between β-catenin/TCF-mediated transcriptional activity and Id2 expression, we transfected HEK-293 cells with an inherently stable truncated form of β-catenin (ΔN89 β-catenin) (10) and determined the level of Id2 protein expression. Levels of Id2 protein were found to be increased in cells overexpressing β-catenin (Fig. 3A) but not in cells overexpressing c-Myc (Fig. 3B). Transfection of cells with ΔN89 β-catenin resulted in an average 3-fold increase in expression of Id2 protein as determined by densitometry in three independent experiments.

To address the mechanism by which β-catenin was increasing Id2 expression we examined the effect of β-catenin on the Id2 promoter. Co-transfection of a −834 to +30 Id2 promoter-CAT reporter construct with the ΔN89 β-catenin expression vector into HEK-293 cells resulted in a 3-fold induction of the Id2 promoter activity compared with co-transfection with an empty vector (Fig. 4A). This induction of the Id2 promoter activity by ΔN89 β-catenin could be prevented by co-transfection of the cytoplasmic domain of E-cadherin (Fig. 4A), a molecule that is known to sequester β-catenin (11). Furthermore, in SW480 cells that have high nuclear β-catenin levels due to a mutation in the APC protein (Table I), reintroduction of the wild type APC protein was found to reduce the levels of Id2 promoter activity (Fig. 4B).

Analysis of the Id2 5′ flanking region sequence (GenBank™ accession number AF270493) demonstrated that the Id2 promoter contains a site on the negative strand (ATCAAG) at −95 that is similar to a reported optimal TCF binding site (WW-CAAAAG where W = A or T) (15). We constructed oligonucleotides containing either the wild type Id2 promoter sequence or the promoter sequence that had been modified to contain an optimal TCF binding site. In vitro synthesized TCF4 was able to bind both these oligonucleotides as determined by electrophoretic mobility shift assay. Similarly, an electrophoretic mobility shift assay was used to demonstrate that nuclear lysates of colon cancer cells contained a protein able to bind oligonucleotides containing the wild type and optimal TCF binding sites but not an oligonucleotide in which the putative TCF binding site had been disrupted (Fig. 5A). Furthermore, the binding of the protein could be competed with unlabeled oligonucleotide (100-fold w/w excess) containing the wild type promoter sequence but not with the oligonucleotide containing the disrupted site (data not shown). Specific retardation of both the optimal TCF probe and the Id2 probe by an anti-TCF4 monoclonal antibody indicated the presence of TCF4 protein. The importance of the putative TCF4 binding site was also demonstrated...
strated using the *Id2* promoter reporter assay. Mutation of the ATCAAG site in the *Id2* promoter to an irrelevant sequence by site-directed mutagenesis rendered the *Id2* promoter refractory to activation by co-transfection of β-catenin/TCF4 (Fig. 5B).

The finding that *Id2* is a target of β-catenin/TCF-mediated transcription suggested a role for *Id2* in colon carcinogenesis. Increased *Id2* protein was demonstrated in colon tumors by Western blotting (Fig. 6). In addition, transient transfection of *Id2* was found to result in a 3-fold increase in clonogenicity of

### Table I

| Cell line | APC gene status | β-Catenin gene status | TCF-mediated transcriptional activity | *Id2* expression |
|-----------|-----------------|-----------------------|---------------------------------------|-----------------|
| LIM1215   | Normal          | T41A                  | +                                     | High            |
| Caco-2    | LOH/aa 1367     | ND                    | +                                     | High            |
| SW480     | LOH/aa 1338     | ND                    | +                                     | High            |
| LIM2405   | Normal          | ND                    | –                                     | Low             |
| LIM2412   | Normal          | ND                    | –                                     | Low             |
| LIM2537   | Heterozygous    | ND                    | –                                     | Low             |

*Assessed by TOPFLASH/FOPFLASH TCF-reporter assay (16). Cell lines were considered positive (+) when there was greater than a 3-fold increase in TOPFLASH reporter activity compared to the FOPFLASH control reporter.

*Personal communication from Dr. R. Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia.

*Allelic loss and mutation of remaining allele resulting in truncation at amino acid 1367 (30).

*Not determined.

*Ref. 31.

*Allelic loss and mutation of remaining allele resulting in truncation at amino acid 1338 (30).

*Heterozygous with one wild type and one mutant allele (E. Vincan, unpublished).
K562 was used as a hybridization positive control (+ve con) investigated by Northern blot analysis. Poly(A) probe labeled with [α-32P]ATP. The erythroleukemic cell line K562 was used as a hybridization positive control (+ve con).

Empty vector and antisense constructs served as controls. We transfected with 2 µg of vector DNA into HT29 carcinoma HT29 cells that express an inducible Id2 construct. Immunoblot analysis of Id2 tagged the doxycycline-inducible Id2 with a V5 tag to differentiate it from the endogenous Id2 protein. Immunoblot analysis of Id2 tagged protein detects only transfected protein and not endogenous Id2 protein.

Induction of Id2 protein by β-catenin can increase the level of Id2 protein and activate the LEF/TCF pathway. In colonic polyps, the pattern of Id2 expression correlates with the pattern of β-catenin expression. Similarly, human colon tumors, which are known to harbor a high frequency of APC and β-catenin mutations that result in increased levels of β-catenin, also have increased levels of Id2.

To confirm these findings we created cell lines of the colon carcinoma cell lines. The expression of Id2 in a series of human colon carcinoma cells lines was investigated by Northern blot analysis. Poly(A) probe labeled with [α-32P]ATP. The erythroleukemic cell line K562 was used as a hybridization positive control (+ve con).

human colon carcinoma cells in semisolid agar (Fig. 7A). However, we were unable to demonstrate increases in Id2 protein levels in the colonies due to the small numbers of cells recovered in these experiments.

To confirm these findings we created cell lines of the colon carcinoma cell line HT29 cells that express an inducible Id2 construct. Empty vector and antisense constructs served as controls. We tagged the doxycycline-inducible Id2 with a V5 tag to differentiate it from the endogenous Id2 protein. Immunoblot analysis of Id2 tagged protein detects only transfected protein and not endogenous Id2 protein.

DISCUSSION

Here we have demonstrated that Id2 is a target of the β-catenin/TCF pathway. Consistent with these findings, human pancreatic tumors, which express increased levels of Id2 (6, 16), have been shown to contain β-catenin and, to a lesser extent, APC mutations (17). It is also of interest to note that transgenic mice overexpressing the related protein Id1 develop intestinal adenomas but not carcinomas (18), a phenotype very similar to that seen in mice carrying an APC mutation (19).
nenous β-catenin. Immunoblotting with an anti-β-catenin antibody failed to detect the expressed protein, presumably due to the interference in the binding of the antibody to its epitope by the C-terminal tag. It is thus not possible to ascertain the true relative increase in β-catenin levels achieved by transfection.

We were, however, able to confirm that the modest increase in Id2 protein was not due to insufficient TCF levels in the transfected cells as co-transfection of TCF4 with β-catenin did not appreciably increase the level of Id2 (data not shown).

β-Catenin/TCF is known to activate transcription of a number of genes including the transcription factor c-myc (20). A recent study demonstrated increased Id2 promoter activity induced by N-Myc or c-Myc in neuroblastoma cells (21). This raised the possibility that the induction of Id2 expression by β-catenin in our study could be secondary to induction of c-Myc. However, the Id2 promoter construct used in our study does not contain any canonical Myc sites. More importantly, we found that, in contrast to β-catenin, c-Myc overexpression in HEK-

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**Fig. 5. Identification of a TCF4 binding site in the Id2 promoter.** A, electrophoretic mobility shift assay of LIM1215 nuclear extracts with [α-32P]dATP-labeled oligonucleotides. Lanes 1 and 4, Id2 promoter oligonucleotide with a C inserted to disrupt the putative TCF binding motif (underlined) (GATCCGCTGCTTGATAGACG); lanes 2 and 5, the Id2 promoter oligonucleotide with a T inserted to give an optimal TCF binding motif (GATCCGCTGCTTGGATAGACG); lanes 3 and 6, oligonucleotide of the wild type Id2 promoter sequence (GATCCGCTGCTTGATAGACG). The different nucleotide sequence (in bold) between each oligonucleotide is indicated below the lane. In lanes 1–3 antibodies to TCF4 were added to the nuclear lysates; lanes 4–6 show lysates to which a nonspecific (NS) irrelevant monoclonal antibody has been added. The TCF4 and supershifted bands are indicated (arrows). B, CAT reporter activity comparing wild type −834 to +30 Id2 promoter (TT) and two independently derived clones with TT → TCT and TT → GGG mutations. HEK-293 cells were co-transfected with wild type or mutant reporter constructs together with an ΔN89 β-catenin (black bars) or empty vector control plasmid (hatched bars). CAT activity is expressed as the -fold increase over the empty vector control following normalization with co-transfected β-galactosidase. Each transfection was carried out in triplicate plates. Shown are means ± S.E. from three independent experiments.

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**Fig. 6. Id2 protein expression in colon carcinoma tissue.** Human colon carcinoma tissue (T) and paired normal colon mucosa (N) from four different patients were analyzed for Id2 protein levels by Western blot. Fifty micrograms of total protein from each sample were resolved by 15% SDS-PAGE and analyzed by blotting with an antibody to Id2. Immunoreactive proteins were detected using a peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence. The same membrane was stripped and subsequently rebotted with antibodies to actin as a control for loading. The LIM1215 cell line was used as a positive control (+ve con).
293 cells did not alter the level of Id2 protein. We also provide evidence that the Id2 promoter contains a functional TCF binding site providing a mechanism for the direct activation of Id2 transcription by β-catenin/TCF. Thus it appears that in colon cells the Id2 gene is a direct target of the β-catenin/TCF pathway.

To investigate the functional consequences of Id2 overexpression, we have analyzed colon cells transfected with Id2 sense and antisense constructs for colony formation in semisolid agar. Colon cells transfected with Id2 sense constructs formed an increased number of colonies. This is particularly interesting since the basal Id2 levels in the cell lines used are already high. However, while this result may appear counterintuitive, it was a reproducible finding with both transient and constitutive expression of Id2. The mechanisms underlying this observed increase in clonogenicity have not been elucidated.

It is interesting to note that in our studies many of the crypts that display enhanced Id2 expression also display morphological and structural similarity to crypts without high levels of Id2 or β-catenin suggesting that Id2 overexpression does not, on its own, affect crypt morphology. This may indicate that further genetic disruptions occur in vivo that result in the dysplastic features of adenomatous polyps. We would therefore suggest that Id2 might act as a “landscaping gene” allowing unrestrained proliferation and the further chance of genetic alteration (29).

In summary, we have demonstrated that the dominant negative HLH transcriptional regulator Id2 is a target of the β-catenin/TCF pathway and suggest that overexpression of Id2 as a consequence of APC and β-catenin mutations may play an important role in human tumorigenesis.

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β-Catenin/TCF Induces Id2 Expression

FIG. 7. Effect of Id2 on the growth of colon cells in semisolid agar. A, SW480 cells were transfected with empty vector (EV) or full-length Id2 sense (S) or Id2 antisense (AS) constructs. Forty-eight hours after transfection 1×10⁶ cells/dish were seeded into semisolid agar and cultured for 10 days after which time the number of colonies was counted. Results are expressed as percentage of the empty vector control. Shown are the means ± S.E. from two experiments performed in triplicate. B, HT29 cells stably transfected with the Tet-inducible Id2 sense and antisense construct. Cells transfected with empty vector (lanes 1 and 4), V5-tagged Id2 sense construct (lanes 2 and 5), and an antisense construct (lanes 3 and 6) treated with (+, lanes 1–3) or without (−, lanes 4–6) 2 μg/ml doxycycline (Dox) for 18 h were solubilized, resolved by 15% SDS-PAGE, and immunoblotted with antibodies to V5 and Id2. Immunoreactive proteins were detected with an appropriate peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence. C, HT29 cells (1×10⁶/dish) were seeded in semisolid agar in the presence of doxycycline (2 μg/ml) and cultured for 10 days after which time the number of colonies was counted. Results are expressed as percentage of the empty vector control. Shown are the means ± S.E. from three experiments performed in triplicate.
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