Optimized Proteomic Analysis on Gels of Cell−Cell Adhering Junctional Membrane Proteins[†]

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ABSTRACT: A high level of structural organization of functional membrane domains in very narrow regions of a plasma membrane is crucial for the functions of plasma membranes and various other cellular functions. Conventional proteomic analyses are based on total soluble cellular proteins. Thus, because of insolubility problems, they have major drawbacks for use in analyses of low-abundance proteins enriched in very limited and specific areas of cells, as well as in analyses of the membrane proteins in two-dimensional gels. We optimized proteomic analyses of cell−cell adhering junctional membrane proteins on gels. First, we increased the purity of cell−cell junctions, which are very limited and specific areas for cell−cell adhesion, from hepatic bile canaliculi. We then enriched junctional membrane proteins via a guanidine treatment; these became selectively detectable on two- dimensionally electrophoresed gels after treatment with an extremely high concentration of NP-40. The framework of major junctional integral membrane proteins was shown on gels. These included six novel junctional membrane proteins of type I, type II, and tetraspanin, which were identified by mass spectrometry and by a database sequence homology search, as well as 12 previously identified junctional membrane proteins, such as cadherins and claudins.

For analyses of functional biosystems, it is important to systematically identify the constituents of specific membrane domains. A potent method for such analyses is the proteomic approach using two-dimensional gels. However, mainly because of the low content and insolubility of membrane proteins, especially when they are integrated into highly structurally organized domains (*1*−*3*), they are not well separated by two-dimensional gel electrophoresis. However, the two-dimensional gel electrophoresis, itself, has superior protein separation ability and is advantageous for presenting the major composition framework of proteins (*4*). Thus, though they show promise, proteomic approaches using gels are not so well-established for comparative analyses of specific functional domain, constitutive membrane proteins.

In epithelial cells, over a very limited area of the plasma membrane, cadherin-based cell−cell adhesion is highly organized into beltlike cell−cell adherens junctions (zonula adherens). These, in combination with claudin-based tight junctions (zonula occludens), form beltlike junctional complexes (*5, 6*). We previously developed a method for the purification of the cell−cell junctional fraction that is based on the bile canaliculi fraction prepared from the liver (*7*). Using this junctional fraction with rat, mouse, and chick origins as antigens for mAb and/or pAb production, we identified novel junctional membrane proteins such as occludin and claudins (*5*) and peripherally associated junctional proteins such as α-catenin, tenuin, and ZO-1 (*8, 9*). Along with the constituents mentioned above, the main general components of adherens and tight junctions have been detected by immunoblotting on one-dimensional gels for enrichment in a junctional fraction of hepatocyte origin, such as membrane integral proteins (E-cadherin, nectins, JAM-1, etc.) and peripheral components (cingulin, β-catenin, etc.) (*10*−*12*). Thus, it was judged that the junctional fraction was a superior source for analyses of junctional components.

However, further immunological analyses of novel constituents of the junctional fraction have been hindered by antigenic problems. These are of particular concern for junctional integral membrane proteins. Two-dimensional analysis combined with mass spectrometry is a potent method for completely and systematically analyzing the major framework of junctional components in a semiquantitative way on gels. However, most known junctional integral membrane proteins, such as E- or N-cadherins, nectins, JAMs, and claudins, cannot be detected on conventional two-dimensionally electrophoresed gels. In this study, we developed a method that could detect highly enriched junctional integral membrane proteins, known or novel, as spots on two-

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dimensional gels. Treatment of the junctional fraction with methyl-β-cyclodextrin (CD), combined with guanidine treatment, was especially useful for obtaining a highly enriched junctional fraction (JF) and then for obtaining a highly enriched junctional integral membrane protein fraction (JMP-F).

We found that the treatment of JMP-F with an extremely high concentration of NP-40 enabled junctional membrane protein (JMP) spots to be detectable on gels. In this way, as many as ~70% of the spots that were previously undetectable became detectable after 10% NP-40 treatment and corresponded to integral membrane proteins. Mass spectrometric identification of JMP spots, selectively detectable by NP-40 treatment, led us to the identification of novel JMPs of types I, II, and tetraspanin. Furthermore, database analyses of these novel JMPs have added other novel JMPs of similar types. Thus, this study presents the framework of major adhering junction constitutive integral membrane proteins that were successfully surveyed on gels. This makes available a simple method for comparative studies of integral membrane proteins.

EXPERIMENTAL PROCEDURES

Preparation of Highly Enriched Junctional Fraction (JF). Junctional fraction was purified from ICR wild-type mouse livers as previously described (*7*). Mouse livers were minced with razor blades, incubated in hypotonic buffer (1 mM $NaHCO_3$ and 2 μg/mL leupeptin) for 30 min, and homogenized with a loose-fitting homogenizer. The homogenate was adjusted to a concentration of 48.45% (w/v) sucrose by adding a 55% sucrose solution. The gradients comprised a 48.45% sucrose layer containing the sample and a 42.9% sucrose layer. After centrifugation for 60 min at 100000*g*, the bile canaliculi were recovered at the 42.9%−48.45% interface.

The bile canaliculi fraction was treated with NP-40 solution [100 mM KCl, 1 mM $MgCl_2$, 0.1% (v/v) NP-40, and 10 mM Hepes (pH 7.5)]. The sample was fractionated by centrifugation at 100000*g* for 60 min on a discontinuous gradient consisting of 20% (w/v) and 50% sucrose. Junctional fraction was recovered at the 20%−50% interface.

To increase the purity of the junctional fraction, it was again separated by centrifugation at 100000*g* for 60 min on stepwise sucrose gradients consisting of 60, 55, 50, 40, and 30% sucrose solutions (w/v). The fraction recovered at the 40%−50% interface was collected, diluted in 4 volumes of the cholesterol depletion buffer [10 mM methyl-β-cyclodextrin (CD) and 10 mM Hepes buffer (pH 7.5)] (*13*), and incubated on ice for 20 min. The resulting CD-treated junctional fraction was then centrifuged through a discontinuous gradient consisting of 60, 55, 50, 45, and 40% sucrose solutions (w/v) at 100000*g* for 60 min. The CD-treated junctional fraction was recovered at the 50%−55% interface, as the highly enriched junctional fraction (JF).

Preparation of Highly Enriched Junctional Integral Membrane Protein Fractions (JMP-F). For the preparation of the highly enriched junctional integral membrane protein fractions (JMP-F), JF was diluted in 4 volumes of dilution buffer [10 mM Tris (pH 7.5)] and centrifuged at 15000*g* for 10 min. The pellet was suspended in the guanidine solution [6 M guanidine-HCl and 10 mM Tris-HCl (pH 7.5)] and incubated for 20 min on ice. The sample was centrifuged in a TLA100.2 rotor (Beckman, Geneva, Switzerland) at 60000 rpm for 10 min. The pellet including the highly enriched junctional membrane protein fraction (JMP-F) was once more repeatedly treated with guanidine and then washed twice with dilution buffer by centrifugation (60000 rpm for 10 min).

Two-Dimensional Gel Electrophoresis. To prepare NP-40-treated JMP-F suitable for two-dimensional gel electrophoresis, a pellet of 400 μg of JMP-F was suspended in a 10% NP-40 solution [10% NP-40 and 10 mM Tris (pH 7.5)], followed by centrifugation for 10 min at 60000 rpm. After the treatment was repeated once more, each 200 μg of JMP-F with or without NP-40 treatment was suspended in 200 μL of sample buffer (DeStreak Rehydration Solution, GE Healthcare, Buckingham, U.K.), with immobilized pH gradient (IPG) buffer added to a final concentration of 2% (v/v) for the selected pH ranges. The sample was loaded into 11 cm IPG-phor strip holders and overlaid with immobilized pH gradient (IPG) strips (GE Healthcare) . IPG strips were covered with an adequate volume of mineral oil and incubated at room temperature overnight. After incubation, IPG strips were rinsed briefly under deionized water and placed in coffins, and hydrated filter wicks were placed between the IPG strips and the electrodes.

Isoelectric focusing (IEF) was conducted as follows: for the strips at pI 4−7, 500 V for 1 h, ramp to 1000 V over 1 h, ramp to 6000 V over 2.5 h, and hold at 6000 V for 30 min and for the strips over pH gradients from 3 to 10 and from 6 to 11, 500 V for 1 h, ramp to 1000 V over 1 h, ramp to 6000 V over 2 h, and hold at 6000 V for 30 min. After IEF, the strips were rinsed briefly with deionized water and blotted with filter paper. Equilibration occurred in 6 M urea, 2% (w/v) SDS, 10% (w/v) DTT, 30% (v/v) glycerol, and 50 mM Tris-HCl (pH 8.8) for 20 min, and for a further 20 min in 6 M urea, 2% (w/v) SDS, 25% (w/v) iodoacetamide, 30% (v/v) glycerol, 50 mM Tris-HCl (pH 8.8), and a trace amount of bromophenol blue for the second SDS−polyacrylamide gel electrophoresis on a 13 cm (width) × 12 cm (height) × 0.7 mm step gradient polyacrylamide gel made up of 9 cm (height) of a 10% acrylamide gel and 3 cm (height) of a 15% gel. Gels were stained with silver nitrate using PlusOne Silver Staining Kit Protein (GE Healthcare), as previously described (*14*).

Mass Spectrometric Identification of Spots on Two-Dimensional Gels. Spectrometric identification of proteins was performed as previously described (*15, 16*). Briefly, after SDS−PAGE, proteins were visualized by silver staining and excised separately from gels, followed by in-gel digestions with trypsin (Promega Corp., Southampton, U.K.) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37 °C. Molecular mass analyses of tryptic peptides were performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using an ultraflex TOF/TOF system (Bruker Daltonics, Billerica, MA) (*15, 16*). Proteins were identified by comparison between the molecular weights determined by MALDI-TOF MS and theoretical peptide masses from the proteins registered in the NCBI database. This was done using the Mascot (Matrix Science, London, U.K.) search engine with a peptide mass tolerance of 0.15 Da and allowance for up to two trypsin miscleavages. Mascot

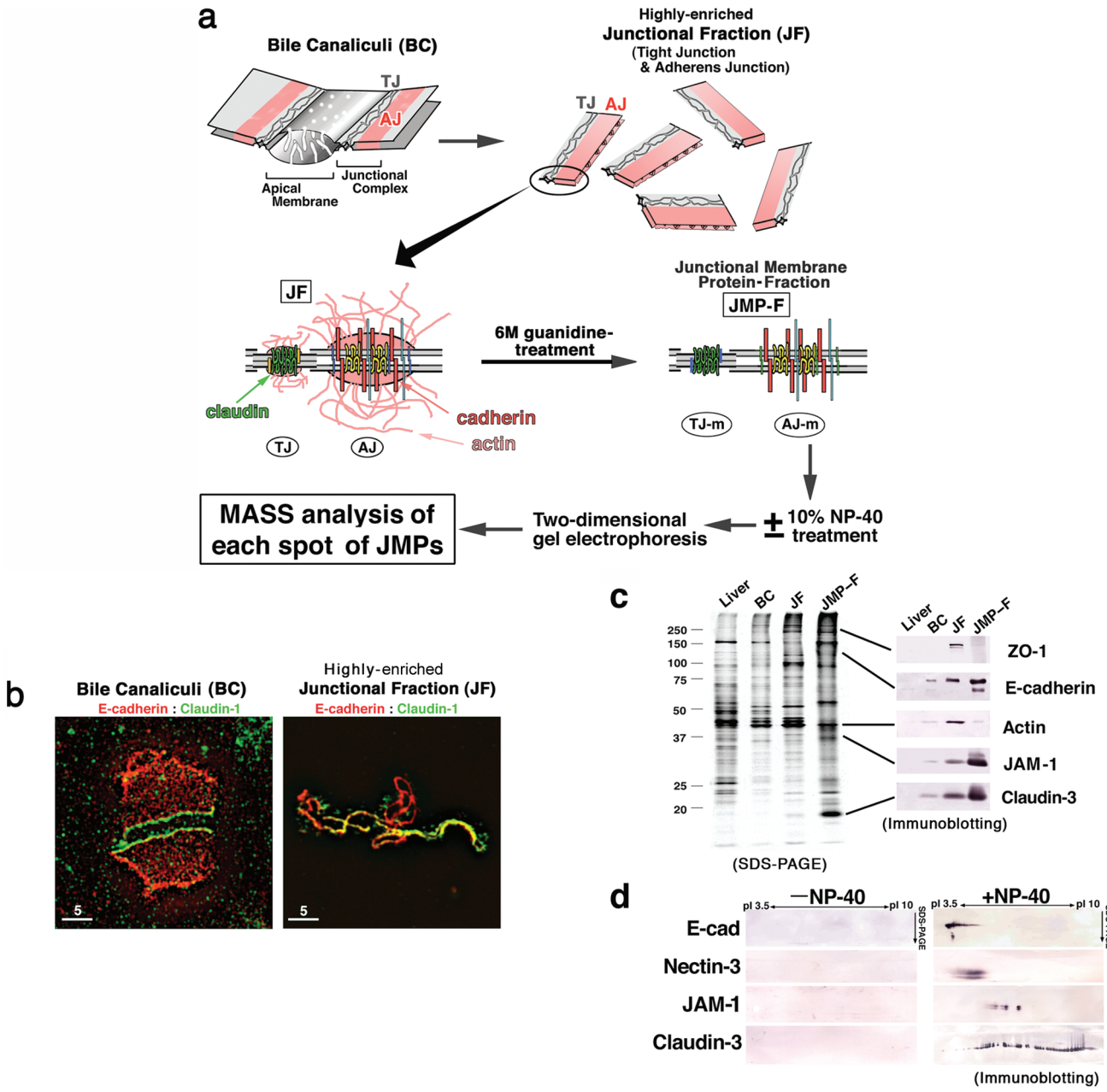

FIGURE 1: Strategy for mass spectrometry-based proteomic analysis of highly enriched cell−cell junctional integral membrane protein fraction (JMP-F). (a) Design and implementation of the strategy. After 0.1% NP-40 treatment of the bile canaliculi (BC) fraction prepared according to previously reported methods, the highly enriched junctional fraction (JF) with tight junctions (TJ) and adherens junctions (AJ) was purified via sucrose density gradient centrifugation in the presence of methyl-β-cyclodextrin (CD) to extract cholesterol from membranes. Treatment with 6 M guanidine removed peripherally associated junctional proteins, whereas highly enriched junctional integral membrane protein fraction (JMP-F) of tight junctional membrane (TJ-m) and adherens junctional membrane (AJ-m) remained. After being treated with 10% NP-40, JMPs were selectively detected in two-dimensionally electrophoresed gels. Mass spectrometry determined the genes of each spot. (b) Immunofluorescence images of purified bile canaliculi and junctional fractions. E-Cadherin staining reveals that the lateral membranes are included in bile canaliculi (BC); however, they are stripped off in the highly enriched junctional fraction (JF). Bars are 5 μm. (c) Evaluation of the purification process for junctional membrane proteins. Fractions of liver, bile canaliculi (BC), junctional fraction highly enriched (JF), and guanidine-treated highly enriched junctional integral membrane protein fraction (JMP-F) were one-dimensionally electrophoresed (left panel). Immunoblotting for E-cadherin, JAM-1, and claudin-3 as representatives of JMPs reveals that JMPs are highly enriched in guanidine-JMP-F (right panel). By contrast, immunoblotted signals for ZO-1 and actin reveal that peripherally associated junctional proteins are largely removed in JMP-F. (d) Detection of spots on two-dimensional gels with extremely high concentrations of NP-40. Immunoblotted signals for E-cadherin, nectin-3, JAM-1, and claudin-3 are not detected in two-dimensional gels without NP-40 treatment (left panel, −NP-40). By contrast, when fractions of JMPs were treated with 10% NP-40, signals for E-cadherin, nectin-3, JAM-1, and claudin-3 are immunologically detectable on two-dimensional gels (right panel, +NP-40).

scores of the identified proteins higher than 64 were considered significant ($p < 0.05$).

Homology Searching Analysis. The proteins related to the novel junctional proteins (gi46402185, gi81881775, and gi22122703) due to the mass spectrometric analyses were searched in the protein database of NCBI BLAST.

Antibodies. Mouse anti-ZO-1 monoclonal antibody (T8-754) and rat anti-E-cadherin monoclonal antibody (ECCD2)

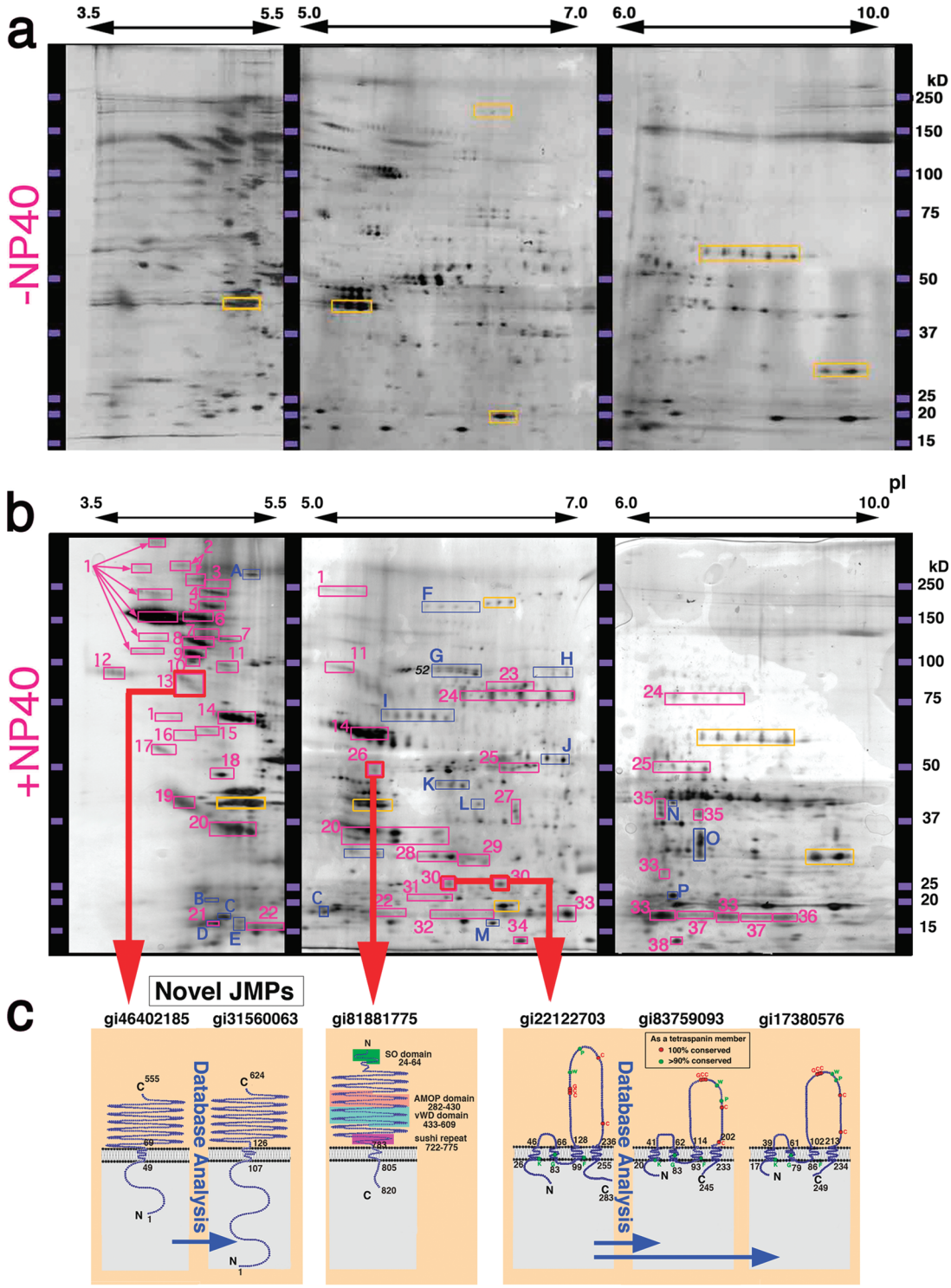

FIGURE 2: Targeted two-dimensional electrophoresis of JMPs. (a and b) Two-dimensional gel electrophoretic patterns of highly enriched junctional integral membrane proteins (JMPs) before (a) and after (b) addition of an extremely high concentration of NP-40 (10%). In panel b, the marked spots cannot be detected before NP-40 treatment at an extremely high concentration (10%) but appear after treatment with 10% NP-40. Junctional integral membrane proteins (JMPs) are marked in pink and numbered in Table 1. Peripherally associated junctional proteins are marked in blue and represented alphabetically in Table 1. Not-yet-identified novel proteins are marked in red. Note the high ratios of integral membrane proteins in this analysis. pl is noted according to the linear pH gradient formation on the isoelectric focusing gels (GE Healthcare). (c) Not-yet-identified novel proteins are schematically drawn, showing that they include integral membrane proteins, type I and type II with a single transmembrane domain, and those with four transmembrane domains. Database analyses on the three not-yet-identified novel proteins reveal related novel proteins, one kind of type II and two kinds of tetraspanin integral membrane proteins.

have been described previously (*17*). Rabbit anti-ERM polyclonal antibody (TK-89) was used as previously described (*18*). Mouse anti-actin antibody was purchased from MP Biomedicals Inc. (Irvine, CA), and rabbit anti-claudin-3 was acquired from Zymed (South San Francisco, CA). Mouse anti-GFP antibody was purchased from Molecular Probes Invitrogen (Carlsbad, CA). Mouse anti-HA antibody was purchased from Covance (Berkeley, CA). Cy2-conjugated

Table 1: Highly Enriched Junctional Integral Membrane Proteins (JMPs) Detected after Treatment with 10% NP-40 in Two-Dimensional Gels for Mass Spectrometric Analyses[a]

	protein name	accession number	function
1	N-cadherin	115424	cell adhesion
	E-cadherin	115419	cell adhesion
2	N-cadherin	115424	cell adhesion
3	protein tyrosine phosphatase, receptor type, S	25092609	signaling
4	integrin α1	13591884	cell adhesion
5	desmoglein 2	22779879	cell adhesion
	calmin	32699547	cell adhesion
6	integrin α5	31560574	cell adhesion
7	integrin β1	45504394	cell adhesion
8	protein tyrosine phosphatase, receptor type, K	26350175	signaling
9	activated leukocyte cell adhesion molecule	31791059	cell adhesion
10	desmoglein 2	22779879	cell adhesion
11	CD10 neutral endopeptidase 24.11	192459	others
12	nectin-like molecule 2	33303727	cell adhesion
13	expressed sequence AI429612	46402185	novel
14	5′-nucleotidase	539794	plasma membrane
15	poliovius receptor homologue (nectin-2)	199786	cell adhesion
16	neurotimin	27151644	signaling
17	Cd1	2624797	others
	serine (or cysteine) proteinase inhibitor, cladeA, member 3K	15079234	others
18	Cd1	2624797	others
19	basigin	34915988	signaling
20	junctional adhesion molecule-1	3462455	cell adhesion
21	claudin-1	6685278	cell adhesion
22	claudin-1	6685278	cell adhesion
	claudin-3	6685291	cell adhesion
23	mKIAA1161	50510819	metabolism
24	CD36	31982474	signaling
25	GPI-anchored cell surface protein hyaluronidase 2	28629213	plasma membrane
26	sushi domain-containing protein 2	81881775	novel
27	Na^+/K^+-ATPase β3 subunit	6680744	transporter
28	syntaxin 4A	30354363	vesicle transport
29	epimorphin	50330	vesicle transport
30	tetraspanin-33	22122703	novel
31	gap junction membrane channel protein β1	22165406	cell adhesion
32	CD81	19526794	signaling
33	PERP	11528520	cell adhesion
34	Golgi autoantigen, golgin subfamily a, 7	16877268	ER/Golgi transport
35	Na^+/K^+-ATPase β3 subunit	6680744	transporter
36	CD72	6680890	signaling
37	claudin-1	6685278	cell adhesion
38	golgin subfamily a, 7	16877268	ER/Golgi transport
A	spectrin α2	63489759	cytoskeleton
B	tubulointerstitial nephritis m s antigen-like	12963691	others
C	23 kDa synaptosomal associated protein	2253401	vesicle transport
D	RAP2C, member of the RAS oncogene family	27369539	signaling
	RAP2A, member of the RAS oncogene family	40254160	signaling
E	RAP2B, member of the RAS oncogene family	54611386	signaling
F	H-ras	7768785	signaling
G	myosin Va	6754784	cytoskeleton
H	catenin α	6753294	cell adhesion
I	transglutaminase 1	31982705	others
J	alkaline phosphatase	22138740	metabolism
K	pacsin3	3559518	vesicle transport
L	NADH dehydrogenase	23346461	metabolism
M	ADP-ribosylation factor 1	21594148	ER/Golgi transport
N	Inpp5a protein	33604082	signaling
O	E2F transcription factor 6	15149491	transcription
P	R-ras	6677819	signaling

[a] The highly enriched integral membrane proteins of cell−cell junctional integral membrane protein fraction (JMP-F) marked in pink in Figure 2 are numbered. Peripherally associated junctional proteins of the highly enriched junctional fraction (JF) marked in blue in Figure 2 are represented alphabetically. Note the high ratios of integral membrane proteins in JMPs.

anti-rabbit IgG, Cy3-conjugated anti-rat IgG, and Cy3-conjugated anti-mouse IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Alexa Flour 647-labeled anti-mouse IgG was obtained from Molecular Probes Invitrogen.

Cell Culture and Transfection. Mouse Eph4 epithelial cell lines were a gift from Dr. Reichmann. Eph4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Aliquots of 1 μg of each expression vector were introduced into Eph4 cells in 1 mL of DMEM using Lipofectamine Plus Reagent (Invitrogen, Paisley, U.K.). After a 24 h incubation, cells were replated and cultured in DMEM containing 10% FCS and 500 μg/mL G418 (Nakalai, Kyoto, Japan) to select stable transfectants.

Immunofluorescence Microscopy. Cells grown on Transwell inserts (Corning) were fixed with cold methanol at −20 °C for 5 min and then blocked with PBS containing 1% BSA for 10 min. BC and AJ were plated on poly-L-lysine-coated glasses and fixed with cold ethanol at −20 °C for 30 min and with acetone at room temperature for 1 min, finally being blocked with PBS containing 1% BSA for 10 min. All were incubated with primary antibodies for 60 min. After being washed with PBS three times, they were incubated with secondary antibodies for 60 min. Samples were washed three times with PBS and then mounted in Fluorescent Mounting Medium (Dako Cytomation, Glostrap, Denmark).

RESULTS AND DISCUSSION

As the first step in the proteomic analyses of highly enriched junctional integral membrane proteins (JMPs), we attempted to increase the purity of the junctional fraction as the source of JMPs. For this, in addition to the established protocol for isolating a junctional fraction (*7*), we increased the specific gravity of mouse junctional fraction by depleting cholesterol with methyl-β-cyclodextrin (CD) (*13*) for further fractionation by sucrose gradient centrifugation. This effectively removed contaminants such as extracellular matrix. Soon after we obtained the junctional fraction from the bile canaliculi (BC) fraction, as previously described, we applied it to stepwise sucrose gradients consisting of 60, 55, 50, 40, and 30% (w/v) solutions and recovered the junctional fraction at the 40%−50% sucrose interface. The junctional fraction was recovered, treated with CD, and subjected to sucrose density gradient centrifugation. It was moved to the 50%−55% sucrose interface, at a higher sucrose concentration compared to that for fractionation without CD (Figure 1a). When we examined immunofluorescence for E-cadherin and claudin-1, junction-like profiles were specifically enriched in this CD-treated junctional fraction, suggesting that we had obtained highly enriched junctional fraction (JF) (Figure 1b).

Next, we used a 6 M guanidine solution to extract the majority of peripherally associated junctional proteins, with most of the integral membrane proteins remaining behind in the highly enriched junctional integral membrane protein fraction (JMP-F) (Figure 1a). We tracked the abundance of proteins among fractions of BC, JF, and JMP-F by one-dimensional gel electrophoresis in combination with Western blotting with respect to well-known integral membrane proteins such as E-cadherin (*5*), JAM-1 (*19*), and claudin-3 (*5*) (as JMPs) and ZO-1 (*5*) and actin (as peripheral junctional proteins). We found that guanidine treatment was noticeably effective for enriching JMPs into JMP-F (Figure 1c). Thus, JMP-F was advantageous for the identification of JMPs in proteomics.

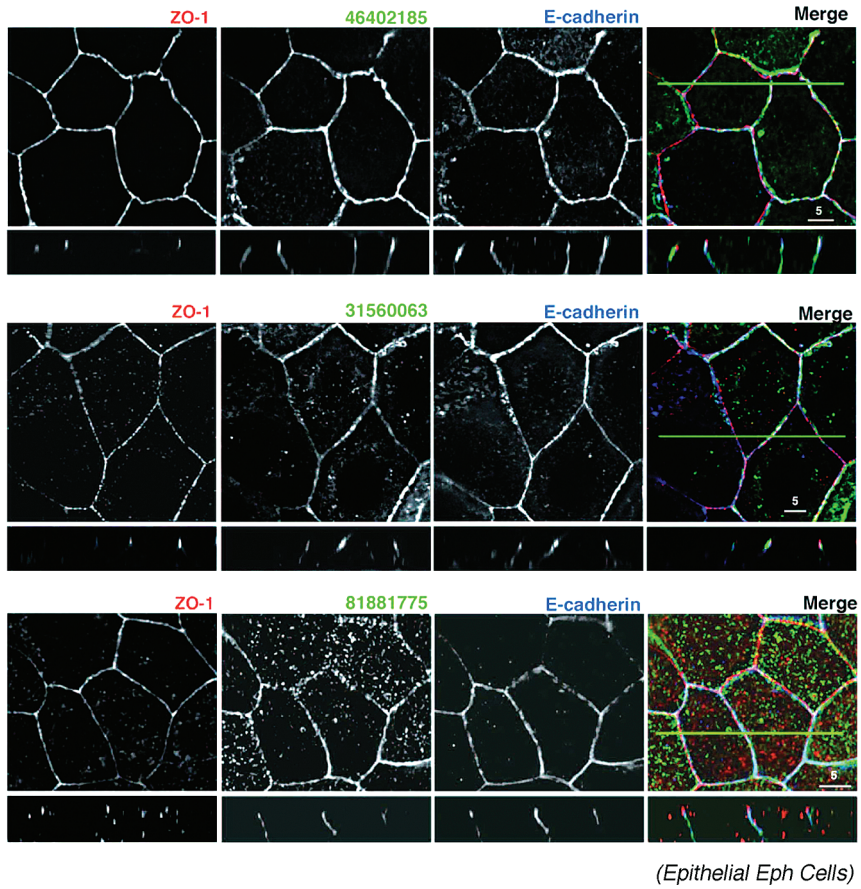

FIGURE 3: Transfection of the tagged construction of novel integral membrane proteins, type I and type II. The genes corresponding to gi46402185 (type II)-GFP, GFP-gi31560063 (type II), and signal-HA-gi81881775 (type I) were transfected into mouse epithelial cells (Eph cells). The transfectants were stained for ZO-1, E-cadherin, and tag. These three novel integral membrane proteins, mass spectrometrically and computationally identified, are enriched in the adherens junction, judged from the colocalization with E-cadherin. The $X-Y$ image (top panel) and a vertical section (bottom panels) cut through the plane of the Z-stack as indicated by the green line in the top right panel are shown: green, tag; red, ZO-1; and blue, E-cadherin. Bars are 5 μm.

For the first isoelectrophoretic focusing on two-dimensional electrophoresis of JMP-F (200 μg) prepared from 20 mice, strips for isoelectric focusing [immobilized pH gradient (IPG) strips] at pI 4−7, 6−11, and 3.5−10 were used. However, no immunoblotted signals for authentic membrane proteins could be detected on conventional two-dimensionally electrophoresed gels for JMPs without strong NP-40 sample treatment. This was most likely because of the high insolubility of the membrane proteins, such as E-cadherin, nectin-3, JAM-1, and claudin-3, in sample buffer for isoelectric focusing (IPG buffer). Hence, to solubilize the membrane proteins for isoelectric focusing and to optimize the conditions for two-dimensional electrophoresis of authentic membrane proteins, we treated JMP-F with various reagents such as NP-40, digitonin, methanol, and methanol with chloroform. Among these reagents, an extremely high concentration of NP-40 (10% NP-40) was the most effective for detecting signals of the given membrane proteins as immunoblotted spots on two-dimensional gels (Figure 1d). Hence, before applying JMP-F to the strips for isoelectric focusing, we treated them twice with NP-40 at an extremely high concentration (10%) in 10 mM HEPES (pH 7.5). Mass spectrometric identification using the silver-stained spots on two-dimensional gels further confirmed that spots of authentic membrane proteins were not present before NP-40 treatment but had appeared selectively after 10% NP-40 treatment (Figure 2). Furthermore, we found that the addition of methanol (10%) to the sample buffer for isoelectric focusing was effective in clearly visualizing the spots, particularly in the acidic region of two-dimensional gels (pI 3.5−5.5). To optimize the condition for two-dimensional analyses of the highly enriched junctional integral membrane protein fraction (JMP-F), we repeatedly performed analyses using more than 50 preparations. We obtained optional conditions at approximately five times. Under these optimal conditions, the reproducibility of spots was greater than 50%. As statistical tools, we used Melanie II (Bio-Rad).

On the basis of the finding that spots of authentic membrane proteins could be detected on two-dimensionally electrophoresed gels in an NP-40 treatment-dependent way, we next took all the silver-stained spots of guanidine-treated JMPs on two-dimensional gels, which could not be detected before 10% NP-40 treatment but were so after 10% NP-40 treatment (Figure 2). Using this strategy, we obtained 54 samples in total. From MALDI-TOF MS analyses of these spots (*16*) with Mascot scores of the identified proteins higher than 64 being considered significant ($p < 0.05$), the following results were obtained (Figure 2 and Table 1). (1) Among spots that could not be detected before but could be detected after 10% NP-40 treatment, the probability of spots corresponding to membrane proteins was 70% [38/(38 + 16)], including 12 known JMPs (Table 1). To examine the localization of noncharacterized membrane proteins other than the 12 known JMPs, we cloned their cDNAs and

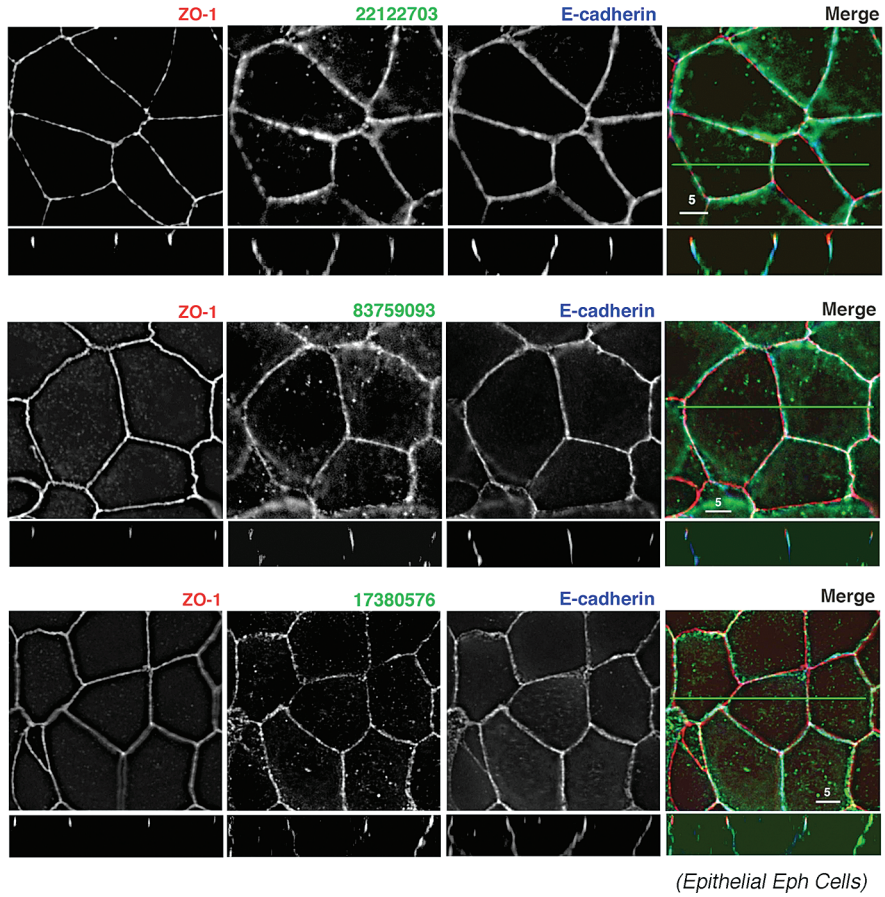

FIGURE 4: Transfection of the GFP-tagged construction of tetraspanin family proteins of gi22122703, gi83759093, and gi17380576. The genes corresponding to gi22122703-GFP, GFP-gi83759093, and GFP-gi17380576 were transfected into mouse epithelial cells (Eph cells). The transfectants were stained for ZO-1, E-cadherin, and GFP. These three of the tetraspanin family proteins, mass spectrometrically and computationally identified, are enriched in the adherens junction, judged from the colocalization with E-cadherin. The $X-Y$ image (top panel) and a vertical section (bottom panels) cut through the plane of the Z-stack as indicated by the green line in the top right panel are shown: green, GFP; red, ZO-1; and blue, E-cadherin. Bars are 5 μm.

constructed expression vectors tagged to exogeneously express them in mouse epithelial cell lines (Figures 3 and 4). As a result, the probability of these membrane proteins being localized in junctions was 45% (17/38), corresponding to 15 species of JMPs, including the 12 known JMPs. The remaining 55% (21/38) were considered to be of nonjunctional localization (such as apical membrane, Golgi apparatus, and nuclear membrane localization), suggesting that the original JF contained these nonjunctional membrane domains as contaminants. (2) Among the known JMPs, JAM-1 (*19*), claudin-1 (*5*), and claudin-3 (*5*) were found to be localized in tight junctions, and desmoglein-2 (*20*) and PERP (*21*) were desmosome components. Only one protein was a gap junction protein (*22*). The six remaining species of known JMPs were cell−cell adherens junction constitutive proteins (E-cadherin, N-cadherin, nectin-2, necl-2, RPTP-K, and CD81). (3) The tagged constructs of three novel JMPs were localized in the cell−cell adhering junctions, mainly in adherens junctions (Figures 3 and 4). These included type I (gi81881775) and type II (gi46402185) integral membrane proteins and one tetraspanin (gi22122703:Tspan-33), though the type I integral membrane protein was also localized in apical microvilli that were positive for ERM proteins (Figure 1S of the Supporting Information).

Furthermore, a database homology search suggested that there were seven integral membrane proteins that were closely related in cDNA sequence to these novel JMPs. The results for gi46402185 suggested a similar sequence, gi31560063, below an Expect value of e^{-41}. From our search of gi22122703 below an Expect value of e^{-20}, we discovered tetraspanin-5,6,7,14,17 and CD63. Among them, as revealed by transfection experiments with the tagged constructs, we found that one novel type II (gi31560063) membrane protein and two tetraspanins (gi83759093:Tspan-6 and gi17380576:Tspan-7) were adherens junction constitutive integral membrane proteins (Figures 3 and 4).

The true TM nature of the novel proteins, which were judged as integral membrane proteins from SOSUI database analyses (*23*), was confirmed as follows. For the novel type II membrane proteins, gi46402185 and gi31560063, the N-terminally GFP-tagged proteins were immunofluorescence-labeled in red with a combination of a mouse anti-GFP antibody and a Cy3-labeled donkey anti-mouse IgG antibody in the presence of 0.1% Triton X-100, but not labeled in red in the absence of 0.1% Triton X-100. By contrast, the C-terminally GFP-tagged proteins were immunofluorescence-labeled in red by a combination of a mouse anti-GFP antibody and a Cy3-labeled donkey anti-mouse IgG antibody in the presence and absence of 0.1% Triton X-100. On the other hand, for the novel type I membrane protein, gi88181775, its signal-HA-tagged construct was used for the localization assay. On the basis of its adherens junctional localization at the plasma membranes in the presence of the signal sequence, it was reasonably judged as transmembrane

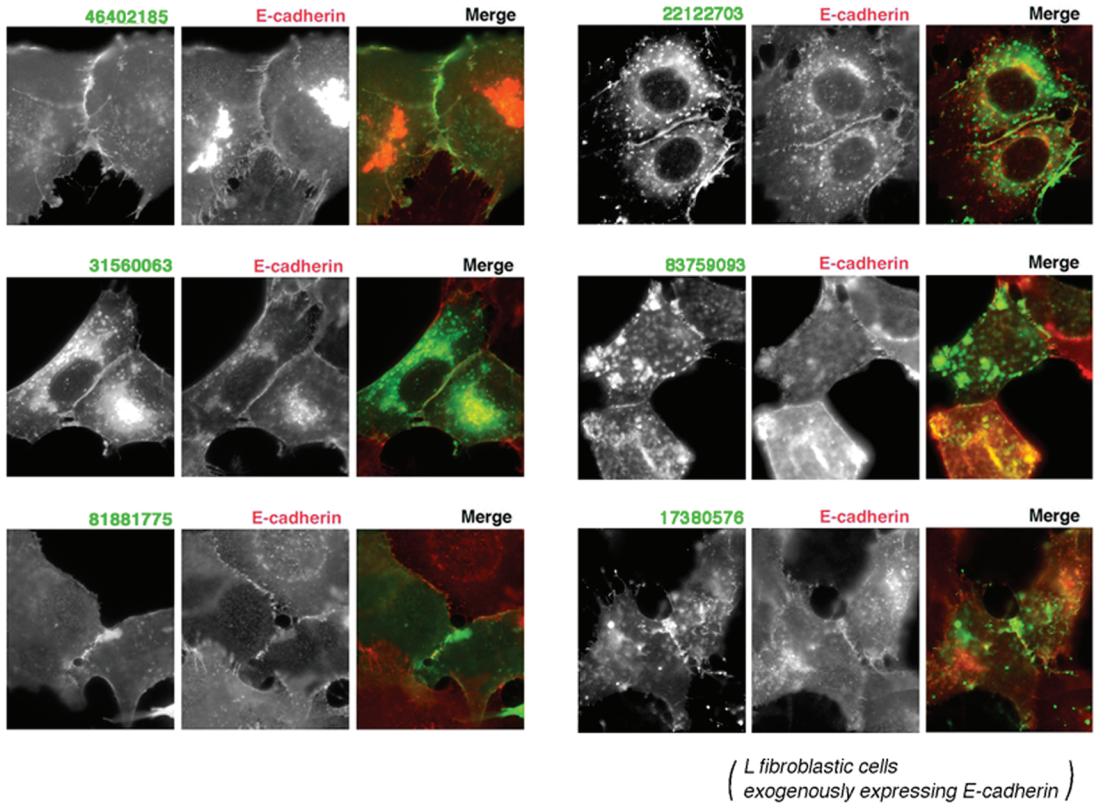

FIGURE 5: Transfection of tagged novel JMPs into L fibroblasts in which E-cadherin is exogenously expressed (EL cells). The tagged constructs of the novel integral membrane proteins (gi46402185-GFP, GFP-gi31560063, signal-HA-gi81881775, gi22122703-GFP, GFP-gi83759093, and GFP-gi17380576) were transfected into EL cells in which the association of JMPs and E-cadherin could be more simply examined than in cultured epithelial cells. The tagged JMPs were immunofluorescently associated with E-cadherin, suggesting the functional link of JMPs with E-cadherin-related cell adhesion.

proteins. Finally, proteins encoded by gi22122703, gi83759093, and gi17380576 were judged as tetraspanins by SOSUI database analyses. The key amino acids of tetraspanins were conserved among them (*24*). They were reasonably judged as transmembrane proteins, on the basis of the consensus that tetraspanins are generally transmembrane proteins.

All six novel adherens junction-constitutive JMPs were so low antigenic, that generation of mAbs in rats and generation of pAbs in rabbits, rats, mice, goats, and guinea pigs against the antigens encoded by mouse, dog, and chicken genes were unsuccessful (*25*). This is why these JMPs have not yet been functionally analyzed, since antibody production is critical for analyses of functions of JMPs. To examine the functional connection between these novel JMPs and E-cadherin, the tagged novel JMPs were transfected into L fibroblasts exogeneously expressing E-cadherin (EL cells) (*26*), in which the association of JMPs and E-cadherin could be more directly examined by immunofluorescence microscopy than could be in cultured epithelial cells. As a result, the tagged JMPs were likely to be closely associated with E-cadherin, suggesting a functional link of the novel JMPs with E-cadherin-related cell−cell adhesion (Figure 5).

In this study, we were able to develop mass spectrum-based, two-dimensional gel electrophoresis-based proteomics. The two main strategies were (1) exhaustive fractionation to isolate and purify cell−cell junctions and (2) solubilization of JMPs with 10% NP-40 to facilitate detection by two-dimensional electrophoresis. The need to use a strong treatment with guanidine to exclude peripherally associated proteins and with NP-40 to solubilize JMPs suggested that the finally detected novel JMPs formed particular complexes for integration into junctions. Along similar lines, cell fractionation has been applied to proteomics for inner cellular substructures such as plasma membrane, endoplasmic reticulum, phagosomes, and Golgi apparatus (*27*). At more delicate levels, caveolae (*28*), clathrin-coated vesicles (*29*), or synaptic vesicles (*30*) have been targets of proteomic analyses. We showed here that application of two-dimensional gel patterning of the integral membrane proteins revealed the framework of the integral membrane proteins of epithelial cell−cell adhering junctions. We found that integral membrane proteins could not usually be detected on gels by conventional methods, consistent with findings that integral membrane proteins are generally undetectable on gels. We treated JMP-F with various types of reagents and found that a high concentration of NP-40 with or without methanol was effective in solubilizing the membrane proteins in two-dimentional gels. Consistent with this type of strategy in solubilizing the membrane proteins using detergents, as reported for other structural domains (*31*), such as cell membranes (*32*) or synaptic vesicles (*29*), strong detergent treatments in JMP-F were effective for sample preparation for two-dimensional electrophoresis.

Considering that functional membrane proteins, including many types of receptors or adhesion molecules, form membrane domains, in many cases structurally integrated, the current style of proteomics, in which proteomic analyses are combined with advanced cell fractionation with sophisticated sample treatment, appears applicable over a wide

range of basic and clinical biological fields as it profiles the major frameworks of membrane protein complexes.

CONCLUSIONS

Cell−cell adhering junctional membrane proteins are highly organized in very limited specific domains of epithelial cells and are thus difficult to be systematically committed by two-dimensional gel electrophoresis combined with mass spectrometric analyses. However, we here presented the total profile of cell−cell adhering junctional membrane proteins in two-dimensional gels. In semiquantitative analyses, E- and N-cadherins were found to be the most abundant in total membrane integral junctional proteins. Also, at least 15 species of membrane proteins were likely to be organized in cell−cell junctional complexes in epithelial-type cells.

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SUPPORTING INFORMATION AVAILABLE

Apical cortical localization of the novel type I integral membrane protein in addition to the cell−cell junctional localization (Figure 1S). This material is available free of charge via the Internet at http://pubs.acs.org.

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