[CANCER RESEARCH 63, 4641–4647, August 1, 2003]

Proteomic Analysis of Intestinal Epithelial Cells Expressing Stabilized β-Catenin[1]

Masahiro Seike, Tadashi Kondo, Yasuharu Mori, Akihiko Gemma, Shoji Kudoh, Michiie Sakamoto, Tesshi Yamada,[2] and Setsuo Hirohashi

Cancer Proteomics Project, National Cancer Center Research Institute, Tokyo 104-0045 [Ma. S., T. K., Y. M., T. Y., S. H.]; Fourth Department of Internal Medicine, Nippon Medical School, Tokyo 113-8603 [Ma. S., A. G., S. K.]; and Department of Pathology, Keio University School of Medicine, Tokyo 160-8582 [Mi. S.], Japan

ABSTRACT

Aberrant accumulation of β-catenin protein because of mutation of either the β-catenin or adenomatous polyposis coli gene plays an essential role in the development of colorectal carcinoma. We established previously a stable clone of the rat small intestinal epithelial cell line IEC6, which is capable of inducing stabilized β-catenin protein lacking NH_2-terminal glycogen synthase kinase-3β phosphorylation site under a strict control of the tetracycline-regulatory system. This clone, IEC6-TetOFF-β-catenin ΔN89, shows *in vitro* polypoid growth on the removal of doxycycline and seems to be an appropriate model for analyzing the molecular mechanisms of early intestinal carcinogenesis. Of >2000 protein spots displayed by newly developed two-dimensional difference gel electrophoresis, 22 were found to be up- or down-regulated on the induction of stabilized β-catenin. The majority of these proteins fell into two categories: (*a*) redox-status regulatory proteins and (*b*) cytoskeleton-associated proteins. Representatively, a key redox-status regulatory protein, manganese superoxide dismutase, up-regulated in IEC6 cells expressing stabilized β-catenin protein, was overexpressed in adenoma and adenocarcinoma cells of familial adenomatous polyposis patients in parallel with the accumulation of β-catenin. These results suggest that aberrant accumulation of β-catenin might contribute to colorectal carcinogenesis by affecting redox status in the mitochondria of intestinal epithelial cells.

INTRODUCTION

More than 80% of sporadic colorectal cancers carry a mutation in the *APC* gene, and half of the remainder carry a mutation in the β-catenin (*CTNNB1*) gene. Aberrant accumulation of β-catenin protein resulting from mutation of either the β-catenin or *APC* gene plays an essential role in the development of colorectal carcinoma (1–4). β-Catenin protein binds to the TCF/LEF[3] transcription factors and functions as a transactivator (5, 6). Several downstream transcriptional targets of the β-catenin–TCF/LEF complex have been reported recently (7–12). However, the whole picture of gene and protein expression profiles governing colorectal carcinogenesis remains to be explored.

We established previously a stable clone, IEC6-TetOFF β-catenin ΔN89, which is capable of inducing stabilized β-catenin protein on the removal of Dox from the culture medium using the tetracycline-inducible promoter system (13). The induction of β-catenin ΔN89 caused nuclear translocation of β-catenin protein and TCF/LEF-specific gene trasactivation. IEC6-TetOFF β-catenin ΔN89 cells developed numerous polypoid foci *in vitro* only when the β-catenin ΔN89 protein was induced and thus seem to provide an appropriate tissue culture model for analysis of the adenomatous proliferation of intestinal epithelial cells.

In the present study, we implemented a proteomic approach to identify a population of proteins, the expression of which is affected by the induction of stabilized β-catenin in IEC6-TetOFF β-catenin ΔN89 cells, by using a newly developed 2D-DIGE technique (14, 15). Because in 2D-DIGE the two protein samples to be compared are labeled with different fluorescent dyes (Cy3 or Cy5) and are coseparated in a single two-dimensional gel, the comparison of the two protein populations is accurate. Here, we report that 22 protein spots were significantly up- or down-regulated as a result of the induction of stabilized β-catenin. Mass spectrometry and a database search determined that many of these proteins are involved in the redox-status regulatory pathway. These results suggest that aberrant accumulation of β-catenin might contribute to the development of colorectal carcinoma by directly or indirectly affecting redox status. We were able to reach this conclusion only by having a bird's-eye view of the proteome, not by conventional molecular approaches or by gene expression profiling.

MATERIALS AND METHODS

Protein Extraction and Labeling with Fluorescent Dyes. A stable clone, IEC6-TetOFF β-catenin ΔN89, and a relevant mock clone, IEC6-TetOFF control, were established as described previously (13). Cells were cultured for 14 days with 0.1 μg/ml Dox, or without Dox, before protein extraction. Cells were washed with ice-cold PBS and treated with 10% trichloroacetic acid for 30 min on ice. The cells were then scraped off and pelleted by brief centrifugation. After washing with PBS, the cell pellets were incubated for 30 min with LB containing 6 M urea, 2 M thiourea, 3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 1% Triton X-100. Insoluble materials were removed by centrifugation. The protein concentration was determined by using a Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Fifty micrograms of cell lysates, adjusted to pH 8.5 with 30 mM Tris-HCl, was labeled with 200 pmol of either Cy3 or Cy5 (Amersham Biosciences, Amersham, United Kingdom) according to the manufacturer's recommendations (14, 15). The labeling reaction was quenched by incubation with 0.2 mM lysine for 10 min on ice. The labeled lysates were then incubated with an equal volume of LB containing 130 mM DTT and 2% ampholine (Amersham) for 15 min on ice. Cy3-labeled samples were pooled, and the aliquots were added into Cy5-labeled samples. The final volume was adjusted to 450 μl with LB containing 65 mM DTT and 1% ampholine, before electrophoresis.

2D-DIGE and Image Analyses. Two-dimensional gel electrophoresis was performed as described previously (16). Briefly, IPGs (Amersham), with pH values ranging from 3 to 10, were rehydrated with labeled protein samples for 12 h at 20°C. Isoelectric focusing was performed using IPGphor (Amersham) for a total of 80 kV/h at 20°C. The IPGs were equilibrated for 15 min in equilibration buffer containing 3 M urea, 50 mM Tris (pH 8.8), 30% glycerol, 1% SDS, and 16 mM DTT and then for 15 min in the same buffer containing 122 mM iodoacetamide instead of DTT. Equilibrated IPGs were transferred onto 9–15% gradient polyacrylamide gels. Twelve gels were run in the EttanDalt II system (Amersham) at 17 W for 15 h at 20°C. Two-dimensional gels were scanned with appropriate wavelengths for excitation and emission by using a two-dimensional 2920 Master Imager (Amersham). Relative quantification of spot intensities and statistical evaluation were carried out with DeCyder software (Amersham). We ran 12 gels (*e.g.*, four samples in tripli-

Received 11/4/02; accepted 6/2/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan. Ma. S. and Y. M. are Awardees of Research Resident Fellowships from the Foundation for Promotion of Cancer Research in Japan.

[2] To whom requests for reprints should be addressed, at Cancer Proteomics Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511, extension 4054; Fax: 81-3-3547-5298; E-mail: tyamada@gan2.res.ncc.go.jp.

[3] The abbreviations used are: TCF, T-cell factor; 2D-DIGE, two-dimensional difference gel electrophoresis; (Mn)SOD, (manganese) superoxide dismutase; DOX, doxycycline; FAP, familial adenomatous polyposis; Cy3, 1-(5-carboxypentyl)-1′-propylindocarbocyamine halide; Cy5, 1-(5-carboxypentyl)-1′-methylindocarbocyamine halide; IPG, immobilized pH gradient; LB, lysis buffer; LEF, lymphoid-enhancer factor.

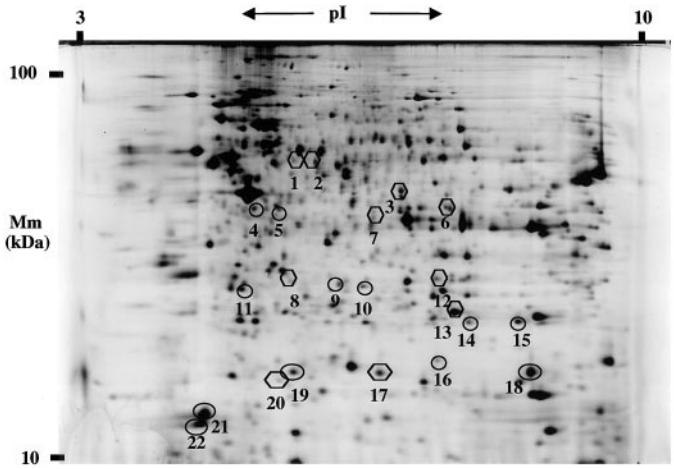


Fig. 1. Representative 2D-DIGE image of Cy5-labeled proteins of IEC-6 cells expressing stabilized β-catenin. The intensities of 22 spots were altered in the proteome of IEC6 cells by the induction of β-catenin ΔN89. The results were consistent in all four independent experiments, and a representative image is depicted. Spots with increased intensity on the induction of β-catenin ΔN89 are marked by *circles*, and spots with decreased intensity are marked by *hexagons*. Spot numbers correspond to those in Figs. 2 and 3*B* and Tables 1 and 2.

cate) in every experiment and confirmed reproducibility by repeating the experiments at least four times.

Protein Identification by Mass Spectrometry. A preparative two-dimensional gel was made as described above, except that the concentration of DTT was increased to 162 mM, and iodoacetamide was replaced by 0.05% acrylamide for equilibration of IPGs. After electrophoresis, the gel was fixed in a solution containing 30% (volume for volume) ethanol and 10% (volume for volume) acetic acid for 60 min, washed briefly with water, and then stained with SyproRuby (Molecular Probes, Inc., Eugene, OR). A two-dimensional image was obtained by scanning with the two-dimensional 2920 Master Imager. The image of the preparative gel was matched to those of analytical gels by using the DeCyder software, and the spots of interest were excised with an automated spot excision robot, SpotPicker (Amersham). In-gel digestion was carried out with trypsin (Promega, Madison, WI) as described previously (17), except that the incubation of the gel with iodoacetamide was omitted. Mass spectrometric analyses of tryptic digests were performed by using Q-STAR Pulsar-*i* (Applied Biosystems, Framingham, MA), and peptide mass mapping was carried out with Analyst QS software (Applied Biosystems). The isoelectric points and molecular masses of protein spots were calculated with PDQuest software (Bio-Rad).

Tissue Specimens. Tissue samples of primary colorectal carcinoma with Dukes' stage B and C and normal colonic mucosa were obtained with informed consent at the time of surgery on 7 patients at the National Cancer Center Hospital.

Western Blot and Two-dimensional Blot Analyses. Fifteen micrograms of lysates were labeled with Cy5 as described above and separated by SDS-PAGE. The gels were scanned with appropriate excitation and emission spectra for Cy5 with Typhoon 8600 (Amersham). The sum of band intensity of the entire lane was used for normalizing the amount of proteins loaded onto SDS-PAGE gels. The proteins were then transferred to nitrocellulose membranes and blotted with antibodies against MnSOD (StressGen Biotechnologies Corp., Victoria BC, Canada), Cu/ZnSOD (StressGen), carbonic anhydrase II (Rockland, Gilbertsville, PA), cofilin (Cytoskeleton Inc., Denver, CO), and β-actin (Abcam Limited, Cambridge, United Kingdom). The blots were detected by using an enhanced chemiluminescence system (Amersham), as described previously (18). The intensity of the bands was quantified using an image analyzer, LAS-1000 (Fujifilm, Tokyo, Japan).

Proteins displayed in two-dimensional gels were transferred to nitrocellulose membranes and blotted with anti-MnSOD antibody, as described above.

MnSOD Activity. After being washed with ice-cold PBS, tissue samples were homogenized in 0.5% Triton X-100/PBS and sonicated twice on ice for 15 s. The soluble fraction was recovered by centrifugation. The activity of MnSOD was determined by measuring the reduction rate of cytochrome *c* with an SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc., Gaithersburg, MD; Ref. 19). The specific activity of MnSOD was assayed in the presence of 4 mM potassium cyanide, which inhibits Cu/Zn SOD activity (20). SOD activity was expressed as units per milligram protein, and one unit was defined as the amount of SOD required to inhibit the rate of cytochrome *c* reduction by 50%. Purified SOD (Sigma Chemical Co., St. Louis, MO) was used to form a standard curve against which the activity of SOD was calculated.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues of colorectal adenoma and adenocarcinoma of 10 patients with FAP were selected from the surgical pathology panel of the National Cancer Center Hospital, as described previously (10). Immunoperoxidase staining was performed with anti-MnSOD polyclonal antibody (StressGen), essentially as described previously (10).

RESULTS

Identification of Proteins whose Expression Is Affected by the Induction of Stabilized β-Catenin. To identify a population of proteins whose expression is regulated by the β-catenin oncoprotein, we compared the global protein expression profiles of IEC6-TetOFF β-catenin ΔN89, cultured in confluence for 14 days with or without Dox. Protein samples were divided into two aliquots, each of which was labeled with one of two kinds of fluorescent dye, Cy3 or Cy5. Cy3-labeled samples were pooled and included into each Cy5-labeled sample as a common internal standard. From each two-dimensional gel, two images were obtained according to specific spectra for excitation and emission: (*a*) Cy5 images unique to each sample and (*b*) Cy3 images common to all gels. The intensity of spots in Cy5 two-dimensional images was normalized to that of the corresponding Cy3 images.

Fig. 1 illustrates a representative Cy5 image unique to IEC-6 cells expressing the β-catenin ΔN89 protein. More than 2000 spots were displayed, and computer-assisted quantitative analyses identified 10 spots with decreased intensity (hexagons; Fig. 1) and 12 spots with increased intensity (circles; Fig. 1), when compared with IEC6 cells not expressing the β-catenin ΔN89 protein.

To exclude artificial differences in spot intensity among the different gels caused by differences in electrophoresis conditions, protein extracts of IEC-6 cells expressing stabilized β-catenin and cells not expressing it were run in triplicate two-dimensional gels. The average

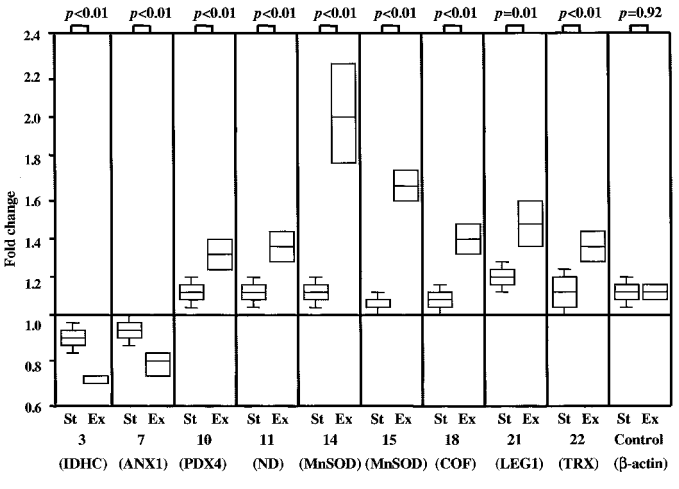


Fig. 2. Statistical validation of differences in spot intensity. Experimental value (*Ex*) indicates the fold difference between IEC6 cells expressing β-catenin and cells not expressing it. Standard value (*St*) indicates the fold differences between each spot in 12 gels loaded with the identical samples. Differences between experimental and standard values in all spots are statistically relevant, with *P*s of <0.01, except for β-actin ($P = 0.92$). Spot numbers refer to those in Fig. 1. *Ex*, experimental values; *St*, standard values; *IDHC*, isocitrate dehydrogenase; *ANK 1*, annexin 1; *PDX4*, perioredoxin 4; *ND*, not identified; *COF*, cofilin; *LEG1*, galectin 1; *TRX*, thioredoxin. Bars, SE.

Table 1 *List of proteins expressed differentially on induction of stabilized β-catenin*

Spot no.[a]	Protein description	Accession no. (SWISS PROT)	Fold change in 2D-DIGE[b]	Theoretical mol. mass (kDa)/pI	Experimental mol. mass (kDa)/pI	Method of identification[c]	Percentage coverage[d]
1	Cytokeratin 8	Q10758	0.60	54.0/5.8	52.2/5.8	MS	39.2%
2	Cytokeratin 8	Q10758	0.79	54.0/5.8	52.5/6.0	MS	36.8%
3	Isocitrate dehydrogenase	P41562	0.81	47.1/6.9	46.7/6.7	MS	44.2%
4	Calponin 3	P37397	1.83	36.6/5.5	43.8/5.4	MS	78.8%
5	Not identified		1.73		43.6/5.7		
6	Not identified		0.79		43.6/7.2		
7	Annexin I	P07150	0.80	39.2/7.3	42.5/6.4	MS/WB	84.4%
8	Not identified		0.72		34.5/5.7		
9	Carbonic anhydrase II	P27139	2.45	29.3/7.2	34.0/7.0	MS/WB	68.8%
10	Perioredoxin 4	O08807	1.27	31.3/7.2	32.8/6.1	MS	44.4%
11	Not identified		1.35		32.5/5.3		
12	Platelet-activating factor acetylhydrolase IB	O35264	0.65	25.9/5.8	32.6/6.2	MS	35.6%
13	Glutathione *S*-transferase P	P09211	0.68	23.8/7.9	30.0/7.3	MS	51.8%
14	Superoxide dismutase	P07895	1.99	24.9/9.2	29.0/7.5	MS/WB	50.0%
15	Superoxide dismutase	P07895	1.66	24.9/9.2	29.1/8.2	MS/WB	36.9%
16	Stathmin	P13668	1.53	17.3/5.8	24.3/5.8	MS	49.0%
17	Destrin	P18359	0.72	18.9/7.6	24.3/6.5	MS	6.7%
18	Cofilin, nonmuscle isoform	P45592	1.34	18.8/8.2	24.3/8.4	MS/WB	74.7%
19	Not identified		1.25		24.3/5.7		
20	Cysteine-rich protein 1	P21291	0.69	21.4/8.5	23.5/5.7	MS	46.3%
21	Galectin-1	P11762	1.39	15.2/5.0	20.8/5.2	MS	58.5%
22	Thioredoxin	P11232	1.25	12.0/4.6	20.0/5.2	MS	77.1%

[a] Spot numbers correspond to those in Fig. 1.
[b] Fold differences between IEC6 cells expressing β-catenin ΔN89 protein and cells not expressing it, calculated by using the BVA mode of the DeCyder software.
[c] Identification was performed by mass spectrometry (MS) and Western blotting (WB).
[d] Percentage cover of the identified peptide in total tryptic digests.

intensity of spots in three Cy5 images normalized to Cy3 images of the same gels was evaluated by the BVA mode of the DeCyder software. The mean intensity of the 22 spots was significantly different between ICE6 cells expressing and not expressing stabilized β-catenin protein, with a $P < 0.05$, confirming that the changes in spot intensity were unlikely to be derived from artificial alterations caused by electrophoresis. Representative statistics are depicted in Fig. 2.

Identification of Proteins by Mass Spectrometry. To identify those 22 differentially expressed proteins, peptide mass fingerprinting of tryptic digests was obtained by mass spectrometry. A database search successfully identified 15 proteins (17 spots) of 22 spots. The identified proteins are listed in Table 1, allied with the corresponding spot numbers in Fig. 1. Spots 15 (Fig. 3*A*) and 14 were identified as MnSOD. To confirm this identification, the protein extract of IEC-6 cells expressing stabilized β-catenin was separated by two-dimensional electrophoresis and blotted with anti-MnSOD antibody. Spots 14 and 15 and an additional protein spot, spot A, reacted with anti-MnSOD antibody (Fig. 3*B*, *top*). Spot A was found at exactly the same location as spot A′ in corresponding Cy5 images (Fig. 3*B*, *bottom*). The intensity of A′ was not affected by the induction of stabilized β-catenin (data not shown). Spots 14, 15, and A (A′) seem to be differently modified forms of MnSOD proteins.

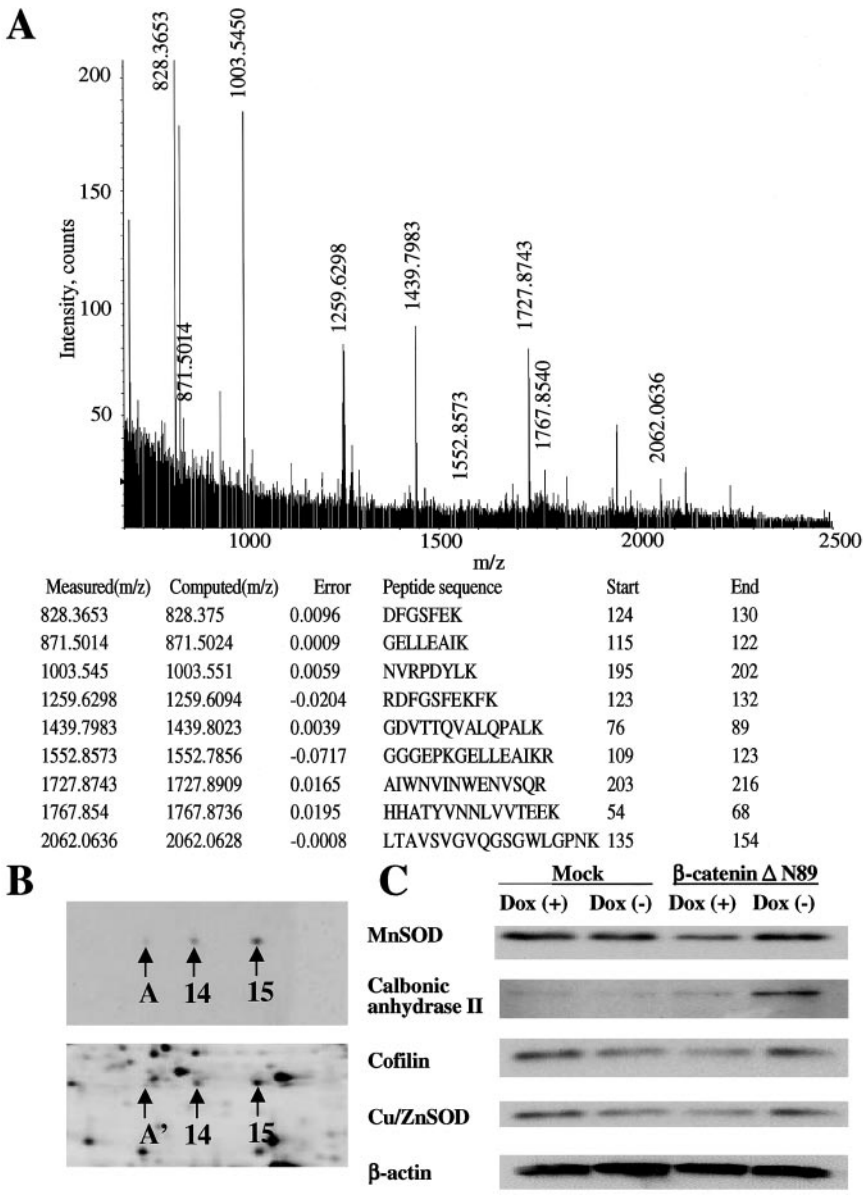
We grouped the 15 identified proteins according to their functions and features, as described in the literature (Table 2). Five of 15 proteins were categorized as redox-status-regulating and related proteins. These included proteins that convert the superoxide anion to water (spots 13–15), reduce cysteine residues of other proteins (spots 10 and 22), and confer resistance against oxidative stress (spot 3). Another major group was the cytoskeleton-associating proteins, which control the polymerization of microtubules (spot 16), regulate the function of the actin cytoskeleton (spots 4, 17, 18, and 21), and are intermediate filament proteins themselves (spots 1 and 2). Although aberrant regulation of cytokeratin 8, platelet-activating factor acetylhydrolase IB, glutathione *S*-transferase-P, MnSOD, stathmin, cofilin, and galectin-1 have been reported previously in various human cancers (21–32), the possible implication of these proteins in the Wnt/β-catenin pathway had not been explored.

Accurate quantification by 2D-DIGE enabled us to study subtle changes in protein expression, even a <2-fold difference. To confirm the degree of change of the spot intensity observed by 2D-DIGE, protein samples labeled with Cy5 were separated by SDS-PAGE and blotted to antibodies against MnSOD, Cu/ZnSOD, carbonic anhydrase II, cofilin, and β-actin. Western blotting analysis revealed that the induction of β-catenin ΔN89 resulted in an increase of MnSOD, carbonic anhydolase II, and cofilin by 2.08-, 1.88-, and 1.71-fold, respectively, when normalized to the total intensity of Cy5 (Fig. 3*C*). On the other hand, the amount of Cu/ZnSOD, another family member of superoxide dismutases located in the cytoplasmic matrix, did not change significantly. The degrees of change observed by Western blot analyses were comparable with those by 2D-DIGE (Table 1).

Overexpression of MnSOD Protein in Colorectal Adenoma and Adenocarcinoma. To explore whether the up-regulation of MnSOD in IEC-6 cells expressing stabilized β-catenin is significant in clinical samples, we performed an immunohistochemical analysis of the tissues of 10 patients with FAP syndrome (Fig. 4). Adenoma (Fig. 4*B*) and adenocarcinoma (Fig. 4*D–F*) cells contained higher amounts of MnSOD protein than did neighboring normal epithelial cells. MnSOD was stained intensely in the cytoplasm of adenoma and invasive adenocarcinoma cells in parallel to the level of accumulation of the β-catenin protein (Fig. 4, *A* and *C*). On the other hand, Cu/ZnSOD and catalase were not exclusively stained in adenoma and adenocarcinoma cells (data not shown). Western blot analyses revealed that MnSOD was accumulated in tumor tissues, but not in normal tissues, in 5 of 7 patients with sporadic colorectal carcinoma (Fig. 5*A*). In tumor tissues, enzymatic activity specific to MnSOD was higher than in normal tissues (Fig. 5*B*). These results indicate that MnSOD was involved in the Wnt/β-catenin pathway and that alterations of redox-status by increased enzymatic activity of MnSOD may have been involved in the development of colorectal adenoma and adenocarcinoma.

DISCUSSION

Downstream target genes of the β-catenin and TCF/LEF complex are likely to be important mediators of intestinal carcinogenesis and are good candidates for chemoprevention by molecular targeting.



Measured(m/z)	Computed(m/z)	Error	Peptide sequence	Start	End
828.3653	828.375	0.0096	DFGSFEK	124	130
871.5014	871.5024	0.0009	GELLEAIK	115	122
1003.545	1003.551	0.0059	NVRPDYLK	195	202
1259.6298	1259.6094	-0.0204	RDFGSFEKFK	123	132
1439.7983	1439.8023	0.0039	GDVTTQVALQPALK	76	89
1552.8573	1552.7856	-0.0717	GGGEPKGELLEAIKR	109	123
1727.8743	1727.8909	0.0165	AIWNVINWENVSQR	203	216
1767.854	1767.8736	0.0195	HHATYVNNLVVTEEK	54	68
2062.0636	2062.0628	-0.0008	LTAVSVGVQGSGWLGPNK	135	154

Fig. 3. Protein identification by mass spectrometry. *A*, peptide mass fingerprinting of protein extracted from spot 15. A representative spectrum of time-of-flight mass spectrometry is depicted (*top*). Ion peak spectra of spot 15 after trypsin digestion matched the nine peptide sequences of MnSOD (*bottom*). *B*, Western blot analysis of proteins separated in a two-dimensional gel, showing reactivity with anti-MnSOD antibody. Note that in addition to two spots identified by mass spectrometry as MnSOD (spots *14* and *15*), one spot was also reactive with anti-MnSOD antibody (spot *A*; *top*). Spot *A* is located in exactly the same position as spot *A*′ in the Cy5 image (*bottom*). *C*, Western blot analyses for validation of alterations. Protein samples extracted from IEC6-TetOFF control cells cultured with or without Dox and from IEC6-TetOFF-β-catenin ΔN89 cells cultured with or without Dox for 14 days were blotted to antibodies against MnSOD, carbonic anhydrase II, cofilin, Cu/ZnSOD, and β-actin. Differences between IEC6-TetOFF-β-catenin ΔN89 cells cultured with and without Dox were 2.08-, 1.88-, 1.71-, 1.28-, and 1.02-fold, respectively. The changes detected by 2D-DIGE were 1.99/1.66-, 2.45-, 1.34-, 1.04-, and 1.03-fold, respectively (Table 1).

Transcriptomic approaches using cDNA microarray techniques have thus been widely taken to identify the immediate target genes of the complex (10, 12). However, the whole picture of the protein expression profiles governing β-catenin-mediated intestinal carcinogenesis has not been explored until now. In our previous study, we established a rat small intestinal epithelial cell clone, namely IEC6-TetOFF-β-catenin ΔN89, which is capable of inducing truncated β-catenin protein under the strict control of the tetracycline-regulatory promoter. This cell line enabled us to pinpoint precisely the molecular changes occurring in intestinal epithelial cells on accumulation of β-catenin. In the present study, we performed 2D-DIGE to obtain comparative protein expression profiles of IEC6 cells after the induction of stabilized β-catenin. We always include a mock clone in every experiment as a biological negative control, to exclude any unexpected effects of Dox.

Although labeling proteins with fluorescent dyes confers a wide dynamic range and high sensitivity to two-dimensional gel electrophoresis, Patton and Beechem (33) pointed out that there could be problems with 2D-DIGE because of the different labeling efficiencies of each dye. We solved this problem by labeling each sample with the same fluorescent dye, Cy5, and mixing it with a pooled Cy3-labeled sample for normalization. Inclusion of a common internal standard in all two-dimensional gels significantly eliminated errors resulting from artifacts of electrophoresis and allowed accurate quantitative analyses. This modification of 2D-DIGE made it possible to detect subtle changes—even a <2-fold difference. In fact, we detected 0.65–2.45-fold steady changes in the intensity of 22 of >2000 spots displayed in a two-dimensional gel. At least 6 of these protein spots have been reported to be involved in redox regulation.

Functional dysregulation of several physiological processes that govern intestinal epithelial cell homeostasis, such as cell cycle progression, cell migration, differentiation, and apoptosis, is thought to give a rise to intestinal adenoma. MnSOD, a tetrameric enzyme localized in the mitochondrial matrix, plays an essential role in the conversion of superoxide anion into H_2O_2 and protects cells from damage by free radicals (34). *In vitro* studies have revealed that MnSOD is a key survival factor and determinant of cell resistance to prooxidant and proapoptotic agents, including tumor necrosis factor-α, interleukin-1, ionizing radiation, and anticancer drugs (35, 36). The inhibition of SOD causes superoxide-mediated mitochondrial

Table 2 *Functional classification of proteins expressed differentially on the induction of stabilized β-catenin*

Classification	Spot no.[a]	Protein description	Fold change[b]	Functions	References
Redox-regulation and related proteins	14/15	Superoxide dismutase	1.99/1.66	Scavenging superoxide	34
	10	Perioredoxin 4	1.27	Thioredoxin-dependent peroxidase	40
	13	Glutathione *S*-transferase P	0.68	Conjugation of reduced glutathione to hydrophobic electrophiles	41
	22	Thioredoxin	1.25	Activation of transcription factor	42
	3	Isocitrate dehydrogenase	0.81	Registant against oxidative stress	43
Cytoskeletal regulation	18	Cofilin	1.34	Actin-depolymerizing activity	44
	17	Destrin	0.72	Actin-depolymerizing activity	45
	4	Calponin 3	1.83	Actin-binding protein Smooth muscle contraction	46
	20	Cysteine-rich protein 1	0.69	Interacts with α-actinin Smooth muscle differentiation	47
	16	Stathmin	1.53	Microtubule destabilizing activity	48
	1/2	Cytokeratin 8	0.60/0.79	Intermediate filament	49, 50
Lipid metabolism	7	Annexin I	0.80	Inhibition of phospholipase A2	51
	12	Platelet-activating factor acetylhydrolase IB	0.65	Phospholipid mediation of inflammation	52
Others	21	Galectin-1	1.39	Lactose-binding protein Cell attachment and migration	53
	9	Carbonic anhydrase II	2.45	Reversible hydration of carbon dioxide	54

[a] Spot numbers correspond to those in Fig. 1.
[b] Fold differences between IEC6 cells expressing β-catenin ΔN89 protein and cells not expressing it, calculated by using the BVA mode of the DeCyder software.

damage and the release of cytochrome *c*, resulting in apoptosis (37). The suppression of MnSOD increases the susceptibility of colorectal cancer cells to apoptosis (36). We found that the amount and activity of MnSOD increased in sporadic colorectal cancer tissues. In FAP patients, the expression of MnSOD was up-regulated in colorectal adenoma and invasive adenocarcinoma, in complete accordance with the accumulation of β-catenin protein. Consistently, several types of malignant tumors, including colorectal cancer, have been reported to show an increased expression and activity of MnSOD (21–23, 38, 39). Accumulation of β-catenin protein up-regulates the expression of MnSOD and may contribute to carcinogenesis by protecting intestinal epithelial cells from programmed cell death.

We could not detect the products of known target genes of the β-catenin and TCF/LEF complex, such as c-*myc* and cyclin D1 (7, 8),

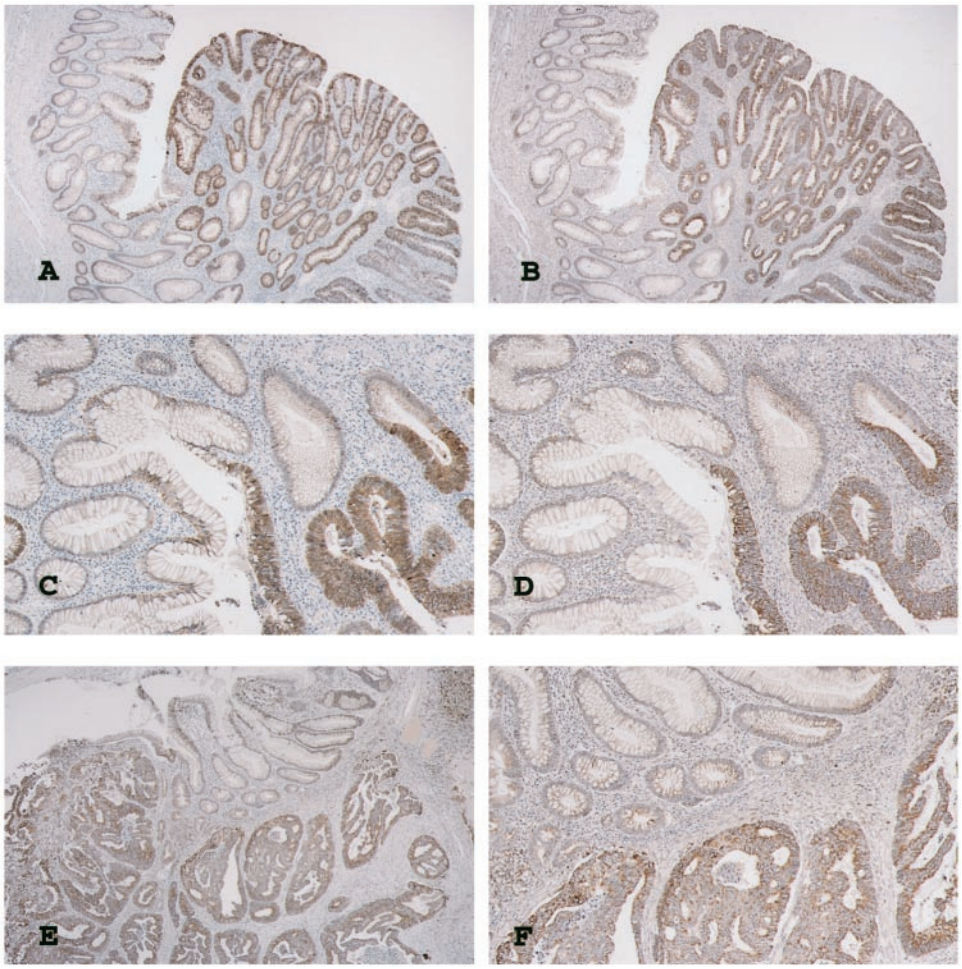


Fig. 4. Immunohistochemical analysis of MnSOD. Immunoperoxidase staining of adenoma (*A* and *B*) and adenocarcinoma (*C–F*) tissues of FAP patients with anti-β-catenin (*A* and *C*) and anti-MnSOD (*B* and *D–F*) antibodies. *C* and *D*, adenocarcinoma replacing part of the normal intestinal epithelium. Original magnification: ×40 (*A*, *B*, and *E*), ×200 (*C* and *D*), and ×100 (*F*).

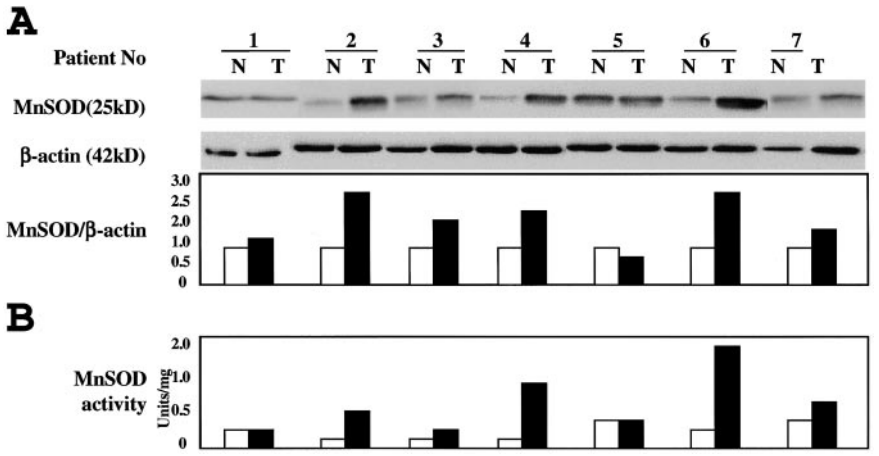


Fig. 5. *A*, increased expression of MnSOD protein in sporadic colorectal cancer. Protein samples extracted from normal (*N*) and tumor (*T*) tissues of colorectal cancer patients were blotted to antibodies against MnSOD and β-actin. In 5 of 7 patients (patients *2*, *3*, *4*, *6*, and *7*), tumor tissues show increased expression of MnSOD protein. Relative expression levels of MnSOD in tumor tissues (*closed columns*) against normal tissues (*open columns*) were calculated after normalizing to the loading control, β-actin (MnSOD/β-actin). *B*, increased enzyme activity of MnSOD in sporadic colorectal cancer. The enzymatic activity (units per milligram) of MnSOD in tumor (*closed column*) and normal tissues (*open column*) of sporadic colorectal cancer patients is depicted. The patients correspond to those in *A*.

using 2D-DIGE. Even today, two-dimensional gel electrophoresis is still the most comprehensive method for protein expression profiling. Using fluorescence dyes and large format gels, we were able to significantly improve the reproducibility and quantification of two-dimensional gel electrophoresis. However, 2D-DIGE is still not sufficiently robust to analyze the entire population of proteins expressed in a given cell. Many hydrophobic or basophilic proteins, as well as proteins with a low cellular concentration, may not be detectable using 2D-DIGE. Recently, we performed global gene expression profiling of IEC6-TetOFF β-catenin ΔN89 using GeneChip microarrays.[4] The MnSOD mRNA level was not affected by the induction of β-catenin ΔN89 (data not shown). MnSOD does not seem to be an immediate target gene of the β-catenin-TCF/LEF complex, and the level of MnSOD seems to be regulated post-transcriptionally by the Wnt/β-catenin pathway. These results imply that although proteomic approaches have certain limitations, they have the potential to provide novel insights that are not possible with transcriptomic analyses using cDNA microarray techniques.

REFERENCES

1. Kinzler, K. W., and Vogelstein, B. Lessons from hereditary colorectal cancer. Cell, *87:* 159–170, 1996.
2. Sparks, A. B., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. Cancer Res., *58:* 1130–1134, 1998.
3. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. Science (Wash. DC), *275:* 1784–1787, 1997.
4. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science (Wash. DC), *275:* 1787–1790, 1997.
5. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature (Lond.), *382:* 638–642, 1996.
6. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell, *88:* 789–799, 1997.
7. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Identification of c-MYC as a target of the APC pathway. Science (Wash. DC), *281:* 1509–1512, 1998.
8. Tetsu, O., and McCormick, F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature (Lond.), *398:* 422–426, 1999.
9. Mann, B., Gelos, M., Siedow, A., Hanski, M. L., Gratchev, A., Ilyas, M., Bodmer, W. F., Moyer, M. P., Riecken, E. O., Buhr, H. J., and Hanski, C. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc. Natl. Acad. Sci. USA, *96:* 1603–1608, 1999.
10. Yamada, T., Takaoka, A. S., Naishiro, Y., Hayashi, R., Maruyama, K., Maesawa, C., Ochiai, A., and Hirohashi, S. Transactivation of the multidrug resistance 1 gene by T-cell factor 4/β-catenin complex in early colorectal carcinogenesis. Cancer Res., *60:* 4761–4766, 2000.
11. Wong, N. A., and Pignatelli, M. Beta-catenin–a linchpin in colorectal carcinogenesis? Am. J. Pathol., *160:* 389–401, 2002.
12. Fujita, M., Furukawa, Y., Tsunoda, T., Tanaka, T., Ogawa, M., and Nakamura, Y. Up-regulation of the ectodermal-neural cortex 1 (*ENC1*) gene, a downstream target of the β-catenin/T-cell factor complex, in colorectal carcinomas. Cancer Res., *61:* 7722–7726, 2001.
13. Yamada, T., Mori, Y., Hayashi, R., Takada, M., Ino, Y., Naishiro, Y., Kondo, T., and Hirohashi, S. Suppression of intestinal polyposis in *Mdr1*-deficient $Apc^{Min/+}$ mice. Cancer Res., *63:* 895–901, 2003.
14. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., and Davison, M. Validation and development of fluorescence two-dimmensional differential gel electrophoresis proteomic technology. Proteomics, *1:* 377–396, 2001.
15. Gharbi, S., Gaffney, P., Yang, A., Zvelebil, M. J., Cramer, R., Waterfield, M. D., and Timms, J. F. Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. Mol. Cell Proteomics, *1:* 91–98, 2002.
16. Gorg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., and Weiss, W. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis, *21:* 1037–1053, 2000.
17. Rosenfeld, J., Capdevielle, J., Guillemot, J. C., and Ferrara, P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal. Biochem., *203:* 173–179, 1992.
18. Naishiro, Y., Yamada, T., Takaoka, A. S., Hayashi, R., Hasegawa, F., Imai, K., and Hirohashi, S. Restoration of epithelial cell polarity in a colorectal cancer cell line by suppression of β-catenin/T-cell factor 4-mediated gene transactivation. Cancer Res., *61:* 2751–2758, 2001.
19. Sutherland, M. W., and Learmonth, B. A. The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase. Free Radic. Res., *27:* 283–289, 1997.
20. Kanbagli, O., Ozdemirler, G., Bulut, T., Yamaner, S., Aykac-Toker, G., and Uysal, M. Mitochondrial lipid peroxides and antioxidant enzymes in colorectal adenocarcinoma tissues. Jpn. J. Cancer Res., *91:* 1258–1263, 2000.
21. Nelson, R. L. Superoxide dismutase in cultured benign and malignant tumors of the colon. Basic Life Sci., *49:* 699–702, 1988.
22. Janssen, A. M., Bosman, C. B., Sier, C. F., Griffioen, G., Kubben, F. J., Lamers, C. B., van Krieken, J. H., van de Velde, C. J., and Verspaget, H. W. Superoxide dismutases in relation to the overall survival of colorectal cancer patients. Br. J. Cancer, *78:* 1051–1057, 1998.
23. Ditzel, H. J., Strik, M. C., Larsen, M. K., Willis, A. C., Waseem, A., Kejling, K., and Jensenius, J. C. Cancer-associated cleavage of cytokeratin 8/18 heterotypic complexes exposes a neoepitope in human adenocarcinomas. J. Biol. Chem., *277:* 21712–21722, 2002.
24. Montrucchio, G., Sapino, A., Bussolati, B., Ghisolfi, G., Rizea-Savu, S., Silvestro, L., Lupia, E., and Camussi, G. Potential angiogenic role of platelet-activating factor in human breast cancer. Am. J. Pathol., *153:* 1589–1596, 1998.
25. Miyanishi, K., Takayama, T., Ohi, M., Hayashi, T., Nobuoka, A., Nakajima, T., Takimoto, R., Kogawa, K., Kato, J., Sakamaki, S., and Niitsu, Y. Glutathione S-transferase-pi overexpression is closely associated with K-ras mutation during human colon carcinogenesis. Gastroenterology, *121:* 865–874, 2001.
26. Bieche, I., Lachkar, S., Becette, V., Cifuentes-Diaz, C., Sobel, A., Lidereau, R., and Curmi, P. A. Overexpression of the stathmin gene in a subset of human breast cancer. Br. J. Cancer, *78:* 701–709, 1998.
27. Price, D. K., Ball, J. R., Bahrani-Mostafavi, Z., Vachris, J. C., Kaufman, J. S., Naumann, R. W., Higgins, R. V., and Hall, J. B. The phosphoprotein Op18/stathmin is differentially expressed in ovarian cancer. Cancer Investig., *18:* 722–730, 2000.
28. Nishio, K., Nakamura, T., Koh, Y., Kanzawa, F., Tamura, T., and Saijo, N. Oncoprotein 18 overexpression increases the sensitivity to vindesine in the human lung carcinoma cells. Cancer (Phila.), *91:* 1494–1499, 2001.

[4] T. Naishiro *et al.*, manuscript in preparation.

29. Melhem, R. F., Zhu, X. X, Hailat, N., Strahler, J. R., and Hanash, S. M. Characterization of the gene for a proliferation-related phosphoprotein (oncoprotein 18) expressed in high amounts in acute leukemia. J. Biol. Chem., *266:* 17747–17753, 1991.
30. Roos, G., Brattsand, G., Landberg, G., Marklund, U., and Gullberg, M. Expression of oncoprotein 18 in human leukemias and lymphomas. Leukemia, *7:* 1538–1546, 1993.
31. Samstag, Y., Dreizler, E. M., Ambach, A., Sczakiel, G., and Meuer, S. C. Inhibition of constitutive serine phosphatase activity in T lymphoma cells results in phosphorylation of pp19/cofilin and induces apoptosis. J. Immunol., *156:* 4167–4173, 1996.
32. Irimura, T., Matsushita, Y., Sutton, R. C., Carralero, D., Ohannesian, D. W., Cleary, K. R., Ota, D. M., Nicolson, G. L., and Lotan, R. Increased content of an endogenous lactose-binding lectin in human colorectal carcinoma progressed to metastatic stages. Cancer Res., *51:* 387–393, 1991.
33. Patton, W. F., and Beechem, J. M. Rainbow's end: the quest for multiplexed fluorescence quantitative analysis in proteomics. Curr. Opin. Chem. Biol., *6:* 63–69, 2002.
34. Fridovich, I. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem., *64:* 97–112, 1995.
35. Hirose, K., Longo, D. L., Oppenheim, J. J., and Matsushima, K. Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. FASEB J., *7:* 361–368, 1993.
36. Kuninaka, S., Ichinose, Y., Koja, K., and Toh, Y. Suppression of manganese superoxide dismutase augments sensitivity to radiation, hyperthermia and doxorubicin in colon cancer cell lines by inducing apoptosis. Br. J. Cancer, *83:* 928–934, 2000.
37. Huang, P., Feng, L., Oldham, E. A., Keating, M. J., and Plunkett, W. Superoxide dismutase as a target for the selective killing of cancer cells. Nature (Lond.), *407:* 390–395, 2000.
38. Ria, F., Landriscina, M., Remiddi, F., Rosselli, R., Iacoangeli, M., Scerrati, M., Pani, G., Borrello, S., and Galeotti, T. The level of manganese superoxide dismutase content is an independent prognostic factor for glioblastoma. Biological mechanisms and clinical implications. Br. J. Cancer, *84:* 529–534, 2001.
39. Chung-man Ho, J., Zheng, S., Comhair, S. A., Farver, C., and Erzurum, S. C. Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res., *61:* 8578–8585, 2001.
40. Okado-Matsumoto, A., Matsumoto, A., Fujii, J., and Taniguchi, N. Peroxiredoxin IV is a secretable protein with heparin-binding properties under reduced conditions. J. Biochem., *127:* 493–501, 2000.
41. Pickett, C. B., and Lu, A. Y. Glutathione S-transferases: gene structure, regulation, and biological function. Annu. Rev. Biochem., *58:* 743–764, 1989.
42. Holmgren, A. Thioredoxin. Annu. Rev. Biochem., *54:* 237–271, 1985.
43. Lee, S. M., Koh, H. J., Park, D. C., Song, B. J., Huh, T. L., and Park, J. W. Cytosolic NADP(+)-dependent isocitrate dehydrogenase status modulates oxidative damage to cells. Free Radic. Biol. Med., *32:* 1185–1196, 2002.
44. Nishida, E., Iida, K., Yonezawa, N., Koyasu, S., Yahara, I., and Sakai, H. Cofilin is a component of intranuclear and cytoplasmic actin rods induced in cultured cells. Proc. Natl. Acad. Sci. USA, *84:* 5262–5266, 1987.
45. Yonezawa, N., Nishida, E., Iida, K., Yahara, I., and Sakai, H. Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. J. Biol. Chem., *265:* 8382–8386, 1990.
46. Applegate, D., Feng, W., Green, R. S., and Taubman, M. B. Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. J. Biol. Chem., *269:* 10683–10690, 1994.
47. Pomies, P., Louis, H. A., and Beckerle, M. C. CRP1, a LIM domain protein implicated in muscle differentiation, interacts with alpha-actinin. J. Cell Biol., *139:* 157–168, 1997.
48. Belmont, L. D., and Mitchison, T. J. Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. Cell, *84:* 623–631, 1996.
49. Marklun, U., Larsson, N., Gradin, H. M., Brattsand, G., and Gullberg, M. Oncoprotein 18 is a phosphorylation-responsive regulator of microtubule dynamics. EMBO J., *15:* 5290–5298, 1996.
50. Dellagi, K., Lipinski, M., Paulin, D., Portier, M. M., Lenoir, G. M., and Brouet, J. C. Characterization of intermediate filaments expressed by Ewing tumor cell lines. Cancer Res., *47:* 1170–1173, 1987.
51. Raynal, P., and Pollard, H. B. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim. Biophys. Acta, *1197:* 63–93, 1994.
52. Prescott, S. M., Zimmerman, G. A., Stafforini, D. M., and McIntyre, T. M. Platelet-activating factor and related lipid mediators. Annu. Rev. Biochem., *69:* 419–445, 2000.
53. Perillo, N. L., Marcus, M. E., and Baum, L. G. Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. J. Mol. Med., *76:* 402–412, 1998.
54. Sterling, D., Reithmeier, R. A., and Casey, J. R. Carbonic anhydrase: in the driver's seat for bicarbonate transport. JOP, *2* (Suppl. 4)*:* 165–170, 2001.