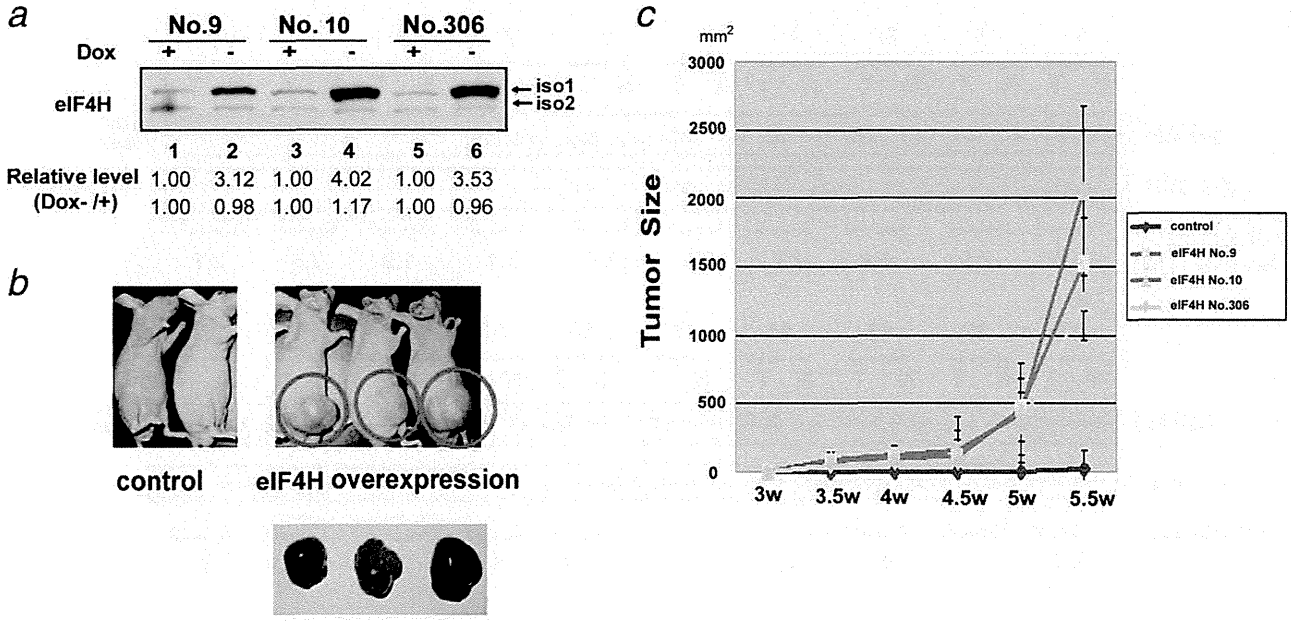


Figure 2. Overexpression of eIF4H isoform 1 led to tumor formation *in vivo*. (*a*) NIH3T3 stable cell lines overexpressing eIF4H isoform 1 (clone no. 9, 10 and 306) were generated using a Tet-Off gene expression system, as described in "Material and Methods" section, and the overexpression of eIF4H isoform 1 was confirmed by Western blotting. eIF4H isoform 1 was overexpressed in the absence of doxycycline (Dox) (lanes 2, 4 and 6) compared with in the presence of Dox (lanes 1, 3 and 5) in stable cell lines. (*b*) eIF4H isoform 1-overexpressing cells (6×10^5 cells) were subcutaneously injected into nude mice and tumor formation was examined for 5.5 weeks. (*c*) NIH3T3 stable clones overexpressing eIF4H isoform 1, no. 9, 10 and 306, were injected into six mice, respectively. Tumor size was measured every 3 days and the mean ± SD of six mice from three independent experiments are shown.

cells using isoform 2-specific siRNA (si201). As expected, si201 did not affect the cell number of LOVO and RKO, in great contrast to si103 and 104 (Figs. 3*c* and 3*d*). These results further supported the eIF4H isoform 1-specific effect on the cell proliferation of colon cancer cells.

Suppression of eIF4H isoform 1 induces apoptosis of colon cancer cell lines

To understand the mechanism of the inhibitory effect on proliferation by eIF4H isoform 1 siRNA, flow cytometric analysis was performed. The treatment of cells with si103 caused a 3-fold increase in the sub-G1 cell population and a decrease in the S phase compared with control cells, which suggests that the growth inhibition of cells by RNAi is due to the induction of apoptosis (Fig. 4*a*). This apoptosis was further confirmed by TUNEL assay (Fig. 4*b*). Both LOVO and RKO treated with si103 showed 60–70% of TUNEL-positive cells compared with about 20% in untreated control cells (Student's *t*-test, $p < 0.05$) (Fig. 4*c*). These results suggest that inhibition of apoptosis might be involved in the transforming activity of eIF4H isoform 1.

Suppression of eIF4H isoform 1 inhibited tumor growth in nude mice

Next, we investigated whether the suppression of eIF4H isoform 1 could also inhibit tumor growth *in vivo*. For the experiment, we created LOVO cell lines that were stably transfected with short hairpin RNA (shRNA) expression plasmids, sh103 and sh104, corresponding to si103 and si104 used in the above experiment. Several clones, sh103-1, 103-2 and 104, showed remarkable reduction of eIF4H isoform 1 compared with control cells (Fig. 5*a*, upper panel). These clones were injected subcutaneously into nude mice and the tumor size was monitored. Strikingly, the tumors formed in mice injected with control LOVO cells grew rapidly, whereas tumors were greatly reduced in mice injected with shRNA stably expressed clones (Figs. 5*a* and 5*b*). These results indicate that eIF4H isoform 1 contributes to the development of colon tumors.

Suppression of eIF4H isoform 1 showed no effect on the growth of immortalized human fibroblasts

The above results have raised the question of whether the decrease of the cell number by eIF4H isoform 1 knockdown is specific to cancer cells or is a more general feature; if it is unique to cancer cells it might be a potential target for cancer therapy. To address this question, we examined the effect of eIF4H suppression on the proliferation of immortalized human fibroblast cell MRC5 using the same siRNA shown in Figure 3. The expression of eIF4H isoforms, especially isoform 1, was greatly suppressed 48 hr after siRNA transfection (Fig. 5*c*). In great contrast with colon cancer cells, there was

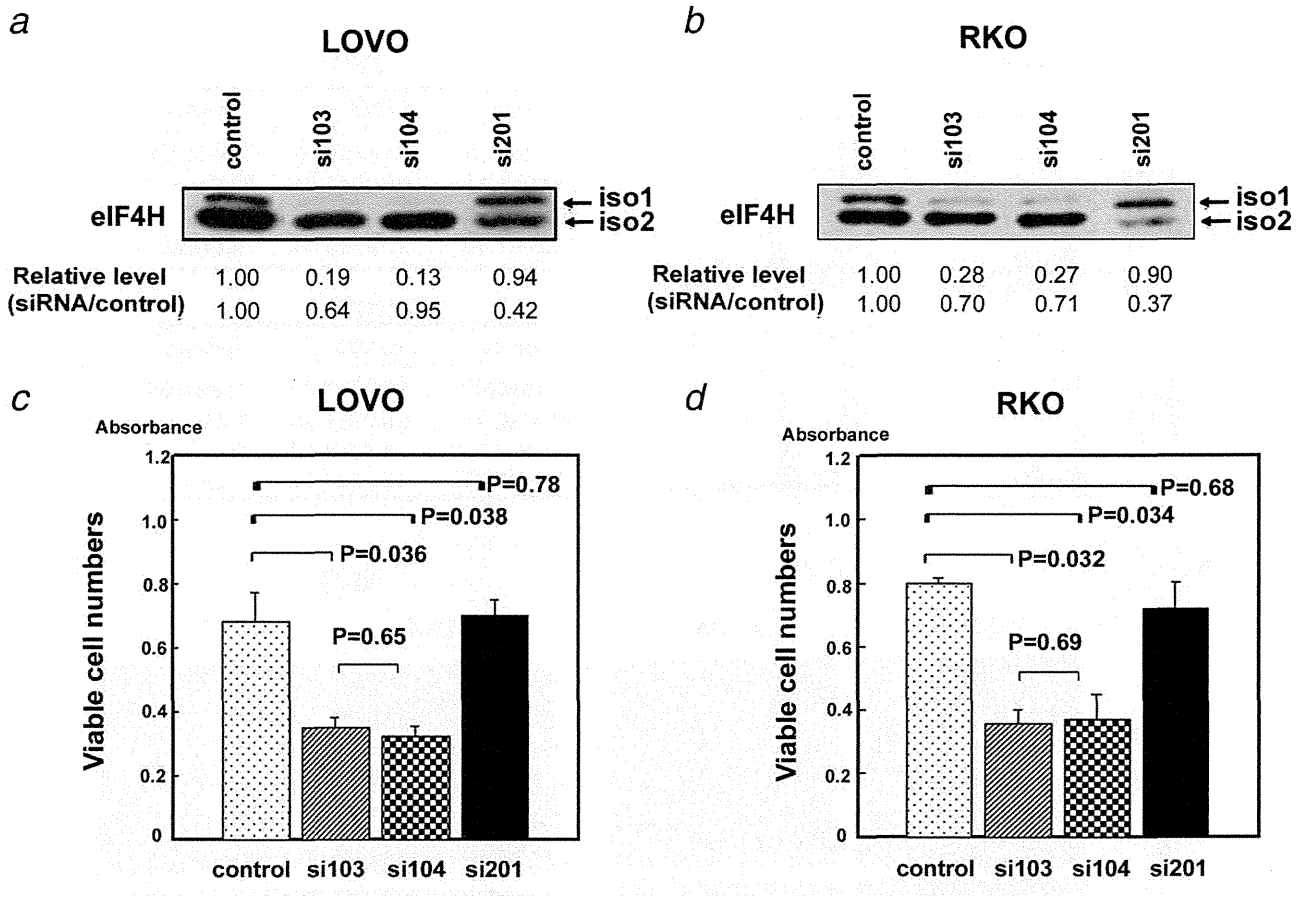


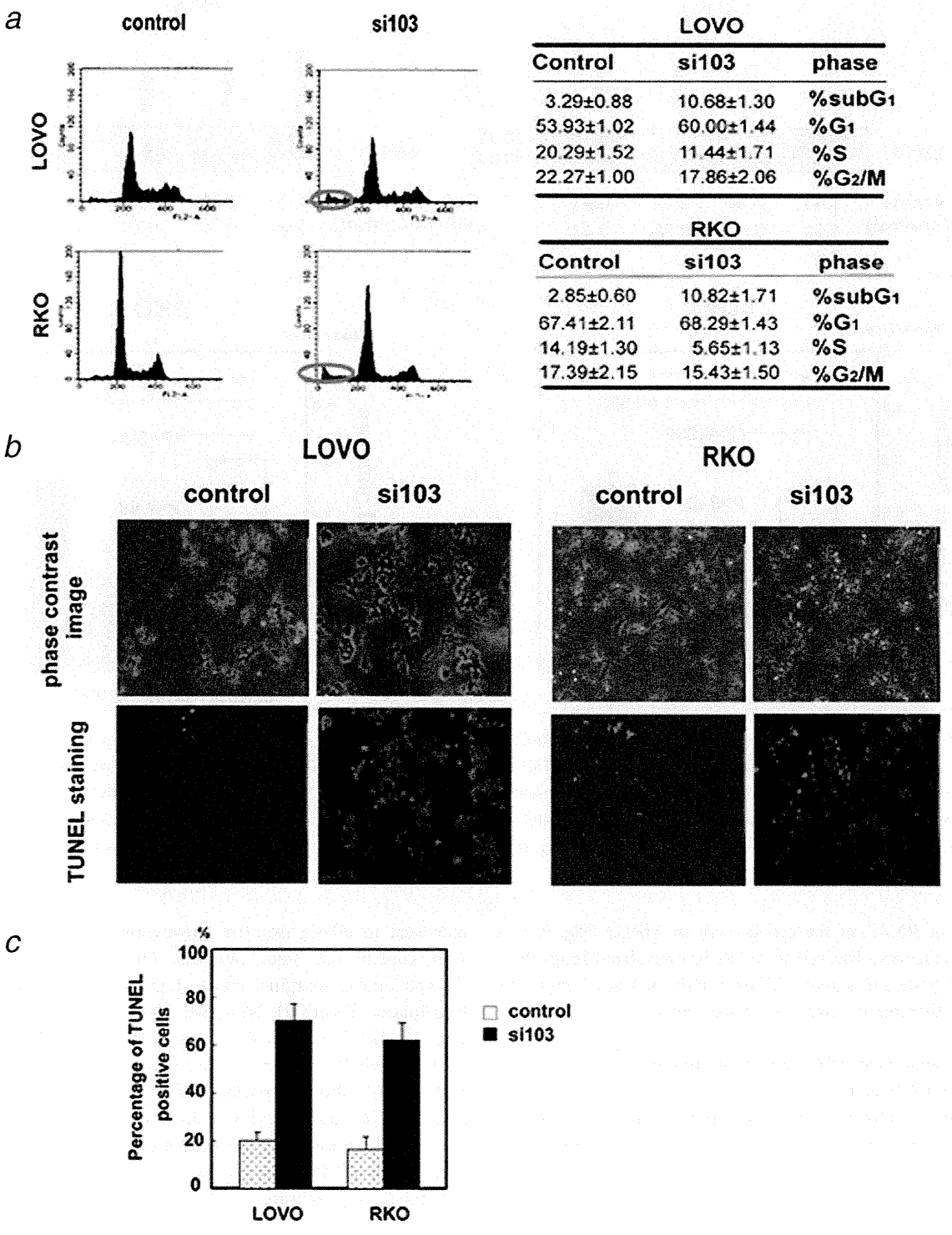
Figure 3. Suppression of eIF4H isoform 1, and not isoform 2, inhibited the growth of colon cancer cell lines. (*a*, *b*) 20 pmol of siRNA (isoform 1 specific: si103, 104; isoform 2 specific: 201) for eIF4H were transfected into LOVO and RKO cells, and the expression of eIF4H isoforms was examined by Western blotting 48 hr after siRNA treatment. The intensity of each band was measured with NIH Image and the relative level of eIF4H isoforms between control and siRNA-treated cells normalized with β-actin was calculated. (*c*, *d*) Viable cell numbers 48 hr after eIF4H siRNA treatment were assessed by MTS assay. Data represent the mean ± SD of eight independent experiments and statistical analysis was performed by Student's *t*-test.

no effect of RNAi on the cell growth of MRC5 (Fig. 5*d*). A similar result was observed in WI38 immortalized lung fibroblast cells (data not shown). Thus, eIF4H isoform 1 might be a suitable therapeutic target for colon cancer.

Possible mechanism of cellular transformation caused by eIF4H isoform 1

What is the mechanism of transformation induced by eIF4H isoform 1 expression? One possible mechanism is that eIF4H isoform 1 leads to the overproduction of potentially oncogenic proteins that stimulate cell proliferation or inhibit apoptosis, such as c-Myc, cyclin D1, VEGF, ODC, Bcl-2 and Bcl-X_L. mRNA of the genes are known to have long and structurally complex 5′-untranslated regions and may require high levels of eIFs, including eIF4H, for their translation. To examine the effect of eIF4H isoform 1 overexpression on the expression of oncogenic proteins, immunoblotting was performed using extracts from NIH3T3 cell lines overexpressing eIF4H isoform 1. Surprisingly, the level of cyclin D1 greatly increased in eIF4H isoform 1-overexpressing cells compared with control cell lines, whereas ODC, Bcl-2 and Bcl-X_L showed only a marginal effect (Figs. 6*a* and 6*b*, Supporting Information Figure 2). Next, we tested whether the suppression of eIF4H isoform 1 decreases the cyclin D1 level. As expected, eIF4H isoform 1-suppressed LOVO cells, sh103-1 and sh103-2, showed marked reduction of cyclin D1 expression (Figs. 6*c* and 6*d*). c-Myc level increased in only one of the eIF4H isoform 1 overexpressing clones and did not decrease in eIF4H stable knockdown cells, which indicates that eIF4H overexpression is not involved in the regulation of c-Myc expression. These results indicate that upregulation of cyclin D1 is involved in the transforming activity of eIF4H isoform 1.

If cyclin D1 upregulation is a direct mechanism of the transforming activity of eIF4H, ectopic expression of cyclin D1 in eIF4H knockdown cells would suppress the inhibitory effect on cell proliferation. To test this, we cotransfected eIF4H isoform 1 siRNA (si104) and cyclin D1 expression



LOVO		
Control	si103	phase
3.29±0.88	10.68±1.30	%subG1
53.93±1.02	60.00±1.44	%G1
20.29±1.52	11.44±1.71	%S
22.27±1.00	17.86±2.06	%G2/M

RKO		
Control	si103	phase
2.85±0.60	10.82±1.71	%subG1
67.41±2.11	68.29±1.43	%G1
14.19±1.30	5.65±1.13	%S
17.39±2.15	15.43±1.50	%G2/M

Figure 4. Suppression of eIF4H isoform 1 induces apoptosis of colon cancer cell lines. LOVO and RKO cells were treated with 20 pmol of si103, siRNA specific to eIF4H isoform 1, for 48 hr and FACS (*a*) and TUNEL (*b*) analysis was performed. (*a*) FACS analysis was repeated three times and the percentage (mean ± SD) of the cell population in each phase of the cell cycle is shown in the right table. (*b*) TUNEL analysis of control or si103-treated colon cancer cells (LOVO and RKO) showed apoptotic cells. (*c*) The number of TUNEL-positive cells was counted from five different fields. The percentage (mean ± SD) was calculated.

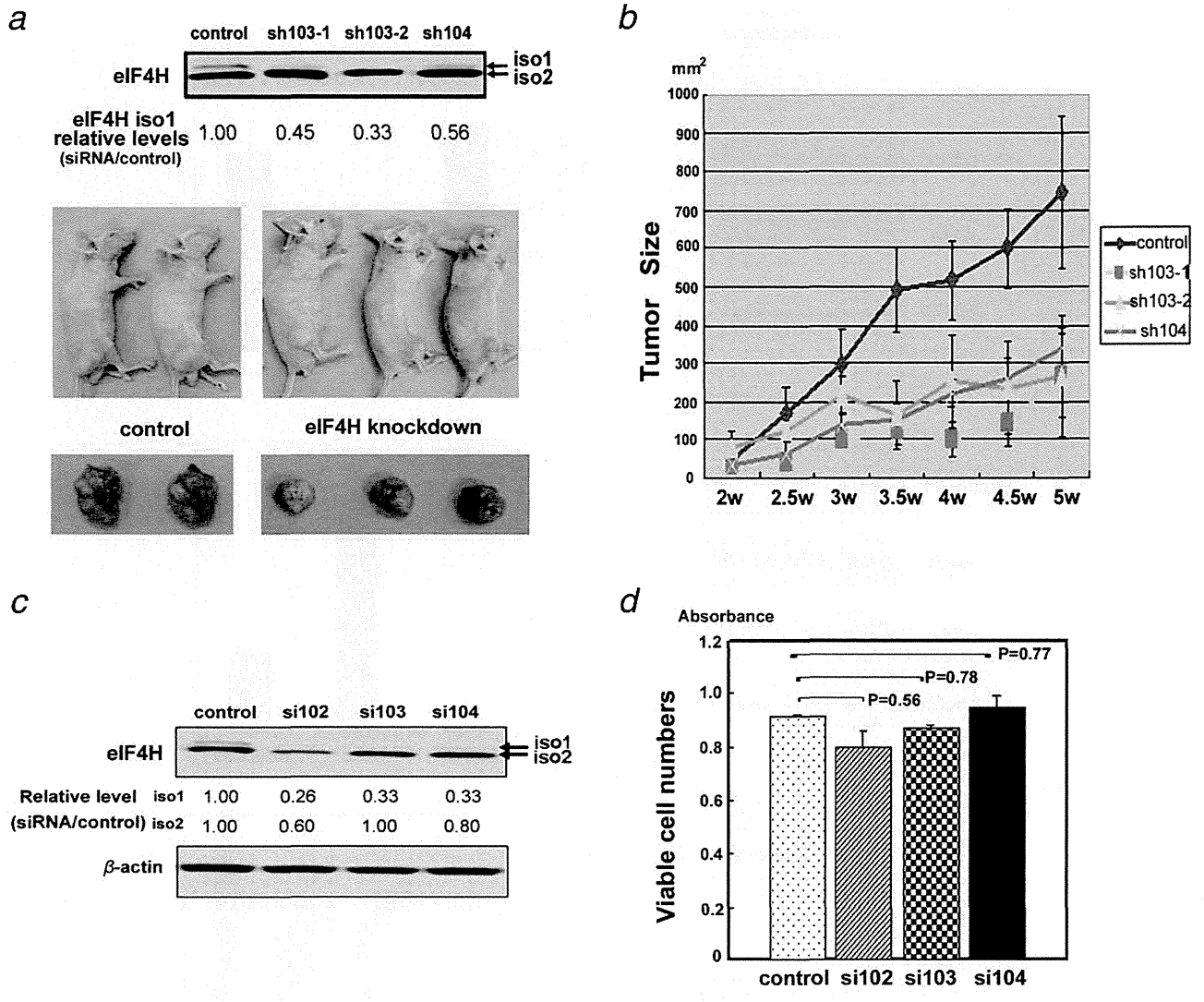


Figure 5. Suppression of eIF4H isoform 1 inhibited tumor growth in nude mice, but showed no effect on the proliferation of immortalized fibroblasts. (*a*) shRNAs (sh103, 104) specific for eIF4H isoform 1 were transfected in LOVO cells and stable knockdown cell lines of eIF4H isoform 1 were generated. sh103-1 and 103-2 are different stable clones generated by sh103 transfection. The suppression of eIF4H isoform 1 in each clone was confirmed by Western blotting. The intensity of each band was measured with NIH image and the relative level of eIF4H isoform 1 between control and stable knockdown cell lines normalized with β-actin was calculated. eIF4H isoform 1 stable knockdown LOVO cells (1×10^6 cells) were subcutaneously injected into nude mice and tumor formation was examined for 5 weeks (lower panel). (*b*) A control and three stable clones, sh103-1, 103-2 and 104, were injected into six mice, respectively. Tumor size was measured every 3 days and tumor growth curves of the mean ± SD of six mice from two independent experiments are shown. (*c*) 20 pmol of siRNA (si102, 103 and 104) for eIF4H were transfected into MRC5 cells and the expression of eIF4H isoforms was examined by Western blotting 48 hr after siRNA treatment. The intensity of each band was measured with NIH image and the relative level of eIF4H isoforms between control and siRNA-treated cells normalized with β-actin was calculated. si102 suppressed both isoform 1 and 2 of eIF4H, whereas si103 and 104 suppressed only isoform 1. (*d*) Viable cell numbers 48 hr after eIF4H siRNA treatment were assessed by MTS assay. Data represent the mean ± SD of eight independent experiments and statistical analysis was performed by Student's *t*-test.

plasmid into a colon cancer cell line, RKO, and measured the viable cell numbers by MTS assay. Expression of the cyclin D1 plasmid was confirmed by Western blotting (Fig. 5*e*). Cotransfection of si104 with control plasmid decreased the cell number, as shown in Figure 3, whereas cotransfection of cyclin D1 expression plasmid reversed the growth suppression effect of si104 (Fig. 5*f*). This result further supports our hypothesis that eIF4H isoform 1 plays an important role in carcinogenesis through the activation of cyclin D1.

The expression of cyclin D1 is well known to be regulated by another eukaryotic translation initiation factor, eIF4E. Regulation is thought to occur at the translational level;

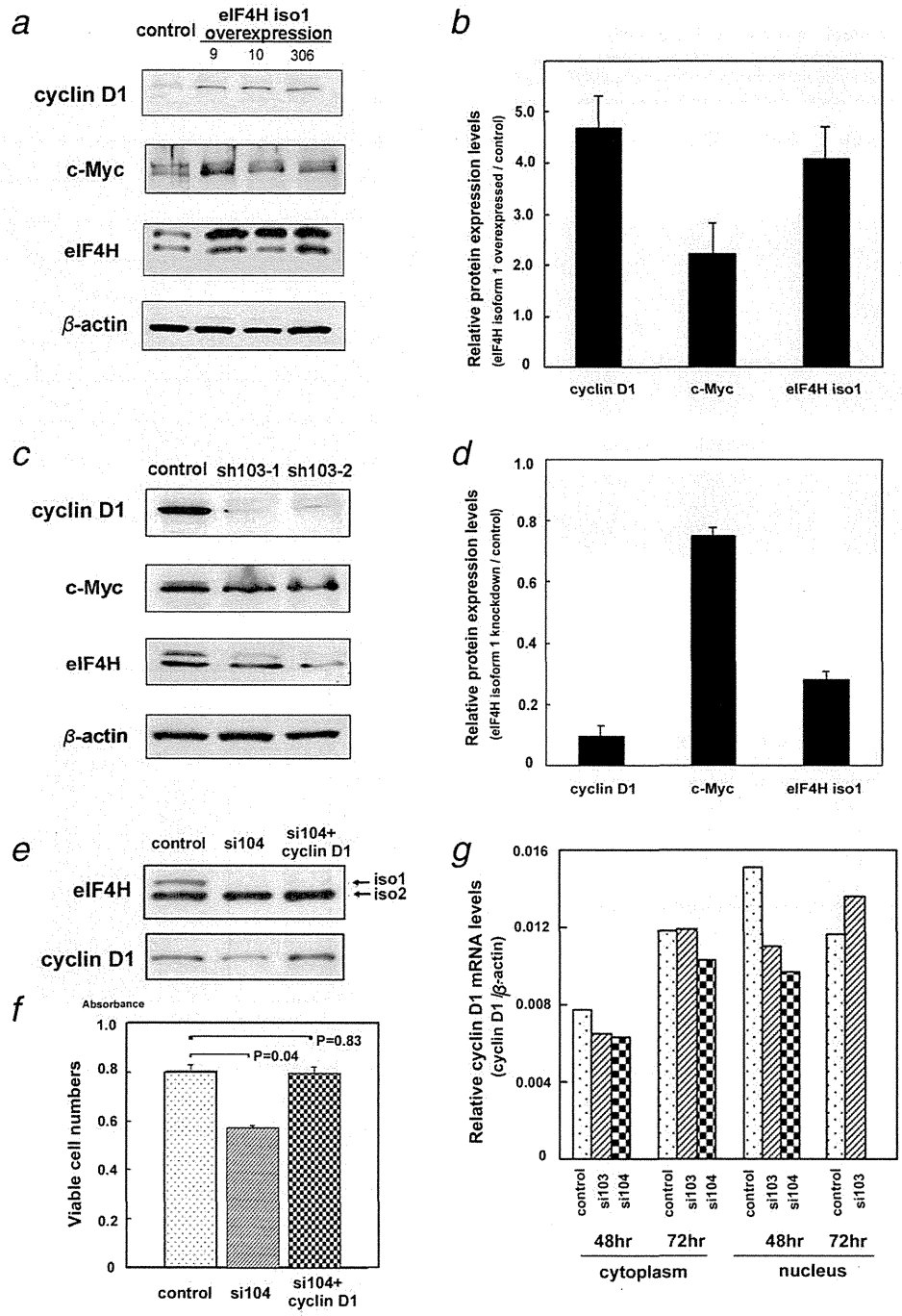


Figure 6. Cyclin D1 upregulation is involved in the transforming activity of eIF4H isoform 1. (*a*) Expressions of cyclin D1 and c-Myc in three different stable clones overexpressing eIF4H isoform 1, no. 9, 10 and 306, were examined with Western blotting. (*b*) The intensity of each band was measured with NIH image and the relative expression levels of each protein normalized with β-actin in eIF4H isoform 1-overexpressing cells compared with control cells are shown. Means and SD values were calculated from the results of the different clones. (*c*) Expression of cyclin D1 and c-Myc was examined in eIF4H isoform 1 stable knockdown LOVO cell lines. (*d*) Data represent the means and SD values calculated from the results of two different clones. (*e*) 50 pmol of eIF4H isoform 1-specific siRNA (si104) with 25 ng of control or cyclin D1 expression plasmid was transfected into RKO cells and the expression of eIF4H isoforms (upper panel) and cyclin D1 (lower panel) was examined by Western blotting 72 hr after siRNA treatment. (*f*) Viable cell numbers 72 hr after transfection were assessed by MTS assay. Data represent the mean ± SD of eight independent experiments and statistical analysis was performed by Student's *t*-test. (*g*) Control or eIF4H isoform 1 siRNA (si103, 104; 50 pmol) was transfected to RKO cells. Total RNA from nucleus and cytoplasm of the cells was extracted 48 and 72 hr after transfection and real-time PCR was performed to examine the cyclin D1 mRNA level. Relative cyclin D1 mRNA levels normalized with β-actin were calculated. Nuclear mRNA level 72 hr after si104 treatment is not shown.

however, recent reports have demonstrated that cyclin D1 mRNA is also controlled at the level of mRNA export from the nucleus, and this transport of cyclin D1 mRNA to the cytoplasm is important for transformation activity.[25–27] Thus, we investigated whether eIF4H also regulates cyclin D1 at the mRNA level. RKO cells were treated with eIF4H siRNA (si103 and 104), and then nuclear and cytoplasmic cyclin D1 mRNA were evaluated by real-time quantitative RT-PCR. Cyclin D1 mRNA levels did not alter either in the nucleus or cytoplasm of siRNA-treated cells (Fig. 6*g*). Although it is possible that eIF4H could control other processes on cyclin D1 mRNA, regulation of cyclin D1 expression at the protein level seems to be the main mechanism, as the decrease of cyclin D1 expression after eIF4H isoform 1 downregulation is more pronounced at protein level.

Discussion

In our study, we investigated whether an alternative splicing form of eIF4H, which is overexpressed in gastrointestinal cancers, contributes to cell proliferation and carcinogenesis. We found that the overexpression of eIF4H isoform 1 in immortalized mouse fibroblasts developed tumors in nude mice. Conversely, silencing of the eIF4H isoform 1 inhibited the proliferation and induced apoptosis of colon cancer cell lines. It is striking that the growth suppression is specific to eIF4H isoform 1 siRNA and not isoform 2. Suppression of eIF4H isoform 1 also inhibited tumor growth in nude mice, although it showed no effect on the proliferation of immortalized human fibroblasts. Moreover, eIF4H isoform 1 upregulated the cyclin D1 level, a protein involved in cell proliferation. Thus, eIF4H isoform 1 stimulates cell proliferation and inhibits apoptosis, both of which are a possible mechanism of the transforming activity of eIF4H isoform 1. These observations provided strong evidence that eIF4H isoform 1 is a novel oncogene and might be a promising molecule target for colon cancer therapy.

Recently, many studies have argued that eIFs play an important role in carcinogenesis.[4,28] eIF4E, an mRNA 5′-cap-binding protein, has been identified to play a pivotal role in the tumorigenesis and metastatic progression of various cancers. Another subunit of eIF-4F complex eIF4G, a scaffolding protein, is also implicated in malignant transformation.[11,29] In our work, we demonstrated that another translation initiation factor, eIF4H, is involved in tumorigenesis, which suggests that translation initiation is a key event for the development of cancer. A mechanism suggested to contribute to tumorigenesis is the translational de-repression of structurally burdened mRNA known to encode proto-oncogenes, *e.g.*, cyclin D1, c-Myc, ODC, FGF and VEGF, through increased levels of eIFs.[30–32] Cyclin D1 was one of the first discovered targets of eIF4E. It has been reported that eIF4E regulates the amount of cyclin D1 not only by translation initiation but by nuclear export of its mRNA.[25–27] Thus, we investigated whether eIF4H isoform 1 is also involved in the nuclear export of cyclin D1 mRNA. Unlike eIF4E, eIF4H isoform 1 showed no effect on the nuclear export (Fig. 6*g*), which suggests that upregulation of cyclin D1 by eIF4H isoform 1 occurs at the translational level.

We also found that suppression of eIF4H isoform 1 induced the apoptosis of colon cancer cells. The induction of apoptosis is associated with the downregulation of translation rates, and underlying changes to the translation mechanism during apoptosis have been elucidated recently.[33] Apoptosis-associated modifications include the specific fragmentation of translational mechanism, such as eIF4G, eIF4B and eIF3j, and alterations in the phosphorylation states of initiation factors, such as eIF2α, eIF4E and eIF4E-BP1,[34] which might change the translation rates of pro- or antiapoptotic proteins. Moreover, eIF4E mediates *myc*-dependent apoptosis by inhibiting mitochondrial cytochrome *c* release through an increase in Bcl-X_L mRNA translation.[35] One explanation for apoptosis occurring by the suppression of eIF4H isoform 1 is that low levels of eIF4H decrease the translational efficiency of antiapoptotic factors, although knockdown of eIF4H isoform 1 showed no effect on Bcl-2 or Bcl-X_L levels (data not shown). Further investigation is needed to clarify the precise mechanism of the induction of apoptosis.

Pre-mRNA splicing is a sophisticated and ubiquitous nuclear process, which is a natural source of cancer-causing errors in gene expression. A large number of cancer-related genes that exhibit alternative splicing have been characterized, including CD44, WT1, BRCA1, MDM2, FGFR and kallikrein family members.[19] As well as being associated with cancer, the nature of alternative gene products is usually consistent with an active role in cancer; for example, several splicing variants of HDM2, the human homolog of MDM2, increase p53 levels and enhance p53 transcriptional activity.[36] Our recent data also showed that a splicing variant of *c-myc* suppressor FIR was frequently found in colon cancer tissues and promoted tumor development by disabling FIR repression to sustain high levels of *c-myc*[37]; therefore, the alternative spliced isoform plays an important role in carcinogenesis and eIF4H isoform 1 is one of these.

In our study, we showed that only eIF4H isoform 1 and not isoform 2 possesses transforming activity. An important question is why only one isoform has oncogenic potential. eIF4H is thought to stimulate protein synthesis by enhancing the helicase activity of eIF4A. The most likely explanation of eIF4H isoform 1-specific activity is that the isoform 1-specific exon 5 causes conformational change of the protein and enhances its helicase activity, which increases the cyclin D1 level, although this was not confirmed. Another possibility is that eIF4H isoform 1 specifically binds to other oncogene products or tumor suppressor proteins and enhances/inhibits their activities. Further studies are needed to elucidate the mechanism of the isoform 1-specific oncogenic potential of eIF4H.

Drugs that can selectively kill pathogenic cells without damaging healthy cells are so-called "magic bullets."[38] Our

observation showed that selective suppression of a splicing variant of eIF4H inhibited the proliferation of colon cancer cells and had no effect on immortalized human fibroblasts. Consequently, eIF4H isoform 1 would be an ideal therapeutic target for colon cancer.

Acknowledgements

We thank Dr. Tetsuo Noda and Dr. Kazuei Igarashi for critical reading of this article, and Ms. Masumi Ishibashi and Ms. Nobuko Tanaka for technical assistance. This work was supported by Grants-in-Aid to T.T. from the Ministry of Education, Science, Sports and Culture of Japan.

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research articles Journal of proteome research

High-Yield Peptide-Extraction Method for the Discovery of Subnanomolar Biomarkers from Small Serum Samples

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Serum proteins/peptides reflect physiological or pathological states in humans and are an attractive target for the discovery of disease biomarkers. However, the existence of high-abundance proteins and the large dynamic range of serum proteins/peptides make any quantitative analysis of low-abundance proteins/peptides challenging. Furthermore, analyses of peptides, including the cleaved fragments of proteins, are difficult because of carrier protein binding. Here, we developed a differential solubilization (DS) method to extract low-molecular-weight proteins/peptides in serum with good reproducibility and yield as compared to typical peptide-extraction methods such as organic solvent precipitation and ultrafiltration. Using the DS method combined with reverse-phase HPLC fractionation followed by MALDI-TOF-MS, we performed high-quality comparative analyses of more than 1500 peptides from 1 μL of serum samples, including low-abundance peptides in the subnanomolar range and containing many peptides bound to carrier proteins such as albumin. We applied this method and successfully discovered four new biomarker candidates of colon cancer, none of which have previously been observed in serum and one of which is a fragment of the protein zyxin that possibly originated from tumor cells. Our results indicate that serum peptide analyses based on the DS method should greatly contribute to the discovery of novel low-abundance biomarkers.

Keywords: biomarkers • colorectal cancer • mass spectrometry • peptide extraction • peptidomics • plasma • serum

Introduction

Serum/plasma contains thousands of different types of proteins and peptides,[1−5] and can provide valuable information about the numerous processes that take place within the body. Quantitative analysis of the proteins/peptides in serum/plasma samples is expected to reveal biomarkers. Therefore, many researchers have attempted to provide disease-specific biomarkers for the early detection of disease, drug susceptibility, and the evaluation of prognosis.[6−9]

The low-molecular-weight (LMW) proteins/peptides in the serum/plasma include members of several physiologically important classes, such as cytokines, chemokines, and peptide hormones, along with proteolytic fragments of larger proteins,[10,11] including those generated by cancer-specific exopeptidase.[12] The LMW region is of great interest in proteomic studies that aim to identify disease-specific proteins. The most widely used approaches for the LMW serum/plasma proteome employ SELDI-TOF-MS[13−17] and MALDI-TOF-MS analysis coupled with simple preparations using solid-phase extraction methods or functionalized paramagnetic beads.[7,12,18] When these approaches are combined with advanced bioinformatic techniques, many key LMW proteins/peptides with putative molecular masses below 20 kDa are identified, some of which could be used to determine the onset of disease.[7,12−17,19−22] These results suggest that the LMW serum/plasma proteome might contain an unexplored archive of histological information, and are expected to yield useful biomarkers for disease detection. However, these approaches can detect only a small portion of the LMW proteins/peptides in the serum/plasma.

Serum/plasma proteins/peptides are present at concentrations that are likely to extend over 10 orders of magnitude or more. So far, 22 major proteins have been defined, constituting approximately 99% of the total serum/plasma proteins.[4,5] Among the remaining 1% are thousands of low-abundance proteins/peptides that are of great interest for proteomic studies, as they include potential biomarkers. However, high-

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Table 1. Clinical Features of CRC Patients

patient no.	age	gender	AJCC stage
D1	49	Male	IIIb
D2	57	Male	IV
D3	51	Female	IIIa
D4	49	Male	II
D5	56	Female	IIIb
D6	59	Male	IV
D7	66	Female	II
D8	54	Male	IIIa

abundance proteins in serum/plasma, such as albumin, transferrin, haptoglobin, immunoglobulins, and lipoproteins, interfere with the detection of low-abundance proteins/peptides. Therefore, the reduction of sample complexity is essential prior to identifying proteins that are present in small quantities. Depletion of the high-abundance proteins using affinity methods has made it possible to characterize low-abundance serum/plasma proteins and to identify novel biomarker candidates.[23−26] One of the fundamental limitations of the depletion methodologies, however, is that they tend to simultaneously remove many important LMW proteins/peptides. The high-abundance protein albumin is known to act as a carrier protein within the blood, and to bind physiologically important proteins/peptides such as hormones, cytokines, and lipoproteins.[27−29] Moreover, even after depletion by affinity methods, the remaining proteins can hamper the detection of LMW proteins/peptides. Hence, there is a great need for an improved fractionation/depletion method that removes only proteins, and not the LMW proteins/peptides that bind to them.

Various methods can be used to analyze LMW proteins/peptides in serum/plasma, including organic precipitation,[30,31] ultrafiltration,[4,32] nanoporous silica particles,[33] ZnO-poly(methyl methacrylate) nanobeads,[34] hollow-fiber membranes[3] and protein Equalizer technology.[35] Reverse-phase (RP) chromatography and isoelectric focusing have also been combined with these peptide separation methods.[36−39] Furthermore, biomarker discovery has been performed using carrier protein-bound affinity-enrichment technology.[40,41]

Although methods to improve the recovery of LMW proteins/peptides and to give better reproducibility have been developed, they remain unsatisfactory, and it is still difficult to concentrate LMW proteins/peptides including those that bind to carrier proteins.

In the current study, we developed a novel method for extracting and concentrating LMW proteins/peptides from serum efficiently and reproducibly, in which the LMW proteins/peptides were isolated by a "differential solubilization (DS) method" under denatured conditions. We evaluated our method in comparison with albumin/IgG affinity depletion, organic precipitation, and ultrafiltration. We demonstrated that the DS method represents a powerful strategy for the discovery of subnanomolar peptide biomarkers, including those bound to carrier proteins from small serum samples.

Materials and Methods

Human Serum Samples. Blood samples were taken from eight CRC patients (Table 1) and eight healthy volunteers (HVs; four males and four females; age range, 23−56 years) with no known history of CRC. The samples were allowed to clot at room temperature for 1 h, and then centrifuged at 2000*g* for 15 min at room temperature. Serum (supernatant) samples were stored in aliquots at −80 °C. Informed consent was obtained from the participants, and the studies were performed with the approval of the Ethics Committee of Chiba University School of Medicine, Japan.

DS Method. A 10-μL serum sample was diluted 1:2 with 20 μL of denaturating solution (7 M urea, 2 M thiourea, and 20 mM dithiothreitol (DTT)), slowly dropped into 900 μL ice-cold acetone, and immediately stirred at 4 °C for 1 h, followed by centrifugation at 19 000*g* for 15 min at 4 °C. The precipitate was taken up in 200 μL of 70% acetonitrile (ACN) containing 12 mM HCl and mixed at 4 °C for 1 h, then centrifuged again at 19 000*g* for 15 min at 4 °C. The LMW proteins/peptides were extracted into the supernatant, which was then lyophilized and stored at −80 °C until it was analyzed by Tricine-SDS-PAGE and fractionated by RP-HPLC.

Organic Precipitation, Ultrafiltration, and Albumin/IgG Affinity Removal Methods. Organic precipitation was performed as described by Chertov et al.[30] A 10-μL sample of serum was rapidly added to 20 μL of ACN containing 0.1% trifluoroacetic acid (TFA) and immediately mixed by vortexing, then centrifuged at 19 000*g* for 5 min at 4 °C. Subsequently, the supernatant was lyophilized and stored at −80 °C until it was analyzed by Tricine-SDS-PAGE and fractionated by RP-HPLC. The ultrafiltration method was performed as described by Tirumalai et al.[4] The centrifugal filter membranes were rinsed and used according to the manufacturer's specifications. The serum samples were diluted 1:5 with 25 mM NH_4HCO_3, pH 8.2, containing 20% ACN, and run on an ultrafiltration centrifuge (MWCO 30 000; Microcon YM-30, Millipore Corporation, Bradford, MA). Centrifugation was carried out at 3000*g* until more than 90% of the input serum had passed through the membrane. The filtrate was lyophilized and stored at −80 °C until it was analyzed by Tricine-SDS-PAGE and fractionated by RP-HPLC. The albumin/IgG affinity removal method was performed using a ProteoExtract Albumin/IgG removal kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. A 60-μL sample of serum was diluted 1:9 with 540 μL of binding buffer, and was allowed to pass the column by gravity flow. The flow-through fraction was collected in a collection tube. To wash the column, 600 μL of binding buffer was added to the column, and was allowed to pass the column by gravity flow. The flow-through fraction was collected in the same collection tube. The combined collection sample (1200 μL) was stored at −80 °C until it was fractionated by RP-HPLC.

Tricine-SDS-PAGE. The lyophilized LMW proteins/peptides extracted from serum were dissolved in PAGE sample buffer (50 mM Tris-HCl, pH 6.8, containing 50 mM DTT, 0.5% SDS, and 10% glycerol), and incubated at 57 °C for 5 min. Tricine-SDS-PAGE[42] was used to analyze the solution along with crude serum and SPM (Peptide Marker Kit; GE Healthcare, Little Chalfont, U.K.). The gel image was converted to a densitogram by Scion Image (http://www.scioncorp.com/). The intensity of each band was estimated from each peak area on the densitogram.

Fractionation of LMW Proteins/Peptides by RP-HPLC. The lyophilized LMW proteins/peptides extracted from 10 μL of serum by the DS method, organic precipitation, and ultrafiltration were dissolved in 80 μL of 0.1% TFA and fractionated by RP-HPLC. A sample of the solution treated by the albumin/IgG affinity removal method, corresponding to 10 μL of serum, was fractionated by RP-HPLC using an ODS column (2.0 i.d. × 150 mm, Cadenza CD-C18; Imtakt Corp., Kyoto, Japan) attached to an HPLC system (Nanospace SI-2; Shiseido Fine

Chemicals, Tokyo, Japan). The column was maintained at room temperature, and the flow rate of the mobile phase was 100 μL/min. The composition of the mobile phase was programmed to change over 95 min by varying the mixing ratios $r = [B]/([A] + [B]) \times 100$ of solvent A (0.1% TFA) and solvent B (90% ACN in 0.08% TFA) as follows: a constant mixing ratio (r = 2%) was used from time $t = 0-10$ min, followed by a linear gradient ($r = 2-72\%$) from $t = 10-80$ min, a linear gradient ($r = 72-95\%$) from $t = 80$ to 81 min, and a final constant mixing ratio (r = 95%) from $t = 81-95$ min. The fractions were collected every minute from $t = 25-85$ min, yielding 60 fractions per sample. The HPLC fractions were then lyophilized and stored at −80 °C until the MALDI-TOF-MS measurements were taken.

MALDI-TOF-MS Analyses of HPLC Fractions and Discovery of Biomarker Candidates. Each lyophilized sample that was fractionated by RP-HPLC was dissolved in 10 μL of 50% ACN containing 0.1% TFA. A 1 μL portion of each sample, corresponding to the LMW proteins/peptides from 1 μL of serum, was spotted onto a MALDI target followed by 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA; Nacalai Tesque, Inc., Kyoto, Japan) prepared in 50% ACN and 0.1% TFA. The mixture was allowed to air-dry and measured by MALDI-TOF-MS (Voyager-DE Pro; Applied Biosystems, Foster City, CA). The laser power was adjusted to ensure that no peak was saturated. Each spectrum was the result of 200 laser shots, delivered in four sets of 50 shots to each of four different locations on the surface of the matrix spot. The raw spectra were scaled to the same noise level. For discovery of biomarker candidates, we measured 16 samples (8 patients and 8 HVs) of each HPLC fraction on one day and normalized them to the noise level. Then, candidate peptides were assigned by comparison with 16 normalized spectra. To confirm the accuracy of the MALDI-TOF-MS data, we repeated the same measurement twice on separate days.

Statistical Analysis. After the assignment of biomarker candidates, P-values were calculated from the peak intensities of candidate peptides using Student's t test.

Identification by MALDI-TOF/TOF-MS/MS. Peptides selected on the basis of statistical differences in ion intensity between the cancer and control groups were analyzed by MALDI-TOF/TOF-MS/MS, using an UltraFlex II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) operated in the 'LIFT' mode. The monoisotopic masses were initially assigned by one-dimensional reflectron-TOF MS. Spectra were obtained by averaging multiple signals. The laser irradiance and the number of acquisitions (typically 100−150) were operator adjusted to yield maximal peak deflections from the digitizer in real-time. Monoisotopic masses were assigned for all the selected and other prominent peaks noted after visual inspection, with the low-end and high-end internal standards used for recalibration. The pass/fail criterion for recalibration was the correct assignment of an m/z value for the "middle" calibrant with a mass accuracy equal to or better than 12 ppm.

Fragment ion spectra from TOF/TOF analyses were transformed into a peak list using the Flexanalysis software (Bruker Daltonics). The peak filter settings were an S/N threshold of 2 and a peak width of 5 m/z. Peptide identifications were made by database comparisons with a "non-redundant" human database (NCBInr; release date, March 25, 2006; 145 766 entries; National Center for Biotechnology Information, Bethesda, MD) using the MASCOT MS/MS ion search program, version 2.0.04 for Windows (Matrix Science Ltd., London, U.K.), with the following search parameters: a monoisotopic precursor mass tolerance of 40 ppm, a fragment mass tolerance of 0.5 Da, and no specified protease cleavage site.

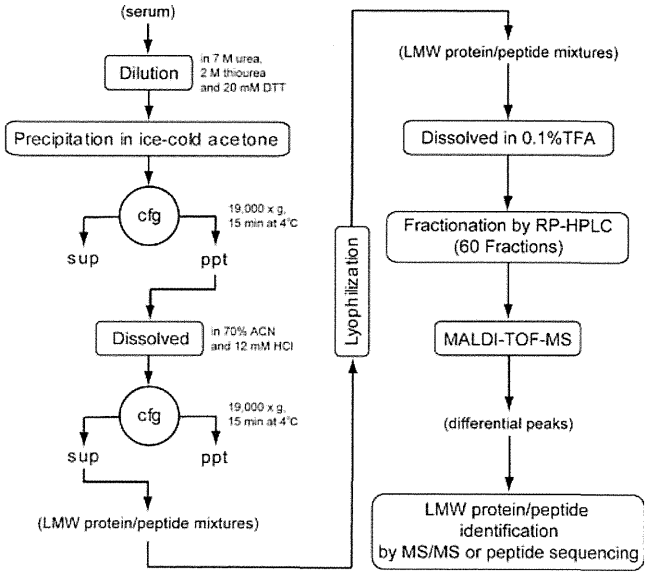

Figure 1. Flowchart for the extraction and quantitative analysis of LMW proteins/peptides in serum or plasma. Abbreviations: cfg, centrifugation; sup, supernatant; ppt, precipitate; DTT, dithiothreitol; ACN, acetonitrile; TFA, trifluoracetic acid.

Synthetic Peptides. Nonlabeled synthetic peptides and the isotope-labeled synthetic peptides were obtained commercially from AnyGen Co., Ltd. (Kwangju, Korea). To prepare the isotope-labeled peptides, four types of ^{13}C,^{15}N uniformly labeled 9-fluorenylmethoxycarbonyl (FMOC) amino acids were used: GLYCINE-N-FMOC ($^{13}C_2$, 98%; ^{15}N, 98%); L-ALANINE-N-FMOC ($^{13}C_3$, 98%; ^{15}N, 98%); L-PHENYLALANINE-N-FMOC ($^{13}C_9$, 98%; ^{15}N, 98%); and L-PROLINE-N-FMOC ($^{13}C_5$, 98%; ^{15}N, 98%). The amino acid sequences of the three peptides were DEAGSEADHEGTHSTKRGHA, VNPFRPGDSEPPPAPGAQRAQ, and SETESRGSESGIFTNTKESSSHHPGIAEFPSRG, with the underlined amino acids synthesized with uniformly labeled FMOC amino acids. The molecular weight increments of these three peptides relative to the nonlabeled peptides were 17, 22, and 16, respectively.

Quantitative Analysis of Candidate Peptides Using Stable Isotope-Labeled Peptides. Serum or plasma was spiked with stable isotope-labeled peptides prior to peptide extraction by the DS method. The concentrations of the spiked stable isotope-labeled peptides with m/z values of 2092, 2188, and 3505 were 25 fmol, 2.5 fmol, and 50 fmol per 1 μL serum or plasma, respectively. The extracted sample was separated into 60 fractions under the conditions used in the discovery protocol. The fractions containing candidate peptides were analyzed by MALDI-TOF-MS. The concentrations of the three peptides in the serum were estimated from the ratios of the peak intensities of the three peptides in the serum to the isotope-labeled peptides.

Results

Extraction and Analysis of LMW Proteins/Peptides. The newly developed DS method is depicted in Figure 1. The most important feature of the DS method is the use of differential solubilization, which concentrates the LMW proteins/peptides in the tissue lysate and gives a much better yield than the

various organic solvent-precipitation methods.[43] The DS method consists of two steps. In the first step, the serum is mixed with a denaturing solution that consists of urea, thiourea, and DTT, and is then dropped into acetone, which causes all of the proteins and peptides to precipitate. In the second step, those LMW proteins/peptides that are easily dissolved in 70% ACN containing 12 mM HCl are separated from most of the other proteins. To evaluate the yield of LMW serum proteins/peptides obtained by the DS method, we used Tricine-SDS-PAGE, and compared the results with those obtained by typical peptide-extraction methods that involve precipitation with organic solvents and ultrafiltration.

When developing a new methodology, it is important to evaluate how well the "finished product" performs. However, the exact content and abundance of each of the LMW proteins/peptides in serum is unknown. Therefore, for the establishment of the DS method, we added a standard peptide mixture (SPM) containing six peptides used as standard PAGE markers (Figure 2AB, lane 1) to the serum prior to peptide extraction as an internal standard, and we evaluated the yield of the LMW proteins/peptides from the rate of recovery of the SPM. This was assessed by Tricine-SDS-PAGE, not by MALDI-TOF-MS, because after lyophilization the extract is easily dissolved in SDS-containing solutions, which permits the extracts to be analyzed quantitatively over a wide range of molecular weights.

In Figure 2AB, lane 2 contained 0.5 μL of untreated human serum; lanes 4, 6, and 8 contained enriched LMW proteins/peptides from 5 μL of human serum; and lanes 3, 5, and 7 contained the same enriched peptides that had been premixed with 4 μg of SPM (an equal amount to lane 1). Comparisons of lanes 1−4 indicated that the SPM peptides in lane 3 had similar intensities to those seen in lane 1. The arrowheads marked a−d in lanes 2−4 indicate the four major LMW proteins/peptides in the serum that were extracted with high yield by the DS method. When the organic-precipitation method was used (Figure 2AB, lanes 5 and 6), most of the SPM peptides and the four major peptides indicated by arrowheads a−d were lost, although half of the 6.2 kDa SPM and small amounts of the 2.5 SPM peptides along with peptide c were observed. Only a small fraction of the 2.5 kDa SPM peptides was recovered by the ultrafiltration method (Figure 2AB, lanes 7 and 8); none of the other peptides in the SPM and serum were detected by Coomassie brilliant blue (CBB) staining (lanes 7 and 8), even though the molecular weight cutoff was 30 kDa. These results indicate that the yield of LMW proteins/peptides obtained by the DS method was much higher than that obtained by the other methods. We evaluated the reproducibility of the DS method for extracting LMW proteins/peptides from serum by Tricine-SDS-PAGE, as shown in Figure 3. The intensities of each of the protein and peptide bands were identical in all of the lanes, confirming that major proteins/peptides were extracted reproducibly by the DS method. This reproducible extraction can be run concurrently for a maximum of 16 serum samples.

We identified major proteins and LMW proteins/peptides in the extracted serum by the DS method (Supplementary Figure S1 and Supplementary Table S1) by LC-ESI-IT-MS/MS. LMW proteins/peptides in bands 3−11 from untreated serum were concentrated and identified in bands 20−23 and 27−31 in extracted serum. This result suggested that LMW proteins/peptides in the serum were extracted with high yield by the DS method.

When establishing the DS method, two crucial points were carefully assessed. In the first step, the ratio of the volume of acetone to that of the sample was important. The recovery rate of the 2.5 kDa peptide of SPM in this step was analyzed using RP-HPLC, and was confirmed to exceed 90% (Supplementary Figure S2). In the second step, the type and concentration of the organic solvent used, and its pH, were important. We were concerned about the possible cleavage of peptides and proteins resulting from the use of an acidic solvent. However, one of the typical peptide-extraction methods used for discovering peptide hormones in tissue employs hydrochloric acid at a 30-fold higher concentration than that used in the DS method.[44,45] Furthermore, the extraction of SPM was not affected by hydrochloric acid concentrations between 12 and 120 mM. Therefore, we judged the possibility of artificial fragmentation during the DS method to be extremely low. In fact, no fragments of high-abundance proteins such as albumin, IgG, and transferrin were observed in the LMW proteins/peptides extracted by the DS method (Supplementary Figure S1 and Supplementary Table S1). Furthermore, most of the bands of the LMW proteins and protein fragments in the treated sample (bands 20−23 and 27−31) corresponded to those in the untreated serum (bands 3−11) as follows: band 3 versus band 20; band 4 versus band 21; band 5 versus bands 22 and 23; band 6 versus band 23; band 7 versus band 27; band 8 versus band 28; band 9 versus band 29; band 10 versus band 30; and band 11 versus band 31.

The RP-HPLC elution profiles of LMW proteins/peptides enriched by the DS method, the organic-precipitation method, ultrafiltration, and the albumin/IgG affinity removal method from 10 μL of human serum in each case are shown in Figure 4A. The fractions of the enriched serum were collected once every minute from 25 to 85 min, resulting in a total of 60 fractions per sample. Examples of the MALDI-TOF-MS spectra from four fractions (4, 16, 22, and 33) are shown in Figure 4B. For retention times between 25 and 35 min, several sharp chromatographic peaks were detected in all of the RP-HPLC elution profiles, and a broad peak was observed in the treated serum by the ultrafiltration method. However, only a few small peaks were observed by MALDI-TOF-MS in this region, as shown in Figure 4B (a). It is possible that these sharp and broad peaks in the RP-HPLC elution profiles were induced by LMW components with an m/z of less than 1000, or by those not ionized by MALDI-TOF-MS. Figure 4B (b−d) shows three typical features of comparative analyses of MS spectra. First, major peaks were observed in serum samples enriched by the DS method and the albumin/IgG affinity removal method, which coincided with each other, and small peaks were also observed in the samples prepared by the organic-precipitation method (Figure 4B (b)). Second, intense peaks were observed in the samples enriched by the DS method, some of which were also observed in the samples prepared by the albumin/IgG removal method (Figure 4B (c)). Third, some intense peaks were observed in the samples enriched by the DS method, but not in the other samples (Figure 4B (d)). The first feature was mainly observed in fractions with numbers below 15. The second and third features were mainly observed in fractions with numbers above 20. These results indicate that many LMW proteins/peptides are specifically extracted by the DS method in addition to those observed in the albumin/IgG affinity removal serum, which suggests that the DS method makes it possible to extract LMW proteins/peptides that interact with albumin/IgG hydrophobically.

High reproducibility of enrichment and fractionation of the LMW proteins/peptides is essential for the discovery of poten-

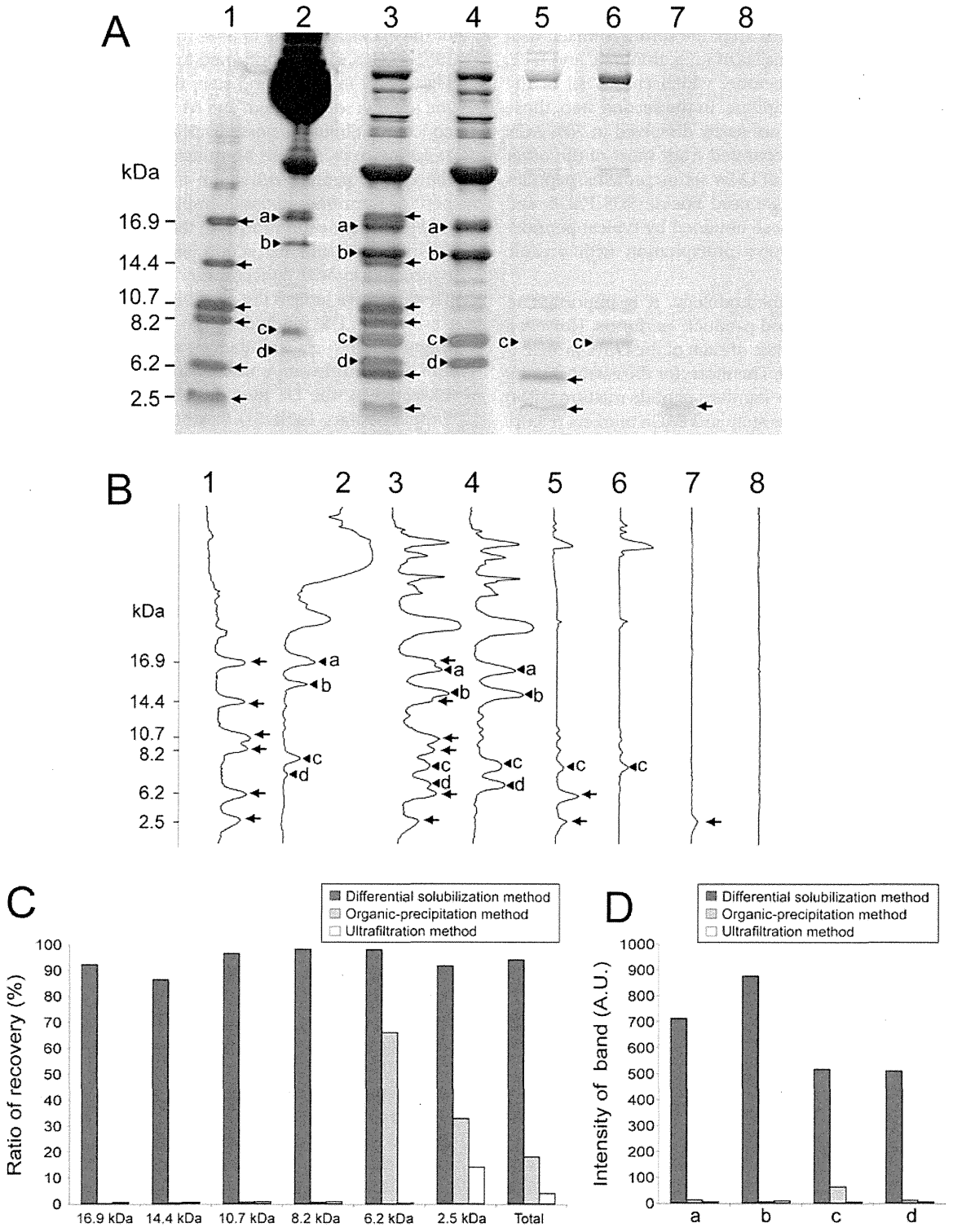

Figure 2. Assessment of the yield of LMW protein/peptide extraction in serum by the DS method. (A) Tricine-SDS-PAGE patterns of LMW proteins/peptides in human serum enriched by three types of peptide-extraction method. Lane 1: SPM 4 μg. Lane 2: untreated human serum 0.5 μL. Lanes 3 and 4: LMW proteins/peptides enriched by the DS method from 5 μL of human serum including 4 μg of SPM and 5 μL of human serum, respectively. Lanes 5 and 6: LMW proteins/peptides enriched by the organic-precipitation method from 5 μL of human serum including 4 μg of SPM and 5 μL of human serum. Lanes 7 and 8: LMW proteins/peptides enriched by the ultrafiltration method from 5 μL of human serum including 4 μg of SPM and 5 μL of human serum, respectively. Lanes 1–8 were stained with CBB. Arrows and arrowheads (a–d) indicate LMW proteins/peptides of SPM and high-abundance ones in serum, respectively. (B) Densitometric analyses of the gel shown in panel A. Lanes 1–8 in panel B correspond to lanes 1–8 in panel A. (C) Recovery rate of SPM treated by three types of peptide-extraction method. The recovery rates of LMW proteins/peptides of SPM were estimated from the densitometric intensities ratio of each LMW proteins/peptides peak of SPM treated by three types of peptide-extraction method (lanes 3, 5, and 7 in panel B) to that of nontreated SPM peak (lane 1 in panel B). (D) Densitometric intensities of high-abundance LMW proteins/peptides peaks (arrowheads (a–d) in panel B) of samples treated by three types of peptide-extraction method (lanes 4, 6, and 8 in panel B).

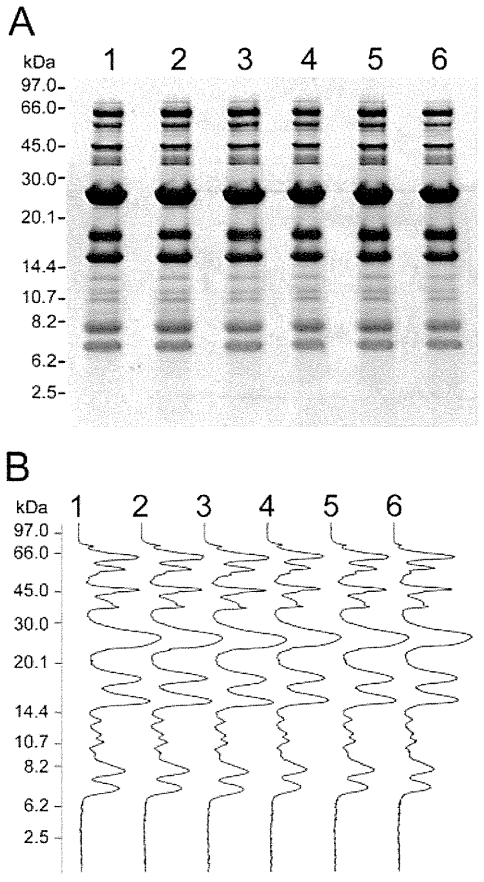

Figure 3. Reproducibility of the enrichment of human serum LMW proteins/peptides by the DS method. Lanes 1−6 contain CBB-stained Tricine-SDS-PAGE profiles of LMW proteins/peptides enriched by the DS method from six portions of serum from an HV (10 μL each). (B) Densitometric analyses of the gel shown in panel A. Lanes 1−6 in panel B correspond to lanes 1−6 in panel A.

tial biomarkers. LMW proteins/peptides were extracted from six portions of the same serum sample and fractionated by HPLC followed by MALDI-TOF-MS analysis (Figure 5). All HPLC profiles and MS spectra are highly coincident. These results indicate that the reproducibilities of the peptide extraction and RP-HPLC fractionation are very high. Figure 6 presents a summary of the MALDI-TOF-MS spectra of the peptides from the serum samples of four HVs found in fractions 21−30 of the RP-HPLC separation. The average number of peaks with different m/z values detected in these fractions in the four serum samples was 320. The four serum samples had 232 peaks in common, and more than 90% (294 peaks) were commonly observed in at least three of the four HVs. These results indicate that the individual peptide content was not higher than expected if we used a highly reproducible method. The total number of peaks with different m/z values was more than 1500 when all of the HPLC fractions in the four serum samples were analyzed by MALDI-TOF-MS.

Discovery and Identification of CRC Biomarker Candidates. We applied our analytical method to serum samples from CRC patients and searched for potential tumor markers. Figure 7A (a−c) shows parts of the MALDI-TOF-MS spectra of fractions 6, 15, and 18 of the RP-HPLC fractions from eight CRC patients (black) and eight HVs (gray). Figure 7B (a−c) shows the average of the eight spectra corresponding to Figure 7A (a−c), respectively. Four peptide peaks were detected only in the serum samples of the CRC patients, with m/z values of 2092 and 2163 in fraction 6; 2188 in fraction 15; and 3505 in fraction 18; and with Student's t-test P-values of 0.0013, 0.0021, 0.00055, and 0.0047, respectively. These four peptides were considered potential markers of CRC.

The four candidate peptides were identified by MALDI-TOF/TOF-MS/MS followed by a Mascot database search (Figure 8A). The peptide with an m/z of 2163 in fraction 6 was a fragment of the fibrinogen α chain, with the amino acid sequence ^{604}ADEAGSEADHEGTHSTKRGHA^{624} (Figure 8A (a)). The peptide with an m/z of 2092 in fraction 6 corresponded to the same region as the peptide with an m/z of 2163, but without the N-terminal alanine (Figure 8A (b)). The peptide with an m/z of 3505 in fraction 18 was also a fragment of the fibrinogen α chain, but its sequence was ^{542}SETESRGSESGIFTNTKESSSHHPGIAEFPSR^{574} (Figure 8A (c)). The peptide with an m/z of 2188 in fraction 15 was a fragment of zyxin with the sequence ^{36}VNPFRPGDSEPPPAPGAQRAQ^{56} (Figure 8A (d)). The Mascot scores for the peptides with m/z values of 2163, 2092, 3505, and 2188 were 106, 126, 162, and 92, respectively. To confirm the identified peptides, we prepared synthetic peptides with the identified sequences and analyzed them by MALDI-TOF/TOF-MS/MS. As expected, the MS/MS spectra of the synthetic peptides corresponded well to the CRC biomarker candidates in serum (Figure 8B (a−c)).

The relationship between the four candidate peptides and CRC has not been previously reported. Fibrinogen α is a major serum protein, and several of its fragments have been reported to be potential biomarkers for various diseases. The three fibrinogen α fragments that we discovered differed from the previously reported biomarkers,[7,12,13,21,46] and might therefore be candidates for CRC-specific biomarkers. The zyxin fragment has been observed in the plasma of HVs[36] but not in serum. The level of zyxin in hepatocellular carcinomas and also CRC has been reported to be up-regulated more than 2-fold compared with that in normal tissues.[47,48] Furthermore, a report by Lopez et al. described zyxin or its fragment as a serum carrier protein-bound peptide related to ovarian cancer.[40] These results suggest that the zyxin in serum might have originated from tumor tissue and that the peptide detected here is possibly a cleaved fragment by a CRC-specific protease.[12]

Discussion

We developed the DS method for extracting LMW proteins/peptides from serum samples with high yield and high reproducibility. RP-HPLC separation combined with the DS method made it possible to analyze quantitatively more than 1500 LMW proteins/peptides from 1 μL of serum using MALDI-TOF-MS with high reproducibility. The yield of serum LMW proteins/peptides was much higher than that achieved with other widely used organic-precipitation methods and the ultrafiltration method, and many LMW proteins/peptides that were not extracted by the albumin/IgG affinity removal method were extracted by the DS method and observed by MALDI-TOF-MS (Figure 4B).

We applied this strategy for comparative analyses of sera from HVs and CRC patients, and successfully detected four CRC-related peptides, none of which have been previously reported. Furthermore, these peptides could be quantitatively evaluated using stable isotope (^{13}C and ^{15}N)-labeled peptides, which were added to serum prior to peptide extraction as an internal standard through the entire process. Figure 9 (a−c) shows the results for

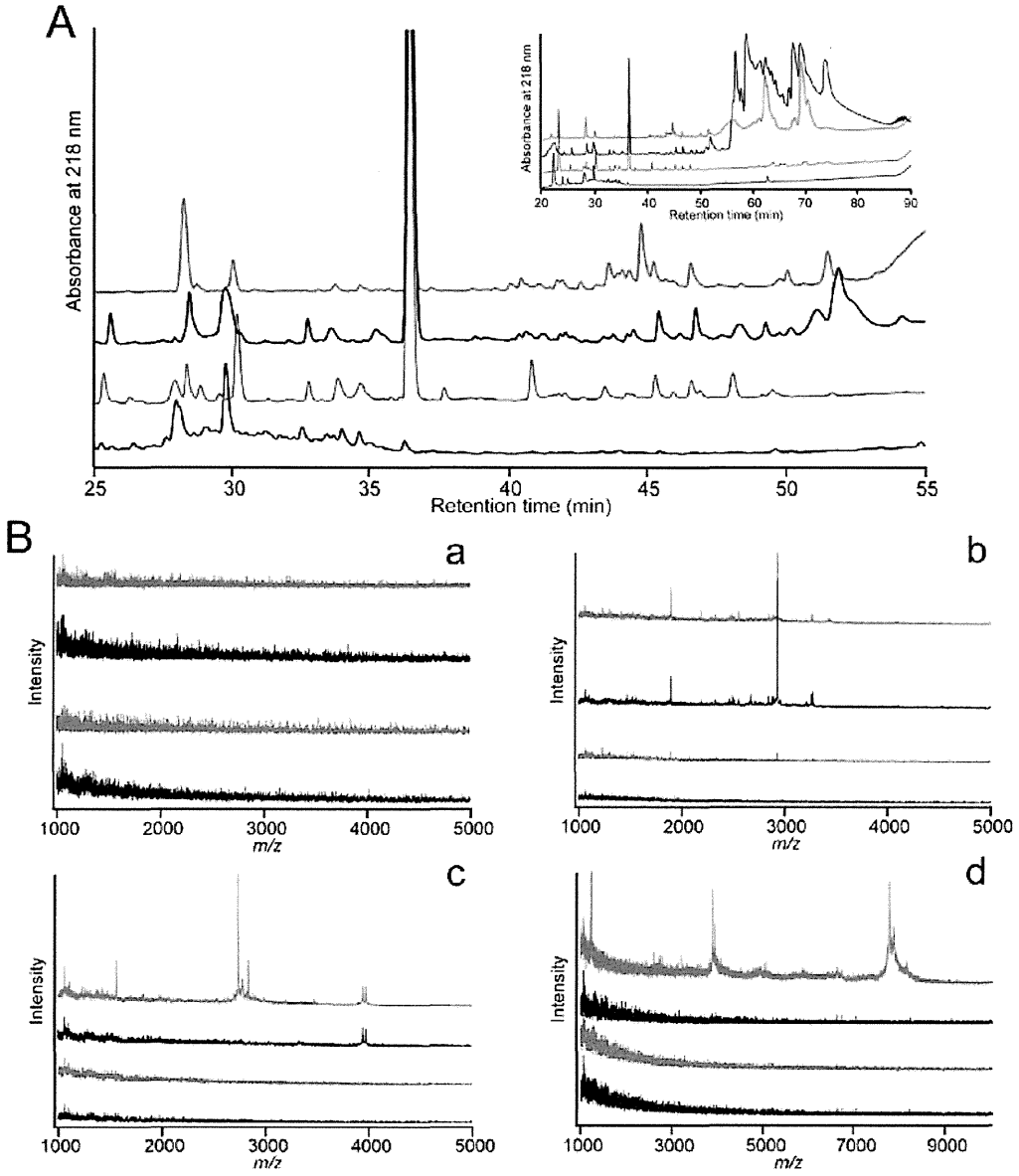

Figure 4. Analysis of LMW proteins/peptides by RP-HPLC and MALDI-TOF-MS. (A) RP-HPLC elution profiles of LMW proteins/peptides extracted by the DS method (red line), the albumin/IgG affinity removal method (black line), the organic-precipitation method (green line), and the ultrafiltration method (blue line) from 10 μL of human serum in each case. (B) MALDI-TOF-MS spectra of LMW proteins/peptides fractionated by RP-HPLC. Spectra (a), (b), (c), and (d) correspond to those of fractions 4 (28−29 min), 16 (40−41 min), 22 (46−47 min), and 33 (57−58 min), respectively. The colors of the spectra correspond to the colors of the RP-HPLC elution profiles.

patient serum. The ratios of the peak intensities of the three peptides in the serum and the isotope-labeled peptides were about 1.2, 0.8, and 0.7, and the concentrations of the three peptides in the serum were estimated to be approximately 32, 2, and 37 fmol/μL (67, 4.5, and 129 ng/mL) using calibration curves (Supplementary Figure S3), respectively.

In our standard protocol, the LMW proteins/peptides were extracted from 10 μL of serum using the DS method and then separated by RP-HPLC. A total of $^1/_{10}$ of each fraction, which corresponded to the LMW proteins/peptides in 1 μL of serum, was used for the MALDI-TOF-MS analysis. The least abundant peptide from among the CRC candidate markers was a fragment of zyxin, the concentration of which was estimated to be on the order of nanograms per milliliter (ng/mL) (Figure 9). This result suggested that the lowest limit of quantitative analysis using our method was in the subnanomolar range from 1 μL of serum samples. As the lowest peptide concentration is known to be 1 pg/mL,[5,49] our method might be able to detect most of the peptides from a 1 mL serum sample using an appropriate separation technique.

By contrast, the dynamic range of MALDI-TOF-MS is on the order of 10^3. This suggests that the detection limit depends on the presence of high-abundance peptides with high ionization efficiencies in each RP-HPLC fraction. The ability to detect low-abundance peptides thus differs in each HPLC fraction. We were able to detect the fragment of zyxin as one of the CRC marker candidates probably because high-abundance LMW proteins/peptides with high ionization efficiencies were absent from the fraction. It might therefore be possible to detect other low-abundance candidate marker peptides if we can further fractionate the LMW proteins/peptides extracted by the DS method using alternative separation methods.

The organic-precipitation method, as shown in lanes 5 and 6 of Figure 2AB, uses final concentrations of ACN and TFA of

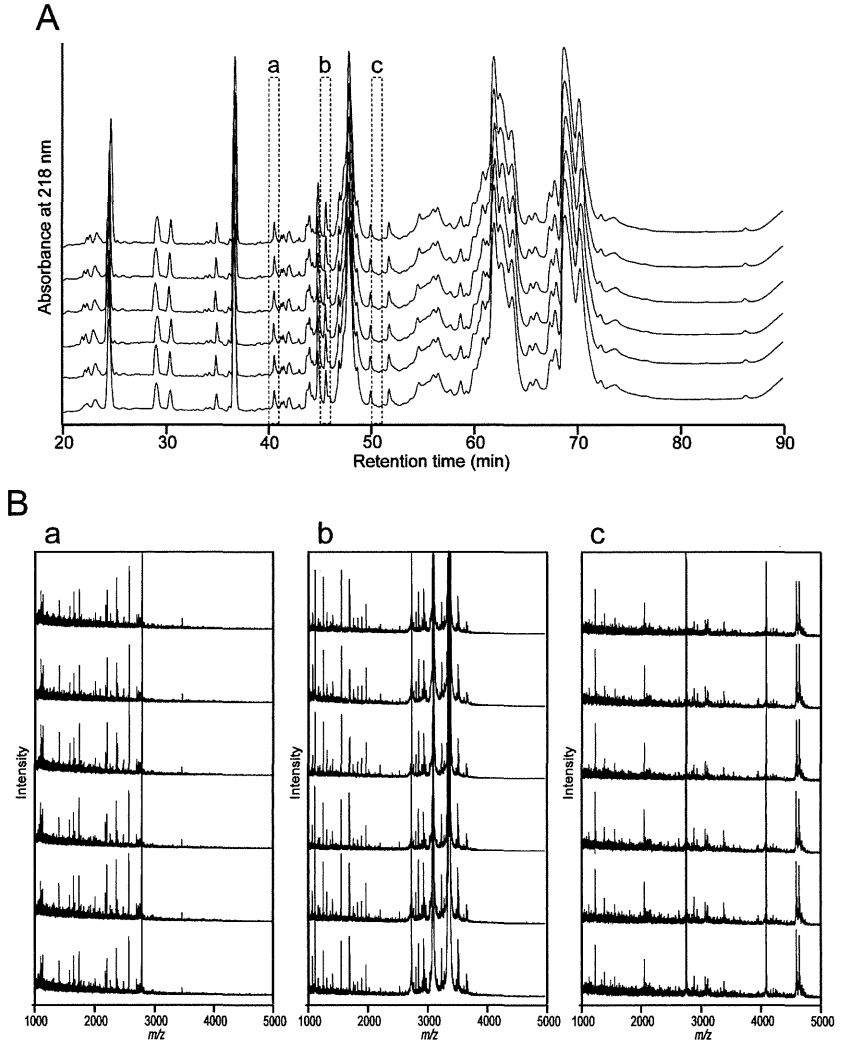

Figure 5. Reproducibility of serum LMW proteins/peptides analyses based on the DS method. (A) RP-HPLC elution profiles of LMW proteins/peptides extracted by the DS method from six portions of serum from a HV (10 μL each). (B) MALDI-TOF-MS spectra of LMW proteins/peptides fractionated by RP-HPLC (A). (a), (b), and (c) show spectra of elutions at retention times a, b, and c, respectively. One-tenth of the LMW proteins/peptides of each fraction (corresponding to LMW proteins/peptides in 1 μL of serum) were analyzed. The six spectra in (a), (b), and (c) were obtained from the six samples in panel A.

66.7% and 0.067%, respectively. Although the concentrations and acidity were similar to those in the DS method, the yield was completely different. Although it was difficult to establish the reason for this, it is possible that the separation of the LMW proteins/peptides from the major proteins and their subsequent solubility were due to their complete denaturation in the serum, which could have occurred in the 7 M urea, 2 M thiourea, and 20 mM DTT solutions prior to acetone precipitation and dehydration by pure acetone. A further advantage of the DS method is the constancy of yield, which is independent of the composition of the proteins/peptides in the sample. In contrast to the DS method, the composition and yield of the LMW proteins/peptides extracted by the organic-precipitation method would be easily affected by coaggregation with proteins. For example, the intensities of the bands corresponding to peptide c and the high-molecular-weight proteins differed in lanes 5 and 6 of Figure 2AB, indicating that the addition of SPM affected the yield of the proteins/peptides upon extraction. The peptides extracted might depend on the abundance of major proteins such as albumin and IgG.

The most important aspects for the discovery of potential biomarkers are the sensitivity, reproducibility, and comprehensibility of the quantitative analysis. Only a few hundred LMW proteins/peptides are observed by the Protein Chip system, even though this method has high throughput. Samples prepared on the surface of Protein Chips include many more proteins/peptides, most of which are not ionized by MALDI, and relatively high-abundance peptides with high ionization efficiencies can be detected. By contrast, comprehensive analyses of peptides aiming to discover biomarkers have used ultrafiltration followed by HPLC fraction techniques. In these studies, however, the small yield of peptides has made it necessary for large serum/plasma samples of more than a few hundred microliters to be employed in order to detect more than 1000 peptides, and they also require complicated methods to separate peptides using micro or capillary HPLC in order to concentrate small amounts of LMW proteins/peptides, which make it difficult to fractionate peptides stably over a long period of time. In contrast to these comprehensive peptidome analyses, our current strategy based on the high yield DS method of extraction allowed us to analyze quantitatively low-abundance peptides using conventional HPLC with a flow rate of 100 μL/min (Figure 4). This made it possible to fractionate LMW

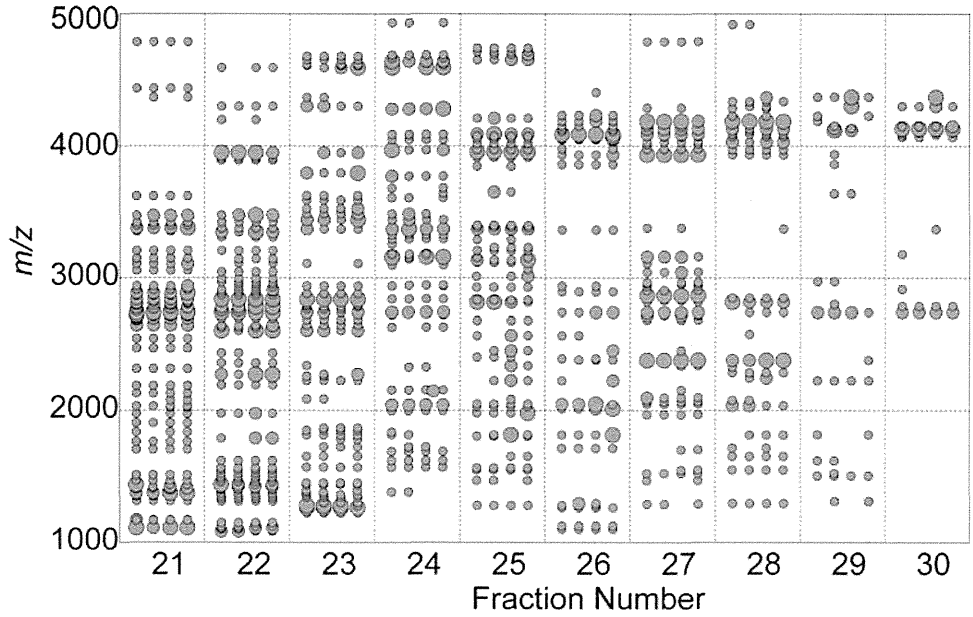

Figure 6. Two-dimensional serum LMW proteins/peptide map of four sera from HVs. Vertical axis: *m/z*. Horizontal axis: RP-HPLC fraction number. Circle size: peak height in MALDI-TOF-MS spectrum of each peptide (small size, S/N = 3−5; medium size, S/N = 5−8; and large size, S/N > 8). In order to show the map for all four HVs, the horizontal axis has been slightly shifted.

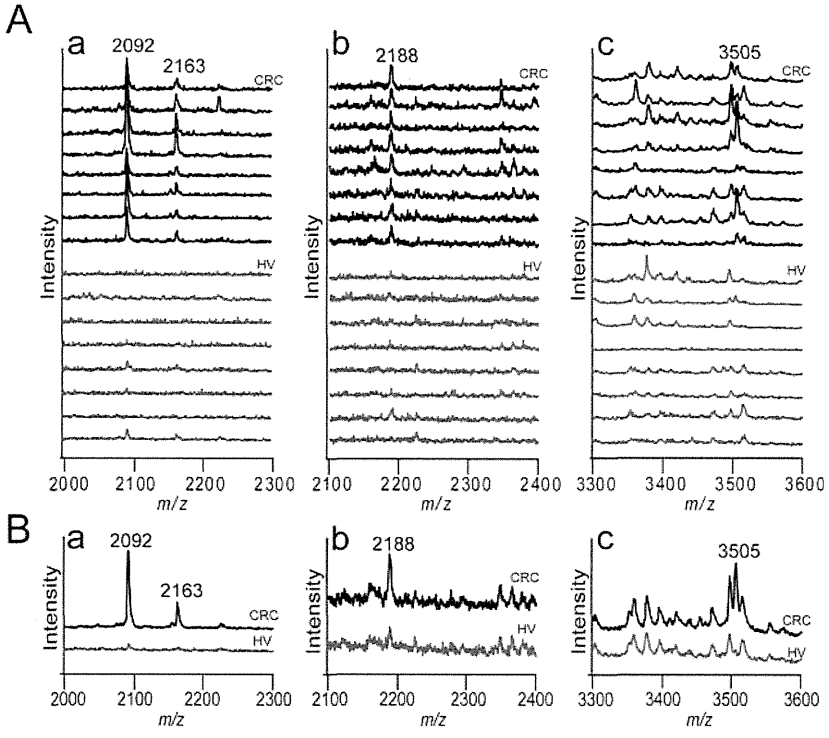

Figure 7. Detection of four biomarker candidate peptides of CRC. (A) MALDI-TOF-MS spectra of eight CRC patients (black) and eight HVs (gray) observed in fractions 6 (a), 15 (b), and 18 (c). (B) Parts (a−c) show the average spectra for the eight CRC patients (black) and eight HVs (gray) in panel A (a−c), respectively.

proteins/peptides in more than 10 samples with highly reproducible elution profiles, which enabled us to compare LMW proteins/peptides by MALDI-TOF-MS with high accuracy, as shown in Figure 6. This high-quality analysis was also supported by the results shown in Figure 7B, in which the average spectra of CRC patients and HVs were highly coincident with each other except for the peaks of candidate peptides.

One of the major peptides in serum, indicated by arrowhead d in Figure 2AB, proved to be a fragment of apolipoprotein C-1, which is thought to bind to albumin, and was not extracted by organic precipitation or ultrafiltration (Figure 2AB, lanes 5−8), but was extracted with high yield by the DS method. This indicates that the LMW proteins/peptides extracted by the DS method might include those bound to carrier proteins, such as albumin, which are difficult to extract using other methods. This notion was strongly supported by three findings. First, many peptides extracted by the DS method were not observed in treated serum using the albumin/IgG affinity removal

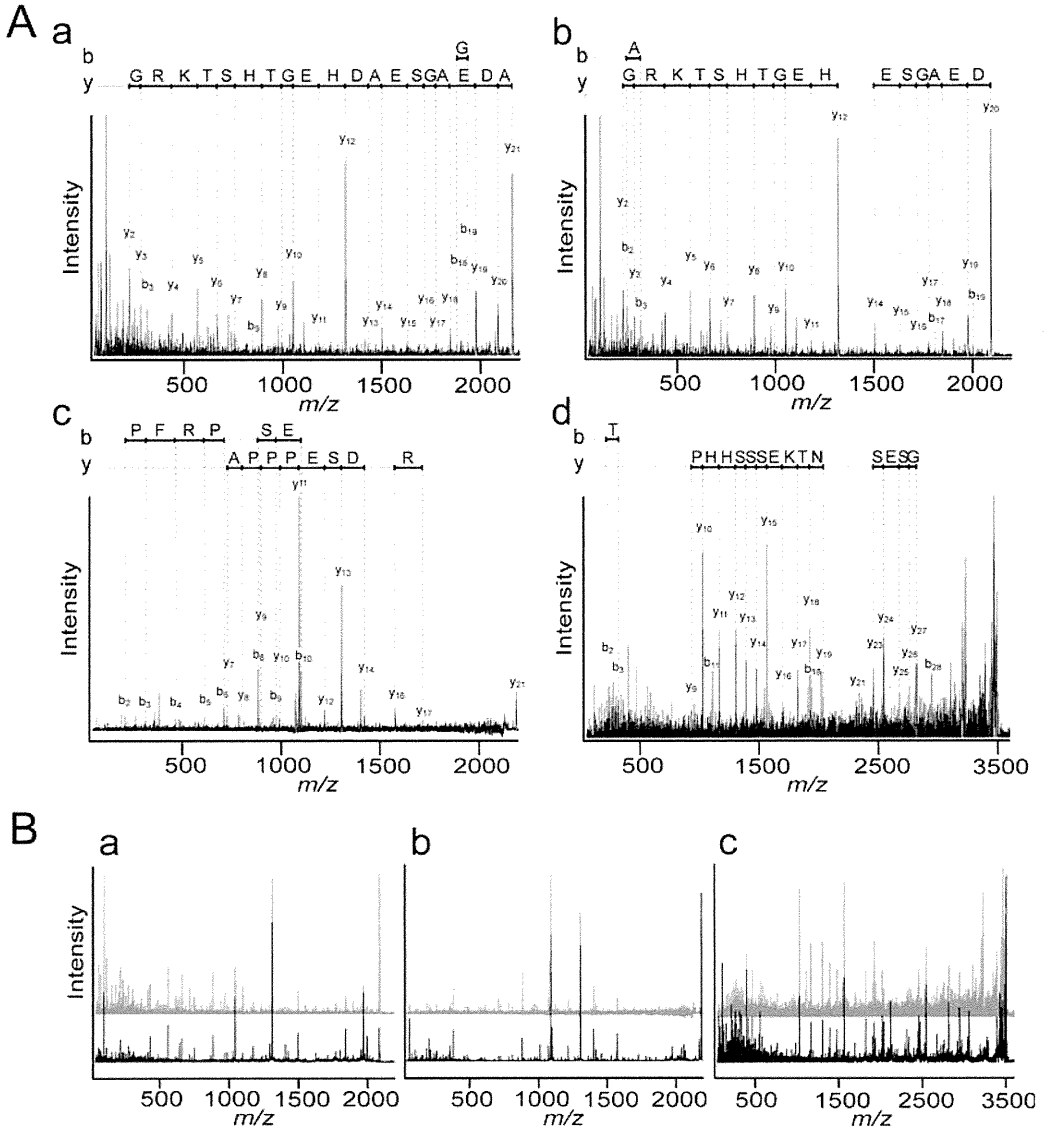

Figure 8. Identification of CRC candidate biomarkers. (A) MS/MS spectra with the following *m/z* sequence assignments: 2163 (a) and 2092 (b) in fraction 6, 2188 in fraction 15 (c), and 3505 in fraction 18 (d). MS was used to analyze the four CRC biomarker candidates along with the ion of choice that was selected for the MS/MS analysis. The fragment ion spectra shown here were taken from a Mascot MS/MS ion search of the human segment of the NR database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein) and retrieved sequences. (B) Correspondence of MS/MS spectra with *m/z* values of 2092 (a), 2188 (b), and 3505 (c) between the CRC biomarker candidates in serum (gray) and synthetic peptides (black).

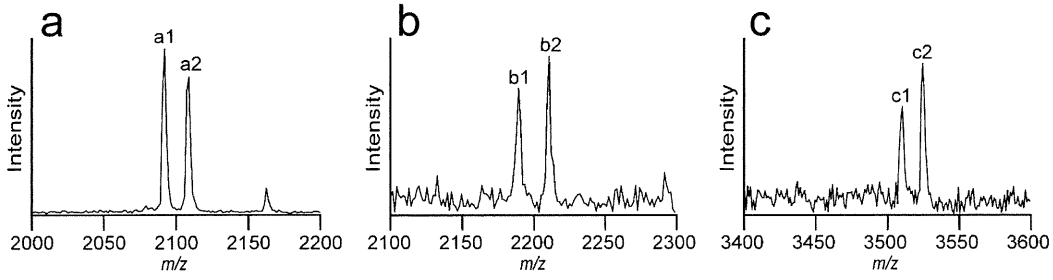

Figure 9. Quantitative analysis of the abundance of the three candidate peptides using stable isotope-labeled peptides with *m/z* values of 2092 (a), 2188 (b), and 3505 (c). The MS peaks a1, b1, and c1 correspond to the peptides in the serum of a CRC patient, and the MS peaks a2, b2, and c2 correspond to the stable isotope-labeled peptides that were spiked to the serum prior to peptide extraction by the DS method. The concentrations of the spiked stable isotope-labeled peptides a2, b2, and c2 were 25 fmol, 2.5 fmol, and 50 fmol per 1 μL of serum, respectively.

method or the other methods (Figure 4B (b–d)). Second, the DS method made it possible to extract many types of apolipoprotein that are thought to bind to albumin (Supplementary Figure S1 and Supplementary Table S1). Third, zyxin or its fragment has been detected from among the proteins collected by carrier protein-bound affinity enrichment.[40]

For clinical use, the DS method is low cost and it is possible to increase the throughput via the optimization of incubation times and by using an automated robot for simultaneous treatment. In addition, the samples treated by the DS method are directly loaded onto an RP-HPLC column. Therefore, the DS method might be suitable not only for biomarker discovery, but also for validation studies of biomarker candidates using high-throughput SRM measurement instead of MALDI-TOF-MS analysis combined with RP-HPLC fractionation.

Conclusions

Technologies of proteome analysis and performance of mass spectroscopy have steadily progressed. However, detailed analysis of LMW proteins/peptides is still challenging because of carrier protein binding and the presence of many high-abundance proteins. The newly developed DS method is a high yielding and highly reproducible extraction method for LMW proteins/peptides in serum, including those bound to carrier proteins. Therefore, this method has the potential to play a prominent role in the analysis of the deepest peptidome zone (less than 1 ng/mL) and should be expected to reveal novel biomarker peptides if used as the standard LMW proteins/peptides extracting method for serum.

Abbreviations: CBB, Coomassie brilliant blue; CHCA, α-cyano-4-hydroxycinnamic acid; CRC, colorectal cancer; DS, differential solubilization; FMOC, 9-fluorenylmethoxycarbonyl; HV, healthy volunteer; LMW, low-molecular-weight; RP, reverse phase; SPM, standard peptide mixture.

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Supporting Information Available: Supplementary Methods; in-gel digestion and MS, Supplementary Figure S1; Tricine-SDS-PAGE patterns of untreated human serum and LMW protein/peptide-enriched serum by the DS method, Supplementary Figure S2; evaluation of extraction yields of premixed 2.5 kDa peptide in SPM by the DS method analyzed by RP-HPLC, Supplementary Figure S3; calibration curves for quantitative analyses of three candidate peptides using stable isotope-labeled peptides, Supplementary Table S1; identification of untreated human serum and LMW proteins/peptides enriched serum by the DS-method. This material is available free of charge via the Internet at http://pubs.acs.org.

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