Int. J. Cancer: **94,** 662–668 (2001)

Publication of the International Union Against Cancer

PURIFICATION AND IDENTIFICATION OF MONOUBIQUITIN-PHOSPHOGLYCERATE MUTASE B COMPLEX FROM HUMAN COLORECTAL CANCER TISSUES

Teruyuki USUBA[1], Yoshio ISHIBASHI[1], Yutaka OKAWA[2,3], Tae HIRAKAWA[2], Koji TAKADA[2]* and Kiyoshi OHKAWA[2]

[1]*Department of Surgery, Jikei University School of Medicine, Nishishinbashi, Minato-ku, Tokyo, Japan*
[2]*Department of Biochemistry 1, Jikei University School of Medicine, Nishishinbashi, Minato-ku, Tokyo, Japan*
[3]*Department of Internal Medicine (Division of Hematology/Oncology), Jikei University School of Medicine, Nishishinbashi, Minato-ku, Tokyo, Japan*

Ubiquitin-conjugated proteins in human colorectal cancer tissues were analyzed by the immunoprecipitation with the antibody FK2 against conjugated ubiquitin followed with SDS-PAGE. In these immunoprecipitable proteins, a 38-kDa protein was abundant in the tumor regions but almost absent in the adjacent normal regions in 17/26 patients, thus we attempted to purify it. Using immunoaffinity chromatography with the antibody FK2 followed by gel filtration and SDS-PAGE, approximately 10 pmol of this protein was separated from 34 g of the pooled cancerous tissue and transferred onto a PVDF membrane. The 38-kDa protein was further digested with *Achromobacter* protease I, resulting in several peptide fragments. Amino acid sequences of these peptides showed complete sequence identity to those derived from either ubiquitin or phosphoglycerate mutase-B, suggesting that the 38-kDa protein is monoubiquitinated phosphoglycerate mutase-B, whose calculated mass is 37,369 Da. Western blot using an antibody against phosphoglycerate mutase-B revealed the presence of the 38-kDa protein in the anti-ubiquitin immunoprecipitates derived from the tumor regions, but not from normal counterparts. In addition, part of non-ubiquitinated phosphoglycerate mutase-B (29 kDa) was also found in the anti-ubiquitin immunoprecipitates, whose levels were higher in the tumor regions than in the adjacent normal regions. These results suggest that monoubiquitination of phosphoglycerate mutase-B as well as formation of a noncovalent complex containing ubiquitin and phosphoglycerate mutase-B increases in colorectal cancer and novel modification of phosphoglycerate mutase-B might have a pathophysiological role.

Key words: *phosphoglycerate mutase; monoubiquitination; affinity chromatography; immunoprecipitation*

The eukaryotic modifier protein, ubiquitin is involved in degradation and regulation of intracellular substrate proteins.[1,2] This process involves multiple steps.[3,4] First, ubiquitin is activated by the ubiquitin-activating enzyme (E1). Second, 1 of ubiquitin-conjugating enzymes (E2s) transfers ubiquitin from E1 to the substrate that is bound to 1 of ubiquitin-protein ligases (E3s). Third, these enzymes catalyze covalent attachment of ubiquitin to the protein substrate, forming the monoubiquitinated protein. In many cases, additional activated ubiquitin moieties are successively transferred to the previously conjugated ubiquitin molecule, forming a multiubiquitin chain adduct. Furthermore, the multiubiquitinated proteins are destined for degradation by 26S proteasome and this proteolytic machinery plays a fundamental role in various cellular events including cell-cycle progression[2,5,6] and apoptosis.[7,8] The monoubiquitin adducts have distinct functions, *e.g.*, a trigger for endocytosis of plasma membrane proteins, thereby regulating growth factor-derived signal input.[9] The ubiquitin-mediated system is often the target of cancer-related deregulation and can underlie carcinogenesis processes[10] and its proteolytic activities are recognized as a new target for cancer chemotherapy.[11]

We and several other investigators have reported that ubiquitin-immunoreactive proteins are accumulated in malignant carcinomas, suggesting a potential relationship with the pathophysiology of cancer.[12–14] By using 2-dimensional gel electrophoresis, 3 of the ubiquitin-immunoreactive proteins have been isolated from tissues of colorectal cancer, breast neoplasm and hepatoma; moreover, cytokeratin 8 fragments,[15] actin family members[16] and glutamine synthetase,[17] respectively, have been identified by amino acid sequencing of their *Achromobacter* protease I (API) digests. In these studies, however, no direct evidence for ubiquitin association, such as the presence of the internal amino acid sequence of ubiquitin in these digests, could be found and, no type of ubiquitin conjugation, whether multiubiquitinated or monoubiquitinated, has been confirmed. Thus, an alternate method is required for the identification of ubiquitin conjugates in cancerous tissues.

Recently, we developed an immunoaffinity technique using an antibody to conjugated ubiquitin,[18] by which ubiquitin-associated proteins can be isolated. Using this technique, several ubiquitin-E2 complexes have been identified in heat-shocked erythroleukemia K562 cells.[18] In the present study, using this method, we analyzed ubiquitin-protein conjugates in colorectal cancer tissues and isolated a 38-kDa protein, whose levels were higher in tumor than normal regions. Amino acid sequencing and immunoblotting analysis enable us to identify this protein as a monoubiquitinated form of phosphoglycerate mutase (PGM) type B.

MATERIAL AND METHODS

Human colorectal cancers

After obtaining informed consent, human colorectal cancers were collected from 26 patients attending Jikei University Hospital of Medicine (Tokyo, Japan) in 1998–99 (Table I) by permission of Ethics Review Committee for Biomedical Research in Jikei University. The patients comprised 17 men and 9 women, whose mean age was 65, ranging between 47–89 years. Immediately after surgical removal, tissue specimens of colorectal tumors and adjacent normal mucosae were washed with chilled phosphate-buffered saline (PBS) and stored at −80°C till use. The histological diagnosis on malignant and adjacent normal tissues was confirmed by pathologic specialists in Jikei University Hospital of Medicine.

Antibodies

The monoclonal antibody FK2 against conjugated ubiquitin,[19] which recognizes not only conjugated ubiquitin but also free ubiquitin in solution,[18] was purified as described previously[20] and covalently coupled to Sepharose using NHS-activated Sepharose

Grant sponsor: Ministry of Education, Science, Sports and Culture of Japan.

*Correspondence to: Department of Biochemistry 1, Jikei University School of Medicine, Nishishinbashi 3-25-8, Minato-ku, Tokyo 105-8461, Japan. Fax: +81-3-3435-1922. E-mail: takada@jikei.ac.jp.

Received 18 March 2001; Revised 6 June 2001; Accepted 22 June 2001

TABLE I – RELATIONSHIPS BETWEEN INCREASES OF THE 38-kDa PROTEIN IN COLORECTAL CANCER AND CLINICOPATHOLOGICAL FACTORS OF 26 PATIENTS

	No. of cases (%)[1]
Location of tumor	
Ascending colon	3/3 (100)
Transverse colon	2/4 (50)
Descending colon	3/3 (100)
Sigmoid colon	2/4 (50)
Rectum	7/12 (58)
Histological type	
Well-differentiated adenocarcinoma	5/11 (45)
Moderately-differentiated adenocarcinoma	9/11 (82)
Poorly-differentiated adenocarcinoma	3/4 (75)
Clinical stage	
I	1/3 (33)
II	9/13 (69)
III	6/9 (67)
IV	1/1 (100)
Dukes stage	
A	1/2 (50)
B	8/14 (57)
C	8/10 (80)
Total	17/26 (65)

[1]Ratio of the 38-kDa protein increase. When a level of the 38-kDa protein in the anti-ubiquitin immunoprecipitate of the tumor tissue region was higher than the adjacent normal region (Fig. 1), the specimen was counted as 'increase.'

4FF (Amersham Pharmacia Biotech, Piscataway, NJ) in accordance with the manufacturer's instructions. The affinity-purified antibody specific to human PGM type B was kindly provided by Oriental Yeast Co., Ltd. (Tokyo, Japan).[21]

Tissue extraction

The tissue specimens were minced and homogenized by a Teflon motor-driven glass homogenizer in 9 vol of ice-cold buffer A (25 mM Tris-HCl, pH 7.4, containing 0.1% CHAPS, 1 mM EDTA and 0.5 mM PMSF) supplemented with other protease inhibitors comprising 0.5 μM antipain, 1 μM aprotinin, 0.1 μM leupeptin, 35 μM chymostatin, 1 μM pepstatin A, 3 μM E-64, 0.5 mM benzamidine, 30 μM phosphoramidon and 0.1 mM bestatin. The homogenates were mixed with a cellulose matrix CDR (Whatmanm, Kent, UK), which adsorbs colloidal materials leaving target proteins in solution, at final concentration of 20 mg/ml and centrifuged at 100,000*g* for 1 hr and the supernatants were collected for the extracts.

Quantitative analysis of protein and ubiquitin

Protein concentration was estimated by the DC protein assay (Bio-Rad, Hercules, CA) standardized with bovine serum albumin. Free ubiquitin and multi-ubiquitin chain were quantified using a competitive radioimmunoassay[22] and a symmetric sandwich ELISA,[20] respectively. The levels of multi-ubiquitin chains were expressed in terms of the multi-ubiquitin chain reference preparation 1 (MUCRP1).[20]

Electrophoretic analysis

Proteins dissolved in the SDS-PAGE sample buffer containing 50 mM dithiothreitol[18] were resolved by SDS-PAGE on 10–25% acrylamide gradient gels in Tris/glycine buffer[23] and visualized using a silver nitrate staining kit according to the manufacture's instruction (2D-Silver Stain II, Daiichi Pure Chemicals, Tokyo). In some cases, the resolved proteins were electrotransferred to nitrocellulose membranes (BA83, Schleicher & Schuell, Dassel, Germany) and ubiquitin- and PGM-immunoreactive proteins were detected by using the antibody FK2[19] and the affinity-purified anti-phosphoglycerate mutase-B (PGM-B),[21] respectively, according to a previously described method.[18,23] Molecular mass estimates of bands were determined by comparing their electrophoretic mobilities with those of the 10 molecular mass marker proteins ranging from 2,500–180,000 (Sigma, St. Louis, MO). Images of the gels and the blots were read with a flatbed image scanner GT-7000U (EPSON, Japan) and then quantitated by densitometry using NIH Image software.

Immunoprecipitation of ubiquitin-protein conjugates

Ubiquitin-conjugated proteins in the tissue extracts were analyzed by immunoprecipitation with the FK2 antibody as described previously.[18] Briefly, 300 μl of the extracts (600 μg protein) were incubated with 30 μl of FK2-immobilized Sepharose (7 mg IgG/ml) in PBS containing 0.1% CHAPS (PBS-CHAPS) at 4°C for 12 hr under constant shaking. Precipitates were washed 3 times with PBS-CHAPS and suspended in the SDS-PAGE sample buffer and then subjected to SDS-PAGE.

Purification of a putative ubiquitinated protein (38 kDa)

Approximately 80 ml of the cancerous extract was applied to a FK2-Sepharose column (5 ml)[18] equilibrated with buffer A at 300 μl/min. After washing with ~25 ml of buffer A, the column was developed with 10 ml of 10 mM Tris-HCl, pH 7.4, containing 3.5 M $MgCl_2$ and 0.1% CHAPS. Fractions (1 ml each) were collected and dialyzed against buffer A and the levels of multi-ubiquitin chains were measured. Similar separations of the extracts (total 340 ml) were repeatedly carried out 3 times. The eluates containing multi-ubiquitin chain were combined and concentrated by ultrafiltration using a Centricon SR3 (Millipore, Bedford, MA) to a volume of 250 μl. The sample was then applied to a Superdex 75 HR10/30 column (Amersham Pharmacia Biotech) equilibrated with buffer A at the flow rate of 500 μl/min and fractions of 500 μl were collected. Fractions containing a 38-kDa protein were combined and concentrated to less than 50 μl and subjected to SDS-PAGE, followed with electrotransfer to a PVDF membrane (Immobilon-P^{SQ}, Millipore) and visualized by stain with Coomassie Brilliant Blue R250.[24]

Enzymatic digestion of purified proteins and separation of digested peptides by HPLC

The 38-kDa protein immobilized on a PVDF membrane was reduced and S-carboxymethylated and then digested with lysylendopeptidase API (Sigma), as described by Iwamatsu.[25] The digests were acidified by adding trifluoroacetic acid at a final concentration of 0.1% and applied to a reverse phase capillary HPLC (173A Microblotter system, Applied Biosystems, Foster City, CA) with the deposition of the peptides on a PVDF membrane after passage through the detector cell. The HPLC separation was performed as follows; a Capillary LC column (C18, 5μ, 300Å, 150 × 0.5 mm, Brownlee Columns, Perkin-Elmer, Norwalk, CT) was used for the separation of the proteins using solvent A (0.1% trifluoroacetic acid) and solvent B (0.085% trifluoroacetic acid with 80% acetonitrile) at the flow rate of 5 μl/min and under the following elution condition: 0–10 min, 6% B; 10–150 min, 6–56% B; 150–180 min, 56% B.

Sequence analysis

Automated Edman degradation for the determination of the amino acid sequence of each peptide was performed with an Applied Biosystems model 492 Protein Sequencer. The obtained sequences were used to identify each peptide by searching the Swiss-Plot protein database with Blast algorithm.

Statistics

Differences in band intensity between pairs were determined by the Wilcoxon matched pairs, signed rank test by using the computer software GraphPad PRISM (GraphPad Software, CA).

RESULTS

Proteins immunoprecipitated by anti-conjugated ubiquitin antibody

To determine whether distinct ubiquitinated proteins are produced in colorectal cancerous tissues, proteins immunoprecipitated with the antibody FK2 to conjugated ubiquitin were analyzed,

revealing that several protein bands were more intensely stained in the tumor tissues than in the adjacent normal tissues (arrowheads in Fig. 1). In addition, a band of monoubiquitin (Ub in Fig. 1*a*) was also abundant in the tumor tissues. Among these bands, we selected a 38-kDa protein for further study, because levels of this protein in the tumor regions were found to be higher than in the adjacent normal regions in 17/26 patient cases (Table I) though the protein levels in both regions were almost equal in 3/26 patients or undetectable in 6/26 patients and, the 17/26 cases comprised 11/17 cases in male patients and 6/9 cases in female patients and obvious differences between both genders were not observed. The 38-kDa bands on each gel were quantified and values of the specimens used in all the SDS-PAGE analyses were standardized to equal 100 arbitrary units. As a result, the 38-kDa protein amounts (mean ± SEM) were significantly higher in the tumor regions (160 ± 20) than in the adjacent normal regions (46 ± 9.0, $p < 0.0001$).

Purification of the 38-kDa protein

In an initial purification of ubiquitin-protein conjugates, immunoaffinity chromatography using the FK2-immobilized Sepharose column was used. Three independent chromatography runs showed similar elution profiles as follows. In the first run, the colorectal tumor extract containing 650 mg of proteins was applied to the immunoaffinity column and the great majority of the proteins did not bind, whereas a small amount (0.99 mg) bound and then were eluted with 3.5 M $MgCl_2$. Most of the multi-ubiquitin chains were adsorbed and 5.89 μg of the multi-ubiquitin chains in terms of the standard MUCRP1, which accounts for over 90% of the initial amount, were recovered in the $MgCl_2$ eluate (Fig. 2). In the second and third runs, the tumor extracts containing 335 and 863 mg of proteins, respectively, were applied to the column and then the $MgCl_2$ eluate fractions, which contained small amounts of proteins (1.09 and 2.18 mg, respectively) and most of the multi-ubiquitin chains (7.52 and 4.33 μg, respectively, in terms of the MUCRP1), were obtained. As a result, total 4.26 mg of proteins were yielded as the affinity-purified fraction and this fraction was subjected to gel filtration using a Superdex 75 HR10/30 column (Fig. 3*a*). The 38-kDa protein was only found in fractions 19 and 20 (Fig. 3*b*), but not in pooled fractions I and III, which mainly contained multi-ubiquitinated proteins and monoubiquitin, respectively (data not shown). Finally, a mixture of the fractions 19 and 20 was analyzed by SDS-PAGE and almost 10 pmol of the 38-kDa protein was transferred onto a PVDF membrane (Fig. 3*c*).

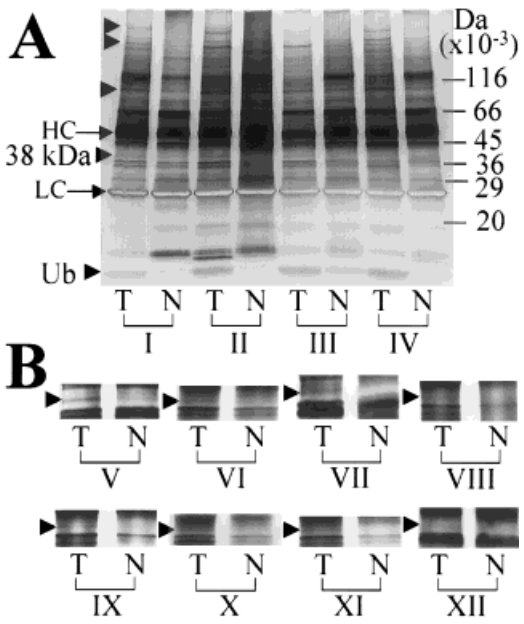

FIGURE 1 – SDS-PAGE of anti-ubiquitin immunoprecipitates of colorectal cancer extracts. Each tissue extract (600 μg protein) obtained from either the tumor regions (T) or the adjacent normal regions (N) was immunoprecipitated with the FK2-antibody-Sepharose (30 μl beads), which recognizes both free and conjugated ubiquitin in solution.[18] The resulting precipitates were solubilized in 60 μl of the SDS-PAGE sample buffer with dithiothreitol and subjected to SDS-PAGE (30 μl/lane). Proteins were visualized using silver staining. (*a*) Four pairs of specimens from the Patients I–IV. Arrowheads indicate proteins, whose levels are higher in the tumor regions than the normal counterparts. Locations of heavy chains (HC) and light chains (LC) of the antibodies leaked from the beads and monoubiquitin (Ub) are indicated to the left. Molecular mass estimates are indicated to the right. (*b*) Arrowheads indicate the 38-kDa protein in 8 pairs of specimens from the Patients V–XII.

Identification of the 38-kDa protein

API digests of the 38-kDa protein was analyzed by reversed-phase HPLC (Fig. 4). Edman degradation of each peptide fragment led to determination of 9 parts of the amino acid sequence: peak 1, TLTGK; peak 2, HGEAQ; peak 3, AMEAVAAQG; peaks 4 and 6, MQIFVK; peak 5, XLEDGXTLSD; peak 7, EGIPPDQQRLIFAGK and DXRYADLTEDQLPS; peak 8, TITLEVEP; and peak 9, XLEGLXEEAIMELXLXXGIXI. The peaks 1, 4–8 showed complete sequence identity to those derived from ubiquitin and the sequences of the peaks 2, 3, 7 and 9 were identical to those derived from human PGM-B (EC 5.4.2.1) (Fig. 5). Neither the calculated mass of ubiquitin (8,565 Da) nor PGM-B (28,804 Da) was consistent with the 38 kDa, but their sum (37,369 Da) was in good agreement with it. These results strongly suggest that the 38-kDa protein is monoubiquitinated PGM-B.

PGM-B-immunoreactive proteins in the colorectal cancer

To confirm the 38-kDa protein identity, a ubiquitinated protein mixture obtained by the immunoprecipitation with the FK2 antibody from the tumor extracts was analyzed by immunoblotting with the antibody against PGM-B and ubiquitin, simultaneously. As a result, a PGM-B-immunoreactive band of 38 kDa (PGMIR-2 in Fig. 6) and a broad ubiquitin-immunoreactive band (UbIR-1 in Fig. 6) comprised several proteins, whose masses are ranging from 35–38 kDa, were found. These immunochemical data support the possibility that the 38-kDa protein is identical to a monoubiquitin-PGM-B complex. It is of interest that another PGM-immunoreactive band of 29 kDa (PGMIR-1 in Fig. 6), which corresponds to

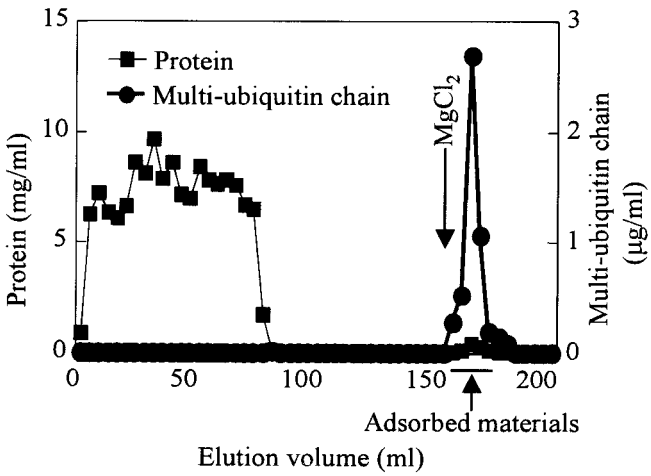

FIGURE 2 – Immunoaffinity chromatography using FK2 antibody-immobilized Sepharose. A pooled extract of colorectal cancer tissues was applied to the FK2-Sepharose column. Multi-ubiquitin chain levels in each fraction were estimated by ELISA and expressed in terms of the standard MUCRP1. After unadsorbed materials were eluted between 4 and 152 ml, adsorbed materials were eluted with 3.5 M $MgCl_2$.

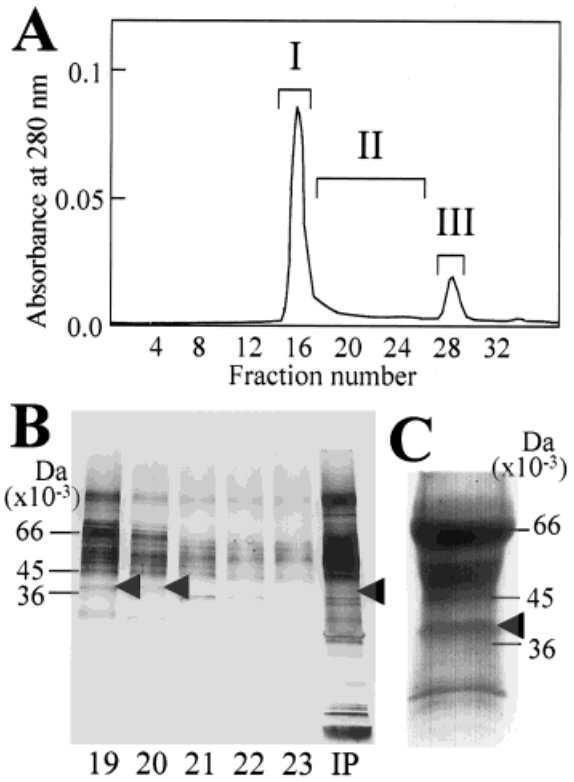

FIGURE 3 – Gel filtration of immunoaffinity-purified proteins. (*a*) The adsorbed materials obtained from the FK2-Sepharose column were fractionated by gel filtration on a Superdex 75 column. Large fractions I, II and III correspond to fractions 15–17, 18–26 and 27–29, respectively. (*b*) SDS-PAGE of proteins in fractions 19–23 and the anti-ubiquitin immunoprecipitates (IP) derived from the colorectal cancer extract (200 μg protein). Small aliquots (5 μl) in each fraction were subjected to SDS-PAGE. Proteins were visualized by silver staining. Arrowheads indicate the 38-kDa protein. Molecular mass estimates are indicated to the left. (*c*) SDS-PAGE of a mixture of the fractions 19 and 20. Resolved proteins were transferred onto a PVDF membrane and visualized by Coomassie Brilliant Blue staining. An arrowhead indicates the 38-kDa protein. Molecular mass estimates are indicated to the right.

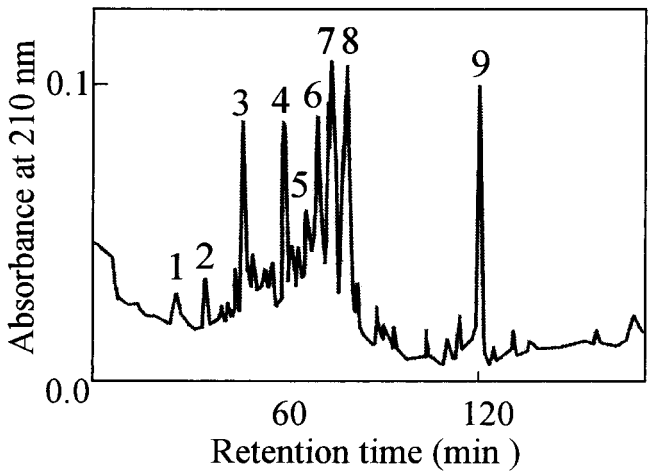

FIGURE 4 – HPLC of peptide fragments of the 38-kDa protein. The 38-kDa protein on a PVDF membrane was digested *in situ* with *Achromobacter* protease I and applied to a reverse phase capillary column and then the absorbance at 210 nm was monitored. Peptide fragment peaks (1–9) were numbered in the order of elution.

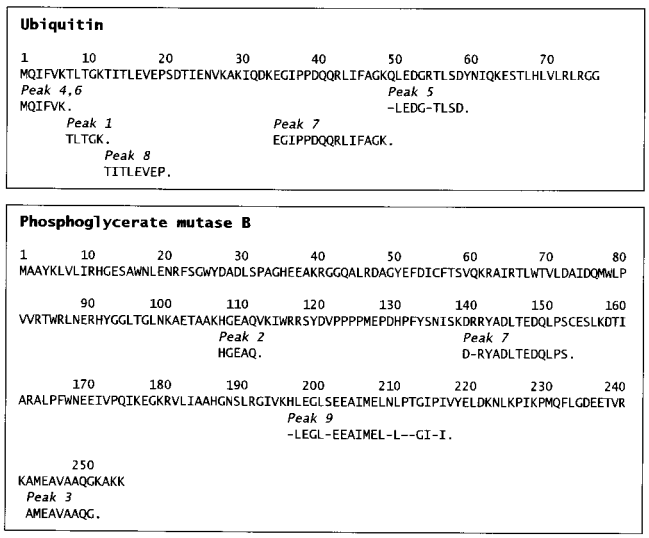

FIGURE 5 – Summary of the amino acid sequences determined in peptide fragment peaks 1–9. Sequences determined by Edman degradation of *Achromobacter* protease I-digests of the 38-k Da protein (Fig. 4). The peak numbers are shown in italics. Sequences are given in 1-letter codes below the sequences of ubiquitin and phosphoglycerate mutase-B. Sequences not identified are indicated by dashes and the dot at the end of the sequence indicates that the amino acid has not been determined from this point.

free PGM-B, was also found in the anti-ubiquitin immunoprecipitates.

The PGM-B-immunoreactive proteins in the tumor regions were compared to those in the adjacent normal regions in representative 2 paired samples. PGMIR-1 and -2 in the anti-ubiquitin immunoprecipitates (Fig. 7*a*) were quantified by densitometry and a value of PGMIR-2 in the tumor region of Patient IV was standardized to equal 100 arbitrary units. PGMIR-2 levels in the tumor regions (100 and 65 in the Patients IV and II, respectively) were higher than those of the normal counterparts (4 and 0 in the Patients IV and II, respectively). Similarly, PGMIR-1 levels in the tumor regions (854 and 830 in the Patients IV and II, respectively) were higher than those of the normal counterparts (575 and 242 in the Patients IV and II, respectively) (Fig. 7*a*). No PGMIR-2 band was detected when we directly analyzed these tissue extracts without the immunoprecipitation (Fig. 7*b*). PGMIR-1 amounts in these non-immunoprecipitated materials were estimated and a value in the Patient IV tumor was set at 100 arbitrary units. As a result, PGMIR-1 levels in the tumor regions (100 and 87 in the Patients IV and II, respectively) were slightly higher than those of the normal counterparts (65 and 62 in the Patients IV and II, respectively) (Fig. 7*b*). In addition, we found large amounts of PGMIR-1, but not PGMIR-2, in the non-immunoprecipitable supernatants of the tumor extracts (data not shown), suggesting part of free PGM-B was immunoprecipitable with the antibody FK2.

DISCUSSION

In our study, we purified the 38-kDa colorectal-tumor-producing protein, which was immunoprecipitable with the antibody FK2 recognizing both free and conjugated ubiquitin in solution[18] and identified the protein as monoubiquitinated PGM-B. To the best of our knowledge, this is the first report of ubiquitination of PGM *in vivo*. A simple explanation of the underlying mechanism for increasing in levels of the monoubiquitinated PGM-B in colorectal tumor tissues may involve either of the 2 following assumptions:

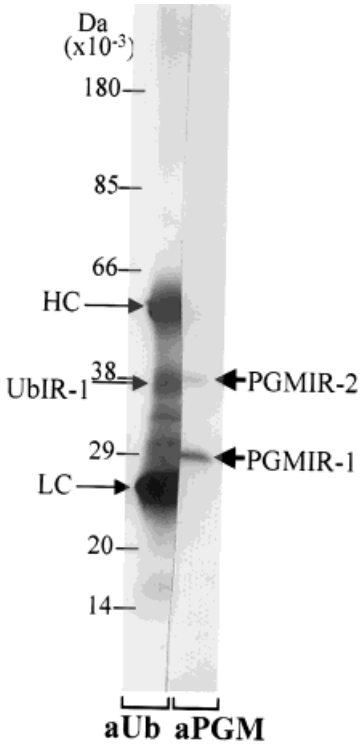

FIGURE 6 – Immunoblotting of the anti-ubiquitin immunoprecipitate of a colorectal cancer extract obtained from the patient III indicated in Figure 1. The tumor extract (600 μg protein) was immunoprecipitated with the FK2-antibody-Sepharose (30 μl beads), which recognizes both free and conjugated ubiquitin in solution.[18] The resulting precipitate was solubilized in 60 μl of the SDS-PAGE sample buffer with dithiothreitol and subjected to SDS-PAGE (30 μl/lane). The resolved proteins were transferred onto a nitrocellulose membrane and a membrane fragment contained in a single lane was separately obtained. This fragment was cut in half vertically and the left and right sides were immunostained with the ubiquitin antibody FK2 (aUb) and the antibody against phosphoglycerate mutase-B (aPGM), respectively. Locations of 2 PGM-immunoreactive proteins are indicated as PGMIR-1 (29 kDa) and PGMIR-2 (38 kDa). Ubiquitin-immunoreactive band-1 (UbIR-1) corresponds to 35–38 kDa. Heavy chains (HC) and light chains (LC) of the antibodies leaked from the FK2 antibody-Sepharose beads and detected by the enzyme-labeled anti-mouse IgG. Molecular mass estimates are indicated to the left.

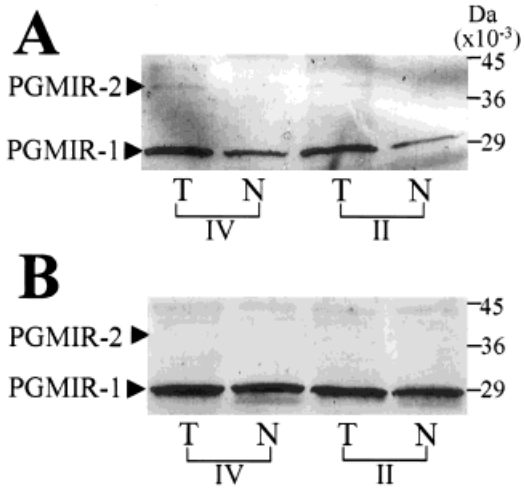

FIGURE 7 – Phosphoglycerate mutase-B (PGM-B)-immunoreactive proteins in the anti-ubiquitin immunoprecipitated or non-immunoprecipitated materials obtained from colorectal cancer extracts. Tumor regions (T) and adjacent normal regions (N) were obtained from the Patients IV and II, indicated in Figure 1. (*a*) Each tissue extract (600 μg protein) was immunoprecipitated with the FK2-antibody-Sepharose (30 μl beads) and the resulting precipitates were solubilized in 60 μl of the SDS-PAGE sample buffer with dithiothreitol. They were subjected to SDS-PAGE (30 μl/lane), followed with immunoblotting using the antibody against PGM-B. (*b*) Each tissue extracts (45 μg protein) were subjected to SDS-PAGE, followed with immunoblotting using the antibody against PGM-B. Locations of 2 PGM-immunoreactive proteins are indicated as PGMIR-1 (29 kDa) and PGMIR-2 (38 kDa). Molecular mass estimates are indicated to the right.

(*i*) PGM-B is monoubiquitinated without further extension of multiubiquitin chains; (*ii*) PGM-B is multiubiquitinated for proteasomal degradation accompanying accumulation of the monoubiquitinated form, which seems to be an intermediate or a partially deubiquitinated product. The fact that any ubiquitin-PGM-B conjugates other than the monoubiquitinated form were not found in the colon tumor tissues (Fig. 6) might support the former. The absence of these conjugates, however, is also possibly caused by proteasomal degradation (or enzymatic deubiquitination) of the conjugates, thus, it is not concluded yet whether PGM-B is recognized as a substrate for monoubiquitination or multiubiquitination in the colorectal tumor tissues.

PGM is an enzyme of the glycolytic pathway, which catalyzes the reversible interconversion of 2-phosphoglycerate and 3-phosphoglycerate.[26] In mammals, this enzyme is a dimeric protein consisting of 2 different subunits, types B and M and PGM-B found in our study is a homodimer of isozyme B. In normal tissues, colorectal and most other tissues have almost exclusively PGM-B, whereas skeletal muscle contains mostly PGM-M (MM isozyme) and heart possesses all of the isozymes (MM, BB and MB).[27] In addition, colorectal, liver, lung and breast carcinoma tissues contain mainly PGM-B and traces of PGM-MB and their PGM enzymatic activities are higher than those of adjacent normal tissues.[28,29] Brain tumours possessed lower PGM activity than normal brain.[30] In fibroblasts, exposure of the cells to hypoxia induces induction of PGM activity, concomitant with elevations of mRNA and the protein levels of PGM-B, indicating that the enzyme activity is regulated mainly at the transcriptional step.[31] Thus, the colorectal cancer specimens analyzed in our study were expected to have high PGM-B contents. In agreement with this supposition, levels of free PGM-B were slightly higher in the tumor region extracts (PGMIR-1 in Fig. 7*b*).

Our present study raises the question of why PGM-B is ubiquitinated. A variety of intracellular proteins are selectively multiubiquitinated for further destruction by 26S proteasome,[1,2] but there is virtually no information regarding the involvement of the ubiquitin system in PGM-B degradation. Several cellular proteins, including histones H2A, H2B and H1,[32,33] G-protein coupled receptors,[34] membrane-bound transporters,[35,36] ubiquitin-conjugating enzymes,[18,37] a yeast kinetochore protein Cbf2p,[38] calmodulin[32] and retrovirus proteins[39] are established as substrates for monoubiquitination *in vivo*. They are not targeted for destruction by 26S proteasome and the monoubiquitination to these proteins regulates diverse cellular events, such as histone regulation, endocytosis and virus budding, *etc.*[33] PGM-B does not share common features with them, but we speculate that this modification might affect activity or location[40] of the enzyme. Involvement of the PGM-B ubiquitination in the progression of colorectal cancer remains to be elucidated.

In our study, PGM-B in colorectal cancer tissues is categorized 3 forms: free PGM-B that is not immunoprecipitable with the antibody FK2, monoubiquitinated PGM-B (PGMIR-2 in Fig. 6 and 7*a*) and immunoprecipitable 'non-ubiquitinated' PGM-B (PGMIR-1 in Fig. 6 and 7*a*). Interestingly, levels of the immunoprecipitated 'non-ubiquitinated' PGM-B were also higher in tumor tissues than normal counterparts (Fig. 7*a*). We predict that this form of PGM-B may be a member of the noncovalent complexes

containing ubiquitin, including ubiquitin thioesters with E2/E3 enzymes containing their substrates, because the antibody FK2 is capable of precipitating ubiquitin-E2 thioesters.[18] Further studies will be required to identify these complexes, which presumably increase in colorectal cancer.

The cellular abundance of the monoubiquitinated PGM-B tended to increase with the stages of colon cancer (Table I), suggesting its correlation with the tumor progression. Thus, the quantification of the ubiquitin-PGM-B conjugates might have diagnostic or prognostic values. The electrophoretic technique employed in our study, however, is less accurate for such applications. Based on our ELISA system for multiubiquitin chains,[20] development of an asymmetric ELISA for the ubiquitinated PGM-B, in which 2 antibodies to ubiquitin and the PGM-B are used for capture and detection of the antigen, respectively, is planned and it will be useful in evaluating the clinical significance.

Accumulation of a 42-kDa ubiquitin-immunoreactive protein in Triton X-insoluble fractions of colorectal cancer tissues have been reported,[15] but we did not make this observation in the anti-ubiquitin immunoprecipitates of the CHAPS-soluble fractions of the corresponding tissues (Fig. 1). An analysis of the CHAPS-insoluble materials will be required to confirm the presence of 42-kDa protein in our specimens.

Several investigators have isolated ubiquitin-immunoreactive proteins accumulated in tumor tissues by using 2-dimensional PAGE, but no internal amino acid sequence of ubiquitin has been found in API digests of the isolated materials, due to API-resistance of ubiquitin immobilized onto PVDF membranes.[15–17] In similar API digests of monoubiquitinated PGM-B in our study and several proteins purified by the immunoaffinity chromatography from K562 cells[18] and various biological materials (unpublished data), we almost consistently found sequences identical to ubiquitin as well as target proteins. A reason for this discrepancy is unknown, but it may suggest the reliability of the methods employed in our study. The unknown immunoprecipitable proteins observed in Figure 1, whose levels were higher (or lower) in the tumor regions than in normal counterparts, will be further identified by the methods in our study to gain a better understanding of the dynamics of ubiquitination or deubiquitination in colorectal cancer.

ACKNOWLEDGEMENTS

Our study was supported in part by a Grant in Aid for Scientific Research on Priority Areas, (Intracellular Proteolysis) from the Ministry of Education, Science, Sports and Culture of Japan. The authors thank members of the laboratory and Prof. T. Aoki (Jikei University) for helpful discussions and suggestions and Mr. Y. Itoh for his technical support. We also thank Dr. H. Yokosawa (Hokkaido University) for his generous supply of FK2 antibody and Drs. K. Uchida and Y. Matuo (Oriental Yeast Co., Ltd.) for their gift of PGM-B antibody.

REFERENCES

1. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425–79.
2. Ciechanover A, Orian A, Schwartz AL. Ubiquitin-mediated proteolysis: biological regulation via destruction. Bioessays 2000;22:442–51.
3. Hershko A, Heller H, Elias S, et al. Components of ubiquitin-protein ligase system. Resolution, affinity purification and role in protein breakdown. J Biol Chem 1983;258:8206–14.
4. Haas AL, Siepmann TJ. Pathways of ubiquitin conjugation. FASEB J 1997;11:1257–68.
5. Glotzer M. Cell cycle. The only way out of mitosis. Curr Biol 1995;5:970–2.
6. Yanagida M, Yamashita YM, Tatebe H, et al. Control of metaphase-anaphase progression by proteolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization. Philos Trans R Soc Lond B Biol Sci 1999;354:1559–69.
7. Dimmeler S, Breitschopf K, Haendeler J, et al. Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway. J Exp Med 1999;189:1815–22.
8. Yang Y, Fang S, Jensen JP, et al. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 2000;288:874–7.
9. Bonifacino JS, Weissman AM. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu Rev Cell Dev Biol 1998;14:19–57.
10. Spataro V, Norbury C, Harris AL. The ubiquitin-proteasome pathway in cancer. Br J Cancer 1998;77:448–55.
11. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999;59:2615–22.
12. Ishibashi Y, Takada K, Joh K, et al. Ubiquitin immunoreactivity in human malignant tumours. Br J Cancer 1991;63:320–2.
13. Kanayama H, Tanaka K, Aki M, et al. Changes in expressions of proteasome and ubiquitin genes in human renal cancer cells. Cancer Res 1991;51:6677–85.
14. Osada T, Sakamoto M, Nishibori H, et al. Increased ubiquitin immunoreactivity in hepatocellular carcinomas and precancerous lesions of the liver. J Hepatol 1997;26:1266–73.
15. Nishibori H, Matsuno Y, Iwaya M, et al. Human colorectal carcinomas specifically accumulate Mr 42,000 ubiquitin-conjugated cytokeratin 8 fragments. Cancer Res 1996;56:2752–7.
16. Iwaya K, Nishibori H, Osada T, et al. Immunoreaction at 43 kDa with anti-ubiquitin antibody in breast neoplasms. Jpn J Cancer Res 1997;88:273–80.
17. Osada T, Sakamoto M, Nagawa H, et al. Acquisition of glutamine synthetase expression in human hepatocarcinogenesis: relation to disease recurrence and possible regulation by ubiquitin-dependent proteolysis. Cancer 1999;85:819–31.
18. Takada K, Hirakawa T, Yokosawa H, et al. Isolation of ubiquitin-E2 (ubiquitin-conjugating enzyme) complexes from erythroleukemia cells using immunoaffinity techniques. Biochem J 2001;in press.
19. Fujimuro M, Sawada H, Yokosawa H. Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. FEBS Lett 1994;349:173–80.
20. Takada K, Nasu H, Hibi N, et al. Immunoassay for the quantification of intracellular multi-ubiquitin chains. Eur J Biochem 1995;233:42–7.
21. Uchida K, Kondoh K, Matuo Y. Recombinant M-, B- and MB-type isozymes of human phosphoglyceric acid mutase: their large-scale production and preparation of polyclonal antibodies specific to M- and B-type isozymes. Clin Chim Acta 1995;237:43–58.
22. Takada K, Hibi N, Tsukada Y, Shibasaki T, Ohkawa K. Ability of ubiquitin radioimmunoassay to discriminate between monoubiquitin and multi-ubiquitin chains. Biochim Biophys Acta 1996;1290:282–8.
23. Takada K, Kanda T, Ohkawa K, et al. Ubiquitin and ubiquitin-protein conjugates in PC12h cells: changes during neuronal differentiation. Neurochem Res 1994;19:391–8.
24. Kennedy TE, Gawinowicz MA, Barzilai A, Kandel ER, Sweatt JD. Sequencing of proteins from 2-dimensional gels by using *in situ* digestion and transfer of peptides to polyvinylidene difluoride membranes: application to proteins associated with sensitization in Aplysia. Proc Natl Acad Sci USA 1988;85:7008–12.
25. Iwamatsu A. s-Carboxymethylation of proteins transferred onto polyvinylidene difluoride membranes followed by in situ protease digestion and amino acid microsequencing. Electrophoresis 1992;13:142–7.
26. Grisolia S, Carreras J. Phosphoglycerate mutase from yeast, chicken breast muscle and kidney (2, 3-PGA-dependent). Methods Enzymol 1975;42:435–50.
27. Durany N, Carreras J. Distribution of phosphoglycerate mutase isozymes in rat, rabbit and human tissues. Comp Biochem Physiol B Biochem Mol Biol 1996;114:217–23.
28. Durany N, Joseph J, Campo E, et al. Phosphoglycerate mutase, 2,3-bisphosphoglycerate phosphatase and enolase activity and isoenzymes in lung, colon and liver carcinomas. Br J Cancer 1997;75:969–77.
29. Durany N, Joseph J, Jimenez OM, et al. Phosphoglycerate mutase, 2,3-bisphosphoglycerate phosphatase, creatine kinase and enolase activity and isoenzymes in breast carcinoma. Br J Cancer 2000;82:20–7.
30. Durany N, Joseph J, Carreras J. Phosphoglycerate mutase, 2,3-bisphosphoglycerate phosphatase and creatine kinase activity and isoenzymes in human brain tumours. Br J Cancer 1997;76:1139–49.
31. Takahashi Y, Takahashi S, Yoshimi T, et al. Hypoxia-induced expression of phosphoglycerate mutase B in fibroblasts. Eur J Biochem 1998;254:497–504.
32. Jennissen HP. Ubiquitin and the enigma of intracellular protein degradation. Eur J Biochem 1995;231:1–30.

33. Hicke L. Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol 2001;2:195–201.
34. Terrell J, Shih S, Dunn R, et al. A function for monoubiquitination in the internalization of a G protein-coupled receptor. Mol Cell 1998;1: 193–202.
35. Galan J, Haguenauer-Tsapis R. Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. EMBO J 1997;16: 5847–54.
36. Lucero P, Penalver E, Vela L, et al. Monoubiquitination is sufficient to signal internalization of the maltose transporter in *Saccharomyces cerevisiae*. J Bacteriol 2000;182:241–3.
37. Gwozd CS, Arnason TG, Cook WJ, et al. The yeast UBC4 ubiquitin conjugating enzyme monoubiquitinates itself *in vivo*: evidence for an E2-E2 homointeraction. Biochemistry 1995;34:6296–302.
38. Yoon HJ, Carbon J. Genetic and biochemical interactions between an essential kinetochore protein, Cbf2p/Ndc10p and the CDC34 ubiquitin-conjugating enzyme. Mol Cell Biol 1995;15:4835–42.
39. Ott DE, Coren LV, Copeland TD, et al. Ubiquitin is covalently attached to the p6Gag proteins of human immunodeficiency virus type 1 and simian immunodeficiency virus and to the p12Gag protein of Moloney murine leukemia virus. J Virol 1998;72:2962–8.
40. Urena JM, Egea G, Grana X, et al. Location of phosphoglycerate mutase in rat skeletal muscle. An immunocytochemical and biochemical study. Eur J Cell Biol 1990;51:151–6.