A Proteomic Approach for the Identification of Cell-surface Proteins Shed by Metalloproteases*

Lin Guo, June R. Eisenman, Rajeev M. Mahimkar, Jacques J. Peschon, Raymond J. Paxton, Roy A. Black, and Richard S. Johnson‡

Proteolytic cleavage (shedding) of extracellular domains of many membrane proteins by metalloproteases is an important regulatory mechanism used by mammalian cells in response to environmental and physiological changes. Here we describe a proteomic system for analyzing cell surface shedding. The method utilized short-term culture supernatants from induced cells as starting material, followed by lectin-affinity purification, deglycosylation, and polyacrylamide gel electrophoresis separation. Relative quantitation of proteins was achieved via isotope dilution. In this study, a number of proteins already known to be shed were identified from activated monocytes and endothelial cells, thereby validating the method. In addition, a group of proteins were newly identified as being shed. The method provides an unbiased means to screen for shed proteins. ***Molecular & Cellular Proteomics 1:30–36, 2002.***

Proteolysis of cell membrane-bound proteins provides a post-translational means of regulating protein function and has been shown to control the production of many soluble cytokines, receptors, adhesion molecules, and growth factors through the process termed ectodomain shedding (1, 2). Abnormal shedding can contribute to diseases such as rheumatoid arthritis and cancer (3). A key player in ectodomain shedding is the ADAM (a disintegrin and metalloprotease) family of metalloproteases (1, 2). ADAMs are characterized by a conserved domain structure that consists of an N-terminal signal sequence followed by the pro-domain, the metalloprotease, and disintegrin domains, a cysteine-rich region usually containing an epidermal growth factor repeat, a transmembrane domain, and a cytoplasmic tail (4).

Tumor necrosis factor-α converting enzyme (TACE/ADAM-17)[1] was the first ADAM family protease to be characterized as a sheddase. It was originally identified by its ability to cleave membrane-bound proTNF-α, the precursor form of TNF-α, resulting in the release of soluble TNF-α from cells (5, 6). Subsequent work, primarily involving TACE knockout mice and cells (7), indicated that the shedding of a number of other proteins is mediated by TACE. These include transforming growth factor-α, L-selectin, p75 TNF recepor, amyloid protein precursor, CD30, IL-6 receptor, Notch 1 receptor, growth hormone-binding protein, and macrophage colony-stimulating factor receptor (7–13). In all these studies, the linkage to TACE was made through a hypothesis-driven approach, rather than via a screening process.

Protein shedding is a post-translational event that is independent of the expression level of mRNA; hence, screening of protein shedding events requires a proteomic approach. To isolate shed proteins, many of which are glycosylated, from cell supernatants, we first utilized a lectin-affinity purification step to isolate glycoproteins. An *N*-deglycosylation step was subsequently used to reduce the heterogeneity of the protein, which enhanced the resolution on a one-dimensional SDS-PAGE (1D-PAGE) gel. To quantitatively compare regulated *versus* constitutive shedding, stable isotope dilution was performed using a novel thiol-alkylating reagent. From mass spectrometric analysis of tryptic fragments, we have identified several metalloprotease-released proteins, including proteins already known to be shed and others that were not.

EXPERIMENTAL PROCEDURES

Dexter-ras-myc (DRM) Cells—The DRM TACE+/+ and TACE−/− cell lines (7) were cultured as described (12). A TACE-encoding retrovirus was generated as described (14) and used to reconstitute functional full-length TACE in TACE−/− DRM cells. The control cells were generated by transfecting TACE−/− DRM cells with retrovirus containing an empty vector. The expression of TACE was confirmed by a functional reconstitution assay. Briefly, DRM TACE−/− monocytes were stimulated with lipopolysaccharide (1 μg/ml), and shedding of TNF and TNF receptor were analyzed by enzyme-linked immunosorbent assay (OptEIA™; Pharmingen).

From the Immunex Corporation, Seattle, Washington 98101-2936

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[1] The abbreviations used are: TACE, tumor necrosis factor-α-converting enzyme; 1D, one-dimensional; 2D, two-dimensional; DRM, Dexter-*ras-myc*; HMVEC, human adult dermal microvascular endothelial cells; WGA, wheat germ agglutinin; MS/MS, tandem mass spectrometry; IC-3, Immunex compound-3; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α; IL, interleukin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; LDL, low density lipoprotein; SHPS-1, SH2 domain-containing tyrosine phosphatase substrate 1; h, human; r, receptor.

Human Adult Dermal Microvascular Endothelial Cells (HMVEC)—HMVECs (BioWhittaker/Clonetics, Walkersville, MD) were grown in EGM2MV media (BioWhittaker/Clonetics) to passage 6. Cultures were fed with fresh media every 2–3 days and passed every 5 days. To pass, 80–90% confluent cultures were gently trypsinized (BioWhittaker/Clonetics), and T175 flasks were seeded at 10,000 cells/cm^2 in 35 ml of media.

Cell Stimulation—Prior to stimulation, DRM cells were expanded in 1-liter spinner flasks, seeded at 2.5×10^5 cells/ml, and grown to ~$2–3 \times 10^6$ cells/ml in 800 ml of growth media. DRM cells were prepared for stimulation by washing twice with cold, serum-free RPMI 1640 (Invitrogen) and once in cold, phenol red-free, serum-free RPMI 1640 (Invitrogen). Washed cells were placed in T175 flasks at 8×10^6 cells/ml in 25 ml of phenol red- and serum-free RPMI 1640. IC-3 (25 μg/ml) and/or PMA (100 ng/ml) (ICN Biomedicals, Inc., Aurora, OH) were added to appropriate flasks. Flasks were incubated for 90 min at 37 °C with 5% CO_2. Supernatants from all flasks were harvested, centrifuged for 10 min at 1200 rpm, 4 °C; 0.22-μm filtered (Corning Glass Inc., Corning, NY) and treated with protease inhibitors (175 μg/ml phenylmethylsulfonyl fluoride, 4.75 μg/ml leupeptin, 6.9 μg/ml pepstatin A, and 2.5 μg/ml EDTA). Supernatants were concentrated (Centricon Plus-80, 10-kDa cut-off; Millipore, Bedford, MA; for volumes up to 80 ml) prior to purification.

For HMVEC stimulation, passage 6, 90% confluent cells were used. Growth medium was gently replaced with EBM-2 basal media (BioWhittaker/Clonetics,) and cultures were incubated for 14 h. Medium was gently replaced again with phenol red-free EBM basal media (BioWhittaker/Clonetics), and half the flasks were supplemented with an inflammatory cytokine mixture for 4 h. The cytokine mixture is composed of 100 ng/ml hCD40L (Immunex, Seattle, WA), 2 ng/ml hIL-1β (Immunex), 2 ng/ml hTNFα (BIOSOURCE International, Inc., Camarillo, CA), 100 units/ml human interferon-γ (BIOSOURCE International, Inc., Camarillo, CA), 30 ng/ml human fibroblast growth factor-basic (Chemicon International, Inc., Temecula, CA), 100 ng/ml hTWEAK (Chemicon International), and 10 ng/ml human vascular endothelial growth factor (Chemicon International). After 4 h, PMA (100 ng/ml) (ICN Biomedicals, Inc.) was added to the cytokine-containing flasks, which were incubated for an additional hour. Supernatants from all flasks were harvested as above. For cytokine-stimulated cells the total supernatant protein yield per 10^8 cells was 6.3 mg, whereas unstimulated control cells yielded 3.0 mg.

Lectin-affinity Purification—To isolate soluble glycoproteins in the cell supernatant, lectin-affinity chromatography using agarose-bound wheat germ agglutinin (WGA) (Vector Laboratories, Inc., Burlingame, CA) was performed. Briefly, 2–4 mg of concentrated supernatant proteins were incubated with 250 μl of washed WGA-agarose beads in 4 ml of HEPES/NaCl buffer (10 mM HEPES, pH 7.5, containing 0.15 M NaCl) in a capped Micro Bio-Spin chromatography column (Bio-Rad, Hercules, CA). After incubating at 4 °C for 1 h on a rotary shaker, the column was washed three times with 5 ml of the HEPES/NaCl buffer. The lectin-binding proteins were then eluted with 3 ml of 0.5 M *N*-acetyl-D-glucosamine in HEPES/NaCl buffer. The excess amount of *N*-acetyl-D-glucosamine was removed from the WGA eluate by 7.5-fold concentration (Centricon YM-10, 10-kDa cut-off; Millipore, Bedford, MA, for volumes up to 2 ml), followed by protein precipitation at room temperature using a method designed for quantitative recovery of protein in dilute solution in the presence of detergents and lipids (15).

Deglycosylation—*N*-Glycans were removed from glycoproteins using recombinant *N*-glycosidase F, also referred to as *N*-glycanase or PNGaseF (Glyko, Inc., Novato, CA). The deglycosylation reaction was carried out as directed by the vendor.

One- and Two-dimensional Electrophoresis—1D-PAGE was performed under reducing conditions using Tris/glycine 4–20% gradient gels (Novex gel; Invitrogen, Carlsbad, CA). The first dimension of the 2D separation was carried out using immobilized 11-cm immobilized pH gradient strips from Bio-Rad (Hercules, CA). The deglycosylated proteins were mixed with rehydration buffer (8 M urea, 2% CHAPS, 45 mM DTT, 0.5% ampholytes, pH 3–10 (Bio-Rad), and 0.0002% bromphenol blue. Isoelectric focusing was performed using the IPGphor system from Amersham Biosciences, Inc. The 4–20% gradient Criterion gels from Bio-Rad were used for the second dimension. Protein bands/spots were detected by staining with Colloidal Blue (Invitrogen).

Protein Reduction, Alkylation, and Digestion—Protein spots/bands were excised from the 1D-PAGE gel, destained by washing with a mixture of 200 mM NH_4HCO_3/acetonitrile (1:1). Proteins were reduced with DTT, alkylated with either iodoacetamide or *N*-ethyl-iodoacetamide (as specified under "Results"), and digested in-gel with trypsin (Promega, Madison, WI) as described (16). *N*-Ethyl-iodoacetamide (either d0 or d5 form) was synthesized from ethylamine hydrochloride (either d0 or d5 form) and iodoacetic anhydride. Tryptic peptides were concentrated by vacuum centrifugation before mass spectrometric analysis.

Mass Spectrometry—Mass spectrometric analysis of tryptic peptides was performed on a Micromass QTOF 1 instrument (Micromass UK Ltd., Wythenshawe, Manchester, United Kingdom). Peptides were sequenced by on-line microcapillary liquid chromatography-electrospray ionization-tandem mass spectrometry (MS/MS) analysis using an LCpackings (San Francisco, CA) 50-μm inner diameter C_{18} column. The gradient was developed using an Eldex Micropro pump (Napa, CA) operating at 5 μl/min, and the flow was split before the injector such that the flow rate through the column was ~250 nl/min. The effluent of the column was directed into an Upchurch (Oak Harbor, WA) micro-tee containing a platinum electrode and a New Objective (Cambridge, MA) uncoated fused silica tip (360-μm outer diameter, 20-μm inner diameter, pulled to a 10-μm opening). The mass spectrometer was operated in a data-dependent MS/MS mode, and proteins were identified by searching a non-redundant protein sequence data base using the Mascot program (17). A second liquid chromotography/MS acquisition (MS-only mode) was performed for each sample to generate accurate ion intensity data for quantitation.

RESULTS

Identification of Metalloprotease-shed Proteins in Monocytes—Many metalloprotease-mediated shedding events are induced by phorbol esters such as PMA and inhibited by hydroxamic acid compounds such as IC-3 (18, 19). To isolate shed proteins, cell supernatants were collected from wild-type bone marrow-derived monocytic (DRM) cells (7) that were stimulated with PMA in the presence or absence of IC-3. From six separate experiments, an average of 4.0 mg of supernatant proteins were derived from 10^9 cells in the presence of IC-3; from nine separate experiments, an average 4.3 mg per 10^9 cells was obtained in the absence of IC-3. Because no statistically significant differences were detected in the total amount of protein in the two samples, it was deduced that shed proteins composed a small fraction of the total and that the majority of the supernatant proteins were derived from normal cell turnover and metabolism. This was confirmed when the supernatant proteins were digested with trypsin and analyzed by MS/MS. These data showed that the most prominent proteins in the cell supernatant were various forms of heat shock proteins, actin, and metabolic pathway enzymes.

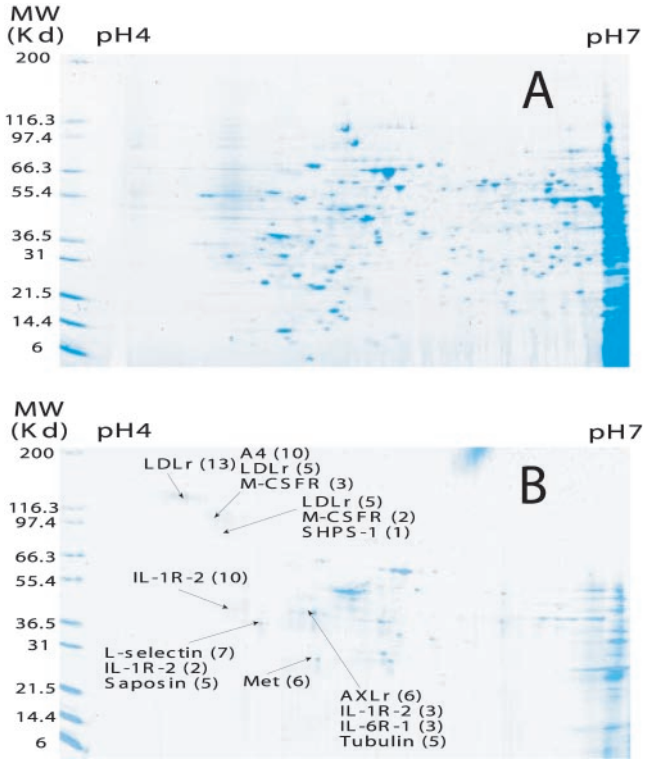

FIG. 1. **2D-PAGE gel of proteins from DRM TACE+/+ cells stimulated with PMA for 90 min in the absence of the metalloprotease inhibitor IC-3.** *A*, 200 μg of supernatant protein, derived from $\sim 5 \times 10^7$ cells, was loaded onto the gel. *B*, from 5.8 mg of total supernatant proteins (derived from $\sim 1.3 \times 10^9$ cells), all of the glycoproteins obtained by WGA lectin-affinity purification were *N*-deglycosylated and loaded onto the gel. Protein assignments were based on data base matches to tandem mass spectra (Table I). The number of peptides identified from each protein is indicated in *parentheses*.

Consistent with this observation, we were unable to discern any differences in the staining pattern on 2D (isoelectric focusing and SDS)-PAGE gels obtained from pairs of cell supernatants (with and without IC-3) (Fig. 1*A* and data not shown). Although 2D-PAGE is widely used and is recognized as a basic tool for proteomics, it seems to display only the most abundant proteins in a complex sample (20, 21). Hence, it was evident that additional protein fractionation would be required to discern quantitative differences between lower abundance proteins in these samples.

Because most cell-surface proteins contain one or more carbohydrate groups, proteins released from cell membranes are likely to be glycosylated. WGA, which contains a group of closely related isolectins, can bind oligosaccharides containing sialic acid or terminal *N*-acetylglucosamine that are common to many mammalian secreted and membrane glycoproteins. Therefore, conjugated WGA-agarose was chosen for the affinity purification of glycoproteins from the cell supernatants. After the lectin-affinity fractionation, the isolated glycoproteins were subjected to *N*-deglycosylation by treatment with recombinant *N*-glycosidase F, which had the effect of reducing glycoprotein heterogeneity and therefore enhancing the protein focusing on SDS-PAGE (1D-PAGE) gels.

The *N*-deglycosylated proteins were analyzed by both 2D- and 1D-PAGE (see Fig. 1*B* and Fig. 2). When compared with the samples from cultures containing IC-3 (data not shown), a few 2D-PAGE spots were determined to be unique or of increased intensity in the supernatants obtained from cells not treated with IC-3 (cells were stimulated with PMA in both cases) (Fig. 1*B*). These spots were not detectable prior to WGA enrichment of glycoproteins (Fig. 1*A*), which apparently was because of their relatively low abundance in the unfractionated cell supernatant. Gel pieces containing these spots were excised, and their protein content was identified by tandem mass spectrometry after in-gel digestion with trypsin (Table I). Except for saposin and tubulin, the proteins that were identified from the 2D-PAGE experiment are type 1 transmembrane proteins (Table I), thus indicating that the lectin-affinity step was reasonably effective in eliminating cytoplasmic proteins. All of the tryptic peptides identified (Table I) were derived from the extracellular domains of the corresponding membrane proteins, as predicted for proteins released by shedding.

Although *N*-deglycosylation reduces protein heterogeneity, it does not eliminate it. Hence, because of differences in isoelectric point and/or molecular mass shifts resulting from *O*-glycosylation and other modifications, most proteins appeared as multiple spots on 2D-PAGE gels, and many of the spots contained more than one protein (Fig. 1). This makes protein quantitation via gel scanning and densitometry quite difficult. To overcome this problem, we established a protein quantitation method that combines 1D-PAGE with stable isotope dilution. Proteins are first fractionated by 1D-PAGE (Fig. 2). Matching pairs of protein bands with the same molecular mass (with and without IC-3) were then excised from the 1D gel. Proteins were reduced with DTT, and cysteines were alkylated with either isotopically light *N*-ethyl-iodoacetamide (d0) or heavy *N*-d_5-ethyl-iodoacetamide (d5) and digested in-gel with trypsin. The tryptic digests were combined and analyzed by mass spectrometry.

Proteins that were identified from the 1D-PAGE gel included all the proteins that were identified in the 2D-gel experiments (see Fig. 1 and Table I). In addition, for those proteins from which data could be obtained for cysteine-containing peptides relative quantitation was determined by comparing the intensity of the d0 and d5 ions (Fig. 2). Two examples of these ion pairs used for quantitation are shown (Fig. 3). Comparison of the d0 *versus* d5 intensity revealed ratios close to 1 for peptides obtained from saposin, heat shock 73 protein, and *N*-glycosidase F (Fig. 2). A ratio of 1 was expected for the *N*-glycosidase F, because an equal amount of *N*-glycosidase F was added to each sample during the deglycosylation treatment. Saposin and heat shock 73 protein were among the most abundant proteins in the cell supernatant before lectin purification and represent non-metalloprotease-mediated shed and secreted proteins, respec-

TABLE I
Peptide sequences identified by tandem mass spectrometry following in-gel trypsin digestion of 2D-PAGE spots (Fig. 1)

Protein designation	Protein description[a]	Peptide sequences identified by MS/MS[b]	
A4	Alzheimer's disease amyloid A4 protein homolog	CLVGEFVSDALLVPDK	mDVCETHLHWHTVAK
		CVPFFYGGCGGNR	STNLHDYGmLLPCGIDK
		EQNYSDDVLANmlSEPR	VESLEQEAANER
	GenPept: P12023	ISYGNDALmPSLTETK	WYFDVTEGK
		mDAEFGHDSGFEVR	YLETPGDENEHAHFQK
AXLr	AXL receptor tyrosine kinase	CELQVQGEPPEVVWLR	DTQTEAGSPFVGNPGD^{C}ITGAR
		VPLQGTLLGYR	ISALQLSDAGEYQCMVHLEGR
	GenPept: NP_033491	GQDTPEVLmDIGLTR	EVTLELR
IL-1R-2	Interleukin 1 receptor, type II	EDLHTDFK	GTTTEPIPVIISPLETIPASLGSR
		EFLSAGDPTR	GNILWILPAVQQDSGTYICTFR
	GenPept: NP_034685	LDSSQLIPRLEGEPVVLR	
		GNKEFLSAGDPTR	VKGTTTEPIPVIISPLETIPASLGSR
		D^{C}ASHCEQmSVELK	LLISD^{C}TSmDDAGYYR
IL-6R-1	Interleukin 6 receptor, α	EWTTTGNTLVLR	SDFQVPCQYSQQLK
		ALEVAD^{C}GTVTSLPGATVTLICPGK	
	GenPept: P22272		
LDLr	Low density lipoprotein receptor	AVGSIGYLLFTNR	LYWVDSK
		CHSGECISLDK	NIYWTDSVPGSVSVADTK
	GenPept: 148623	IGSECLCPSGFR	NVVALDTEVTNNR
		IYWSDLSQK	SEYTSLLPNLK
		LAHPFSLAIYEDK	SWVCDGEADCK
		TILEDENR	LTGSDVNLVAENLLSPEDIVLFHK
		LHSISSIDVD^{C}GGNR	
L-selectin	Selectin, lymphocyte	EIEYLENTLPK	SKEDCVEIYIK
	GenPept: NP_035476	QNYTDLVAIQNK	SPYYYWIGIR
		MWTWVGTD^{C}K	QD^{C}YTDLVAIQNK
		AALCYTASCQPGSCD^{C}GR	
M-CSFR	Macrophage colony-stimulating factor 1 receptor	ASEAGQYFLmAQNK	VIIQSQLPIGTLK
		KLEFITQR	VLDSNTYVCK
		TVYFFSPWR	
	GenPept: P09581		
Met	Met proto-oncogene (hepatocyte growth factor receptor)	TGPVLEHPDCLPCR	YIHAFESNHFIYFLTVQK
		ETLDAQTFHTR	DNINmALLVDTYYDDQLISCGSVNR
		FCSVDSGLHSYmEmPLECILTEK	
	GenPept: NP 032617	FINFFVGNTID^{C}SSYPPGYSLHSISVR	
SHPS-1	SHP substrate 1 protein	LLIYSFTGEHFPR	
	GenPept: JC5289		
Saposin	Saposin precursor	EVVDSYLPVILDmIK	QLESNKIPEVDmAR
	GenPept: JH0604	LVSDVQTAVK	VVAPFmSNIPLLLYPQDHPR
		TD^{C}SSFIQGFVDHVK	
Tubulin	Tubulin, β5	YLTVAAVFR	lmNTFSVVPSPK
	GenPept: NP_035785	LHFFmPGFAPLTSR	ALTVPELTQQVFDAK
		GHYTEGAELVDSVLDVVR	

[a] Protein descriptions were obtained from Entrez, www.ncbi.nlm.nih.gov:80/entrez.
[b] Lowercase m indicates methionine sulfoxide.
[c] *N*-Glycosylation site, Asn is enzymatically converted to Asp because of the *N*-glycosidase F treatment.

tively. In contrast, several membrane proteins, including LDLr, amyloid A4 protein, AXLr, SHPS-1, and CD14, were determined to be in greater abundance in the sample lacking IC-3 (Fig. 2). We conclude that these proteins were shed via a metalloprotease that can be inhibited by IC-3.

This experiment was repeated several times, and the 1D-PAGE gel patterns were very reproducible with the exception of a very high molecular mass protein (>200 kDa) named hybrid receptor SorLA (GenPept: O88307). In most cases, the staining pattern indicated that SorLA was shed in the absence of IC-3 but not in the presence of the metalloprotease inhibitor. In a few cases (Fig. 2), the shedding of SorLA was not apparent. The reason for the absence of SorLA in this particular gel is unknown, but it may be because of variability in gel quality or that this large protein may not migrate reproducibly.

Identification of TACE-mediated Shedding in Monocytes—To link the above shedding events specifically with TACE activity, TACE−/− DRM cells (7) were reconstituted with full-length TACE. Comparison of the protein shedding profiles of the TACE-reconstituted cell line with that obtained

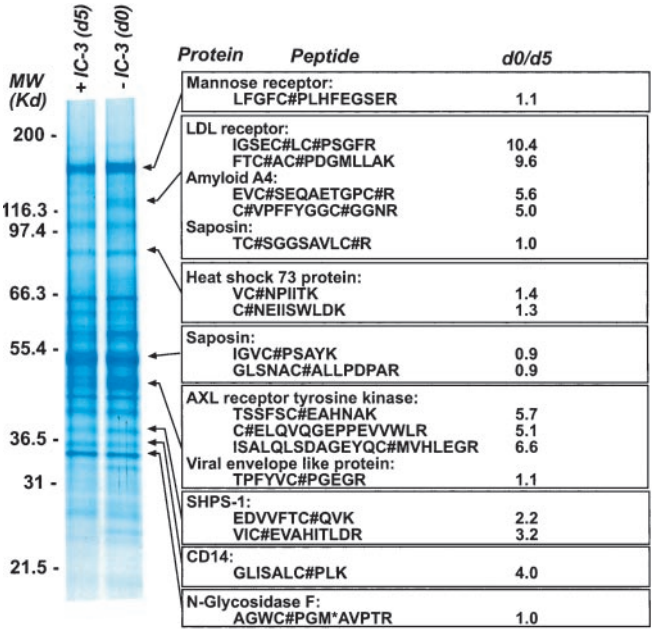

FIG. 2. **1D-PAGE gel of supernatant proteins from DRM TACE+/+ cells after WGA lectin-affinity purification and *N*-deglycosylation.** DRM TACE+/+ cells were stimulated with PMA for 90 min in the presence or absence of the metalloprotease inhibitor IC-3. Proteins obtained from equal numbers of cells (~1×10^9 cells) were loaded in each lane. Matching protein bands were excised from the gel, reduced with DTT, alkylated with either isotopically light (d0)- or heavy-form (d5) *N*-ethyl-iodoacetamide, and digested in-gel with trypsin. The peptides from matched bands were combined and analyzed by mass spectrometry. Ion intensity measurements were used for the determination of the d0/d5 ratios, which reflect the relative protein quantities in the mixtures. The staining pattern was reproducible with the exception of a band >200 kDa identified as hybrid receptor SorLA (*e.g.* see Fig. 4). In most cases, the gel staining showed that SorLA was shed in the absence of IC-3 and that shedding was inhibited by IC-3, indicating that this protein is also a metalloprotease-shed receptor. *C#* designates an alkylated cysteine; M* designates methionine sulfoxide.

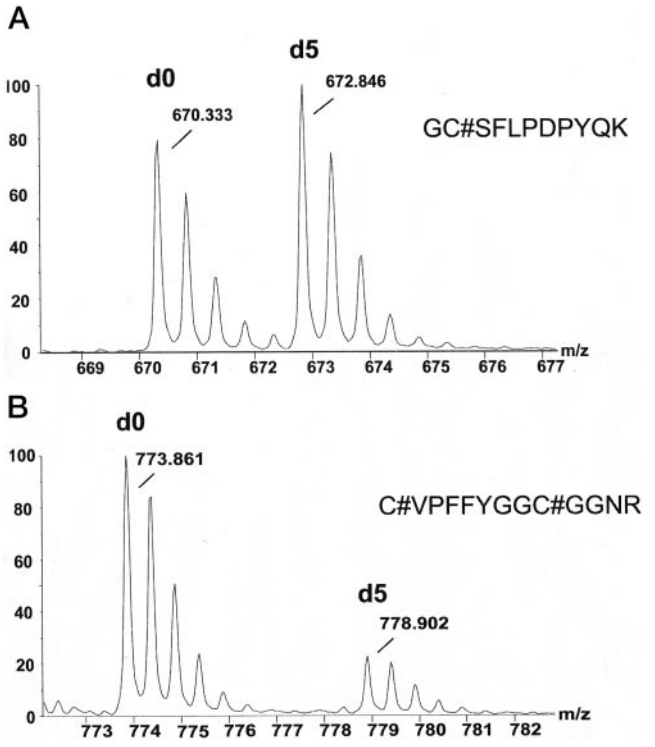

FIG. 3. **Expanded section of mass spectra showing examples of ion pairs used in the quantitation of peptide.** Mass difference of 5 or 10 Da were typically observed for the ion pairs, depending on the number of cysteines in a given peptide. *A*, the $(M + H_2)^{2+}$ ion of the peptide GC#SFLPDPYQK from saposin (see Fig. 4). *B*, the $(M + H_2)^{2+}$ ion of the peptide C#VPFFYGGC#GGNR from amyloid A4 (see Fig. 4). *C#* designates an alkylated cysteine.

from TACE−/− cells transfected with an empty vector revealed visible differences by 1D-PAGE (Fig. 4). Quantitative analysis of selected areas cut from the 1D-PAGE gel showed changes in peptide quantities for several proteins, including hybrid receptor SorLA, LDLr, amyloid A4, AXLr, IL-1R-2, and IL-6R-1. These proteins are therefore most likely shed by TACE.

Identification of Metalloprotease-shed Proteins in Endothelial Cells—To determine whether this approach can be used to identify proteins shed by other cell types, we carried out a study with HMVECs. HMVECs were treated with a mixture of inflammatory cytokines followed by PMA to induce shedding, and the cell supernatant was compared with supernatant from unstimulated HMVECs. After lectin-affinity purification and *N*-deglycosylation, the supernatant proteins were analyzed by 1D-PAGE (Fig. 5). Overall, the two protein profiles were very similar, and some of the discrepancies could be attributed to the cytokines added as part of the cell stimulation (*e.g.* the band labeled interferon-γ). However, two HMVEC-derived proteins, Jagged 1 and endothelial cell protein C receptor, were identified from protein bands that appear to be of greater staining intensity in the cytokine/PMA-treated sample (Fig. 5). Protein quantification using the isotope-coded differential cysteine labeling method demonstrated that these two proteins were indeed more abundant in the stimulated cell supernatant (Fig. 5). Although we did not determine the effect of IC-3 on their release, both are transmembrane proteins and thus likely to be released by shedding. In fact, endothelial cell protein C receptor was previously identified as a metalloprotease-shed protein in endothelial cells (22), thus validating the method as applied to HMVECs.

DISCUSSION

Several proteins already known or implicated as metalloprotease-shed proteins were identified in this study using two different cell systems. These include amyloid A4 protein, IL-1R-2, IL-6R-1, L-selectin, M-CSFR, SorLA, AXLr, and endothelial cell protein C receptor (7, 8, 12, 13, 22–27). Thus, this proteomic technique was validated as a method that can be applied in studies of protein shedding. In addition, this study implicated a number of additional proteins as being shed by metalloproteases, including LDLr, SHPS-1, and Jagged 1.

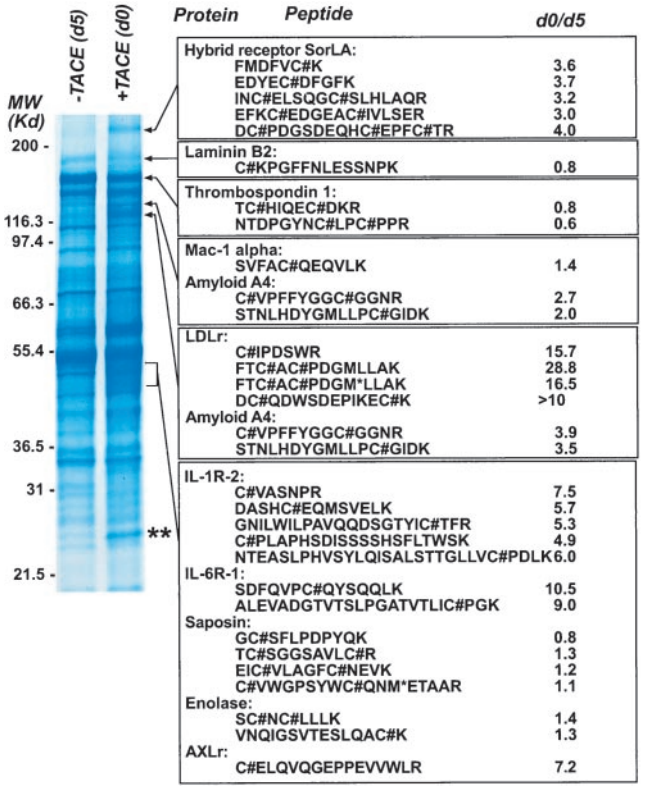

FIG. 4. **1D-PAGE gel of supernatant proteins from PMA-stimulated DRM.** TACE−/− cells and PMA-stimulated DRM TACE−/− cells reconstituted with full-length TACE, following WGA lectin-affinity purification and *N*-deglycosylation, are shown. Proteins obtained from equal number of cells (~1×10^9 cells) were loaded in each lane. Matching protein bands were excised from the gel, reduced with DTT, alkylated with either isotopically light (d0) or heavy form (d5) *N*-ethyl-iodoacetamide, and digested in-gel with trypsin. Tryptic peptides were combined and analyzed by mass spectrometry. Ion intensity measurements were used for the determination of the d0/d5 ratios, which reflects the relative protein quantities in the two protein mixtures. The protein band marked with ** apparently contained protein(s) that were more abundant in TACE-containing cells in comparison to the control cells. Proteins identified from this band include peroxiredoxin 1 (P35700), endothelial protein C receptor (Q64695), and oncostatin M (S64719). Because none of the cysteine-containing peptides were recovered from these proteins, no quantitative measurement could be derived from the data. *C#* designates an alkylated cysteine.

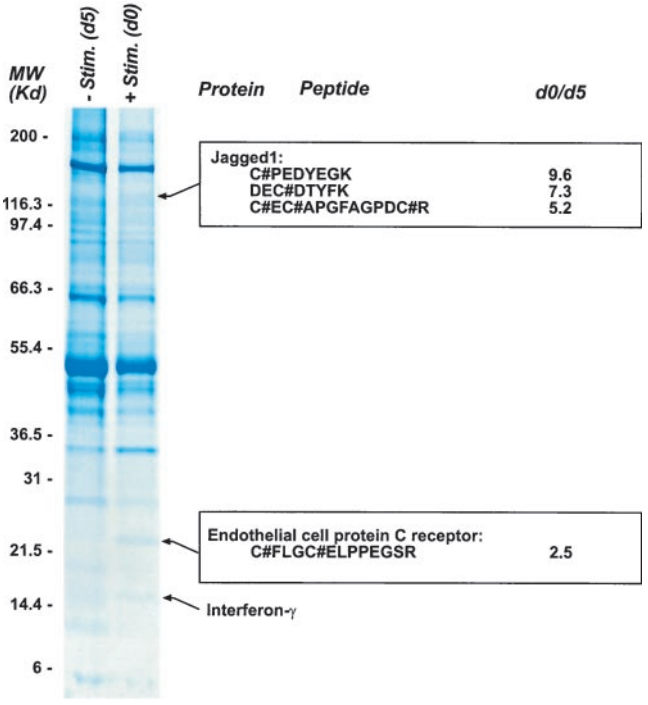

FIG. 5. **1D-PAGE gel of supernatant proteins from HMVECs following WGA lectin-affinity purification and *N*-deglycosylation.** HMVECs were either untreated or stimulated with cytokines followed by PMA to induce shedding. Proteins obtained from 8×10^6 cells were loaded in each lane. Matching protein bands were excised from the gel, reduced with DTT, alkylated with either isotopically light (d0) or heavy form (d5) *N*-ethyl-iodoacetamide, and digested in-gel with trypsin. Tryptic peptides were combined and analyzed by mass spectrometry analysis. Ion intensity measurements were used for the determination of the d0/d5 ratios, which reflect the relative protein quantities in the two protein mixtures. *C#* designates an alkylated cysteine.

TACE was shown to be the responsible protease in the case of the LDLr and some of the previously identified shed proteins (*e.g.* AXLr and hybrid receptor SorLA) for which the sheddase had not been determined.

LDLr is known as a cell-surface receptor that binds to LDL, the major cholesterol-carrying lipoprotein in plasma, and transports LDL into cells by endocytosis (28). Other LDLr gene family proteins, including SorLA (see Fig. 4, a shed protein found here to be released by TACE) have been found to engage in a wide range of biological functions (29). The transmembrane glycoprotein SHPS-1 is a physiological substrate for protein-tyrosine phosphatase SHP-2 and belongs to an inhibitory receptor superfamily. SHPS-1 is abundantly expressed in macrophages and neural tissue and has been implicated in regulating intracellular signaling events downstream of receptor protein-tyrosine kinases and integrin-mediated cytoskeletal reorganization and cell motility (30). Jagged 1 is a ligand for the receptor Notch 1. Jagged 1 signaling through Notch 1 has been shown to play a role in hematopoiesis. The functional significance of metalloprotease-mediated shedding of LDLr, SHPS-1, and Jagged 1 remains to be explored further.

Global proteome displays on 2D-PAGE may largely be limited to the more abundantly expressed and stable proteins (20, 21), but applying targeted protein isolation and modification procedures prior to 2D-PAGE may yield meaningful results. As demonstrated here (Fig. 1), a group of low abundance proteins, most of which serve as immunoregulatory proteins, can be effectively displayed on a 2D-PAGE if the starting material (short-term cell supernatants in this case) is carefully selected and the electrophoresis is preceded by a lectin-affinity fractionation and deglycosylation. Moreover, even 1D-PAGE, a low cost, reproducible, and rapid method

for comparing and characterizing proteins, was found to be effective with these samples. By combining appropriate sample preparation, 1D-PAGE, isotope dilution, and mass spectrometry, we have demonstrated a method for comparing the relative abundance of proteins in complex mixtures.

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‡ To whom correspondence should be addressed. Tel.: 206-381-6412; Fax: 206-621-5440; E-mail: JohnsonR@immunex.com.

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