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Abstract

In contrast to the knowledge regarding the function of chimeric Ewing sarcoma (EWS) fusion proteins that arise from chromosomal translocation, the cellular function of the RNA binding EWS protein is poorly characterized. EWS protein had been found mainly in the nucleus. In this report we show that EWS protein is not only found in the nucleus and cytosol but also on cell surfaces. After cell-surface biotinylation, isoelectric focusing of membrane fraction, avidin-agarose extraction of biotinylated proteins, and SDS-polyacrylamide gel electrophoresis, EWS protein was identified by matrix-assisted laser desorption ionization and nanoelectrospray tandem mass spectrometry of in-gel-digested peptides. These analyses revealed that the protein, having repeated RGG motifs, is extensively asymmetrically dimethylated on arginine residues, the sites of which have been mapped by mass spectrometric methods. Out of a total of 30 Arg-Gly sequences, 29 arginines were found to be at least partially methylated. The Arg-Gly-Gly sequence was present in 21 of the 29 methylation sites, and in contrast to other methylated proteins, only 11 (38%) methylated arginine residues were found in the Gly-Arg-Gly sequence. The presence of Gly on the C-terminal side of the arginine residue seems to be a prerequisite for recognition by a protein-arginine N-methyltransferase (PRMT) catalyzing this asymmetric dimethylation reaction. One monomethylarginine and no symmetrically methylated arginine residue was found. The present findings imply that RNA-binding EWS protein shuttles from the nucleus to the cell surface in a methylated form, the role of which is discussed.
Exposure on Cell Surface and Extensive Arginine Methylation of Ewing Sarcoma (EWS) Protein*

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In contrast to the knowledge regarding the function of chimeric Ewing sarcoma (EWS) fusion proteins that arise from chromosomal translocation, the cellular function of the RNA binding EWS protein is poorly characterized. EWS protein had been found mainly in the nucleus. In this report we show that EWS protein is not only found in the nucleus and cytosol but also on cell surfaces. After cell-surface biotinylation, isoelectric focusing of membrane fraction, avidin-agarose extraction of biotinylated proteins, and SDS-polyacrylamide gel electrophoresis, EWS protein was identified by matrix-assisted laser desorption ionization and nanoelectrospray tandem mass spectrometry. This analysis revealed that the protein, having repeated RGG motifs, is extensively asymmetrically dimethylated on arginine residues, the sites of which have been mapped by mass spectrometric methods. Out of a total of 30 Arg-Gly sequences, 29 arginines were found to be at least partially methylated. The Arg-Gly sequence was present in 21 of the 29 methylation sites, and in contrast to other methylated proteins, only 11 (38%) methylated arginine residues were found in the Gly-Arg-Gly sequence. The presence of Gly on the C-terminal side of the arginine residue seems to be a prerequisite for recognition by a protein-arginine N-methyltransferase (PRMT) catalyzing this asymmetric dimethylation reaction. One monomethylarginine and no symmetrically methylated arginine residues were found. The present findings imply that RNA-binding EWS protein shuttles from the nucleus to the cell surface in a methylated form, the role of which is discussed.

While investigating a 90-kDa anti-cyclophilin (anti-CyP)1 immunoreactive band we noticed that anti-CyP antibodies recognized the RNA-binding Ewing sarcoma (EWS) protein and not a cyclophilin. The EWS gene is involved in tumor-related chromosomal translocations that associate part of EWS gene with various genes encoding transcription factors (1). The N-terminal transcriptional activation domain of EWS is fused to C-terminal DNA binding domains of corresponding partners. The translocation produces chimeric EWS proteins with transforming potential (2–7). The EWS gene of Ewing sarcoma and primitive neuroectodermal tumor is translocated to one of different members of the ETS (erythroblastosis virus-transforming sequence) family that contains the highly conserved DNA binding ETS domain. Often the ETS domain is derived from Fli-1 (Friend leukemia integration-1) and in rare cases from ERG (ETS-related gene), ETV-1 (ETS translocation variant-1), E1AF (E1A factor), or FEV (fifth Ewing variant). In malignant melanoma of soft parts, EWS is fused to ATF-1, in intrabdominal desmoplasmic small round-cell tumor to WT-1, in myoid liposarcoma to CHOP, and in myocard chordrosarcoma to CHN (8).

The cellular role of wild-type EWS protein remains less clear. The EWS protein is a nuclear protein with unknown function containing a C-terminal RNA binding motif and a N-terminal activation domain (9–11). The IQ domain of the EWS protein is involved in calmodulin binding and protein kinase C phosphorylation (12). EWS protein interacts with an SH3 domain of Bruton’s tyrosine kinase and has been identified in B cells as a phosphotyrosine-containing protein (13). G-coupled receptor signaling and other stimuli of tyrosine kinase Pyk2 block the interaction between EWS protein and Pyk2. Partitioning of the EWS protein into a ribosome-associated fraction indicated that the role for EWS in gene expression includes an extranuclear action (14).

In the present investigation we show the EWS protein is not only localized in the nucleus and cytosol but also on the surface of cells and that it is posttranslationally methylated at arginine residues. The identified \( \omega^\text{N}-\) dimethylarginine residues of EWS protein let us modify a previously reported consensus sequence for asymmetric dimethylarginine formation in proteins.

EXPERIMENTAL PROCEDURES

Materials—Stock cultures of Jurkat cells, a tumor T lymphoma cell line, were kindly provided by Dr. J. Kemler-Carraro (Abteilung Klinische Immunologie, Universitätsklinikum Zürich). Peripheral blood mononuclear (PBM) cells and human cutaneous T lymphoma cell line (H9) were obtained from the National Center for Retroviruses (University of Zürich). Purified polyclonal antibodies raised against human CyPA and CyPB (anti-CyP) were kindly provided by G. W0erly (Toxikologisches Institut der Universitat Zürich, Schwerzenbach). Polyclonal antibody 677 against the N terminus of EWS protein (anti-EWS) was a generous gift from Dr. Olivier Delattre (Institut Curie, Pathologie Moléculaire des Cancers, Paris Cedex).

Cell Cultures—Human Jurkat cells were grown in RPMI 1640 medium (Sigma) supplemented with 5% newborn calf serum (Life Technologies), 15 mM HEPES, 2 mM l-glutamine, 50 \( \mu \)g/ml streptomycin, and 1% (v/v) penicillin and streptomycin (Life Technologies) in a humidified 5% CO\(_2\) atmosphere at 37 °C. PBM cells were isolated by Ficol-Hypaque gradient centrifugation. The cells were washed with phosphate-buffered saline, pH 7.4 (PBS), and stimulated with 2 \( \mu \)g/ml phytohemagglutinin in RPMI 1640 medium supplemented with 20%
fetal calf serum (Life Technologies), 15 mM HEPES, 2 mM t-glutamine, 50 μM β-mercaptoethanol, and 1% (w/v) penicillin and streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. Human H9 cell line was grown in RPMI 1640 medium supplemented with 20% fetal calf serum, 15 mM HEPES, 2 mM t-glutamine, 50 μM β-mercaptoethanol, and 1% (w/v) penicillin and streptomycin in a humidified 5% CO₂ atmosphere at 37 °C.

Biotinylation of Cell Surface Proteins—Cell-surface biotinylation was performed as described (15) with some modification. 2 × 10⁶ cells were washed three times with ice-cold PBS, suspended in PBS (25 × 10⁶ cells/ml), and incubated with 0.5 mg/ml sulfosuccinimidobiotin (Calbiochem). After 15 min of incubation, the biotinylation procedure was stopped replacing the labeling solution with culture medium. After incubation for 10 min at 4 °C, cells were washed twice with PBS. Biotinylated or non-labeled cells were solubilized (25 × 10⁶ cells/ml) in lysis buffer containing 1% (w/v) Triton X-100, PBS, protease inhibitor mixture (20 μg/ml pancreas extract, 5 μg/ml Pronase, 0.5 μg/ml thymolysin, 3 μg/ml chymotrypsin, 330 μg/ml papain; Roche Molecular Biochemicals), 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol for 40 min on ice with occasional vortexing. The cell lysates were centrifuged at 480 × g for 5 min followed by 15000 × g for 15 min. Obtained supernatants were then subjected to isoelectric focusing followed by avidin-agarose extraction or directly to avidin-agarose extraction and immunoprecipitation experiments. The Bio-Rad protein assay was used to determine protein concentrations.

Isolation of Membrane-enriched Fractions—All steps were performed at 4 °C. Biotinylated or nonlabeled cells were washed (108 cells/ml) with ice-cold PBS. Pelleted cells were suspended (5 × 10⁸ cells/ml) in homogenizing buffer containing 25 mM Tris-HCl, pH 7.6, 10% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and protease inhibitor mixture. Cells were homogenized with a Dounce homogenizer (20 strokes), and the suspension was centrifuged at 500 × g for 15 min, and the supernatant was centrifuged at 15,000 × g for 10 min, and a membrane pellet obtained at 150,000 × g for 1 h was used for further purification. This supernatant was designated as cytosolic fraction. Pellets were solubilized in 8 M urea, 2% (w/v) CHAPS, 15 mM dithiothreitol, 2% (v/v) Bio-Lyte ampholytes (Bio-Rad), pH 3–10, for 40 min at room temperature. Insoluble material was removed by centrifugation at 10,000 × g for 15 min, and the supernatant was designated as membrane fraction.

Isoelectric Focusing—Isoelectric focusing using a Mini Rotofer cell (Bio-Rad) was done as recommended by the supplier. After cell-surface biotinylation, part of the detergent lysates (17 mg) or solubilized membrane fraction (15 mg) were added to the Rotophor cell containing 13 ml of 8 M urea, 2% (w/v) CHAPS, 15 mM dithiothreitol, 2% (v/v) Bio-Lyte ampholytes, pH 3–10, after prefocusing for 1 h at 12 W. Focusing was carried out at 12 W for 5 h at 10 °C. The 20 fractions were harvested, and their pH values were measured. After Western blot analysis, the fractions of interest were pooled and concentrated with Centricron (YM-30, Millipore), and biotinylated proteins were extracted with avidin-agarose.

Avidin-Agarose Extraction—Either the detergent lysate or membrane fraction pooled after isoelectric focusing was incubated with avidin-agarose (Sigma) overnight on a rotary device at 4 °C. The affinity gel was then washed three times with 1% (w/v) Triton X-100, 0.2% (w/v) SDS, 5 mM EDTA in PBS followed by a wash without SDS and one with water. The captured biotinylated proteins were eluted from the agarose with reducing 2-fold Laemmli buffer heated for 5 min at 95 °C.

In-gel Digestion and Mass Spectrometry—Proteins were separated by SDS-PAGE (7.5%, 0.75 mm thickness) and stained with 0.2% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) ethanol and 10% (v/v) acetic acid for 30 min. Protein bands of interest were excised from the gel and in-gel-digested with trypsin (Promega) or chymotrypsin (Roche Molecular Biochemicals) following the procedure of Shevchenko et al. (16). Upon reduction of the disulfide bonds of the protein with tris(2-carboxyethyl)phosphine) hydrochloride (Pierce), cysteines were alkylated with iodoacetamide (Sigma). Digestions with trypsin (400 ng) were carried out overnight at 37 °C in 100 mM ammonium bicarbonate buffer, pH 8.3, and 4 mM calcium chloride. Digestions with chymotrypsin (500 ng) were performed overnight at 25 °C in the same buffer, pH 8.0. The resulting peptides were extracted from the gel pieces and desalted using pipette tips with C₂⁺ discs (Millipore). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra of the entire digests were recorded on a Bruker Biflex instrument in the reflector mode employing pulsed ion extraction. α-Cyano-4-hydroxycinnamic acid (Fluka) was used as the matrix. The MALDI mass spectra were mass calibrated using ion signals mainly from autoprotopeolytic fragments of trypsin and chymotrypsin, respectively. Electrospray ionization mass spectra (MS) and tandem mass spectra (MS/MS) were acquired on an API III+ triple-quadrupole instrument (PE-Scieix, Ontario, Canada) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark).

Immunoprecipitation—The detergent lysates of cells were preincubated with protein A-Sepharose (Amersham Pharmacia Biotech) for 2 h. Anti-CyP antisera or anti-EWS antisera were added to the supernatants, and the mixture was incubated at 4 °C for at least 12 h, and then protein A-Sepharose was added for 2 h. The agarose pellet was washed three times with 1% (w/v) Triton X-100, 2 mM EDTA, and 10 mM Tris-HCl, pH 7.4, and the antigen was eluted from the gel with reducing 2-CyP antibodies (C). The molecular masses of marker proteins are indicated (in kDa).

Western Blotting and Protein Detection by Enhanced Chemiluminescence—Proteins separated on 7.5% SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane (Schleicher and Schuell) as described (17). After transfer, the membrane was blocked for 40 min with 5% (w/v) milk powder dissolved in PBS and 0.25% (w/v) Tween 20 and incubated with the first antibodies anti-CyP or anti-EWS, respectively, for 1 h at room temperature, followed by incubation with goat anti-rabbit antibody coupled to horseradish peroxidase (Sigma) for 1 h at room temperature. The detection was performed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

RESULTS

Partial Purification of a 90-kDa Cell-surface Protein—Cell-surface proteins of Jurkat and PBM cells were labeled under physiological conditions with the impermeable biotinylation reagent. Labeled surface proteins were extracted with avidin-coupled agarose and separated by SDS-PAGE. Western blot analysis with anti-CyP antibodies showed a strong signal of a protein band with a molecular mass of about 80 kDa (Fig. 1A). To obtain a pure protein in amounts high enough for identification, a membrane fraction of Jurkat and PBM cells was prepared by differential centrifugation after cell-surface biotinylation. The solubilized proteins were further purified by isoelectric focusing under denaturing conditions. The anti-CyP reactive protein was found in a pH range from 7.2 to 9.3. These fractions were pooled, and the biotinylated proteins extracted with avidin-agarose and subjected to SDS-PAGE. The Coomassie-stained band (Fig. 1B) containing the immunoreactive 90-kDa protein of interest (Fig. 1C) was clearly separated from other proteins and was sufficiently pure to be subjected for an identification procedure.
Identification of the 90-kDa Band—The 90-kDa Coomassie-stained gel band containing the protein of interest was excised and in-gel-digested with trypsin, and the resulting peptides were analyzed by MALDI-MS (see "Experimental Procedures"). A protein sequence database search (Mascot Search) performed with the obtained peptide masses (Table I) did not provide an unequivocal result. RNA-binding protein EWS (1) was retrieved with the highest score; however, some of the most intense signals of the MALDI mass spectrum could not be assigned to this protein. To confirm the identification of the protein, nanoelectrospray MS/MS sequencing of selected peptides was performed. Four MS/MS spectra were in complete agreement with the predicted fragmentation pattern of peptides 269–292, 411–424, 425–439, and 472–486 from RNA-binding EWS protein and, thus, unambiguously verified the identification of this protein (Fig. 2, Table I).

Assignment of Arginine Methylation Sites—The tandem mass spectrum shown in Fig. 3 appeared to match peptide 615–632 from this protein, but the observed mass was 28 Da higher than the mass calculated from the amino acid sequence. Detailed analysis of the spectrum indicated that this peptide most likely contains a dimethylated arginine residue located at Arg-471.

### Table I

**Summary of mass spectrometric data of proteolytic fragments from the in-gel-digested protein (see Fig. 1B)**

<table>
<thead>
<tr>
<th>Residue numbers in EWS protein</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg-503 was not methylated in this peptide.</td>
<td>Dimethylation of Arg-503, which is a potential methylation site; Arg-471 is mono-methylated in this peptide.</td>
</tr>
<tr>
<td>Assignment confirmed by MS/MS.</td>
<td>Dimethylation of Arg-490 (potential methylation site); Arg-903 was not modified in this peptide.</td>
</tr>
<tr>
<td>Dimethylation of Arg-615 confirmed by MS/MS.</td>
<td>Dimethylation of Arg-415 confirmed by MS/MS.</td>
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<table>
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<tr>
<th>Observed mass</th>
<th>Predicted peptide mass</th>
<th>Digestion with chymotrypsin</th>
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<td>18683</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
</tr>
<tr>
<td>2897.46</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
</tr>
<tr>
<td>1777.83</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
</tr>
<tr>
<td>3226.65</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
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</table>

<table>
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<tr>
<th>Observed mass</th>
<th>Predicted peptide mass</th>
<th>Digestion with trypsin/</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1149.56</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
<td></td>
</tr>
<tr>
<td>1177.53</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
<td></td>
</tr>
<tr>
<td>1507.74</td>
<td>GQDPRTSQQPQGGMF</td>
<td>Assignment confirmed by MS/MS.</td>
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</tbody>
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<th>Notes</th>
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</thead>
<tbody>
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<td>Arg-615 confirmed by MS/MS.</td>
<td>Dimethylation of Arg-581 confirmed by MS/MS.</td>
</tr>
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<td>Dimethylation of Arg-581 confirmed by MS/MS.</td>
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</tr>
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<td>Dimethylation of Arg-615 confirmed by MS/MS.</td>
<td>Dimethylation of Arg-415 confirmed by MS/MS.</td>
</tr>
</tbody>
</table>

**Identification of the 90-kDa Band**

Dimethylarginines in EWS Protein
position 615 (Table I). Another MS/MS spectrum was found to be consistent with peptide 465–486 containing a dimethylarginine residue at position 471. Re-examination of the MALDI mass spectrum of the tryptic digest revealed the presence of eight additional dimethylarginines located in the C-terminal arginine- and glycine-rich domain of the protein (Fig. 2). Arginines 464, 471, 490, and 503 appear to be dimethylated to a large extent but not completely. Trypsin does not cleave after methylated arginines (18, 19). Analyses of tryptic peptides indicated cleavages at these four arginines that were found to be modified in other peptides. In addition, peptides containing the non-methylated arginines 471 and 503 were identified. A small fraction of arginine 471 (∼15%) also occurs in the monomethylated form (Table I, peptide 465–486).

Fig. 2. Amino acid sequence of the EWS protein. The C-terminal arginine- and glycine-rich domain of RNA-binding protein EWS contains 30 Arg-Gly sequences. Completely dimethylated arginine residues are in bold and underlined, and arginines found to be either dimethylated or non-methylated are only in bold. Arginine 471 (shown in bold and in italics) is either dimethylated or non-methylated and also monomethylated to a small extent. No proteolytic peptide containing arginine 330, which represents a potential site of arginine methylation, had been identified. Regions of protein covered by peptide fragments from MS data (Table I) are highlighted.

Fig. 3. Tandem mass spectrum of the tryptic peptide 615–632 of EWS protein. Nanoelectrospray tandem mass spectrum of the triply charged ion at m/z 593.5 corresponding to tryptic peptide RGGPGGPPGGLMEQMGGR (R, dimethylarginine). The singly and doubly charged fragment ions are labeled according to the Roepstorff-Biemann nomenclature (38).
Immunoprecipitated proteins were subjected to Western blot analysis with antibodies (Fig. 4) as well as with anti-EWS antibody (lane 2) was performed. Immunoprecipitated proteins were subjected to Western blot analysis using the anti-CyP antibodies (A) or the anti-EWS antibody (B). For quantification of the bands, the ECL films were scanned, and intensities of the bands were calculated by using Multi-Analyst software (Bio-Rad). The large band at 55 kDa (lanes 1 and 2) corresponds to the heavy chain of the antibodies. Molecular mass standards (kDa) are indicated.

Cross-reactivity of EWS Protein with Anti-CyP Antibodies—The detection of an immunoreactive band with anti-CyP antibodies (Fig. 4) as well as with anti-EWS antibody (Fig. 4B). In any case a stronger signal was obtained in the Western with anti-EWS antibody even if immunoprecipitation was done with anti-CyP antibodies, indicating cross-reactivity of both antibodies with EWS protein. No signals were observed if (when) goat anti-rabbit antibody alone or rabbit antibodies against several other proteins was used.

Excision of the immunoprecipitated 90-kDa Coomassie-stained bands, in-gel digestion with trypsin, and MALDI-MS analysis of tryptic peptides confirmed the presence of methylated RNA-binding protein EWS in anti-CyP and anti-EWS-precipitated samples. Occurrence of Cell Surface-localized EWS Protein in Different Cell Lines—To ascertain that EWS protein is indeed present on the cell surface, Jurkat, H9, and PBMC cells (PBMC) were biotinylated with sulfo-LC-biotin. Equal amounts of cell proteins were extracted as described in Fig. 1A. Aliquots were separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot using anti-EWS antibody (A). Samples obtained by cell surface biotinylation, membrane fractionation, and isoelectric focusing followed by avidin-agarose extraction (see Fig. 1B) were separated on 7.5% SDS-PAGE and subjected to Western blot analysis with anti-EWS antibody (B). Molecular mass standards (kDa) are indicated.

DISCUSSION

Our data show that the anti-CyP immunoreactive protein located on the surface of T cells is not a cyclophilin but the EWS protein. The anti-CyP antibodies cross-react with the EWS protein as demonstrated by immunoprecipitation experiments. The cause of the cross-reactivity is not obvious. Global alignment of the EWS protein sequence either with human CyPA or CyPB using the program LALIGN revealed a low degree of identity (8.8%) in both cases, and some of the identity seems to be due to numerous glycines present in the proteins. The cross-reactivity led us, however, to the finding that the EWS protein is not only exposed on the cell surface of different cells but also that its arginine residues are extensively and asymmetrically dimethylated.
Dimethylargininines in EWS Protein

<table>
<thead>
<tr>
<th>Gly/Phe</th>
<th>Gly</th>
<th>Gly</th>
<th>Arg</th>
<th>Gly</th>
<th>Gly</th>
<th>Gly/Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>~3</td>
<td>~2</td>
<td>~1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>EWS protein (29 sites)</td>
<td>45/30</td>
<td>52/15</td>
<td>38/100</td>
<td>100/100</td>
<td>72/100</td>
<td>14/14</td>
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<td>Review (20 sites) (from Ref. 22)</td>
<td>35/15</td>
<td>75/100</td>
<td>80/100</td>
<td>100/100</td>
<td>75/100</td>
<td>55/100</td>
</tr>
<tr>
<td>hnRNP A1 (3 sites) (from Ref. 29)</td>
<td>33/33</td>
<td>33/100</td>
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<td>33/33</td>
<td></td>
</tr>
<tr>
<td>High molecular weight fibroblast growth factor-2 (8 sites) (from Ref. 30)</td>
<td>50/0</td>
<td>38/50</td>
<td>88/100</td>
<td>100/88</td>
<td>0/180</td>
<td>0/18</td>
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<tr>
<td>Total (60 sites)</td>
<td>42/15</td>
<td>57/100</td>
<td>61/100</td>
<td>98/100</td>
<td>65/100</td>
<td>27/18</td>
</tr>
</tbody>
</table>

a–e Other amino acid residues found in these positions (%); a Asp, 3; Leu, 3; Met, 3; Asn, 7; Pro, 7; Arg, 28; b Asp, 3; Glu, 3; Phe, 3; Met, 14; Pro, 7; Arg, 10; Ser, 3; c Asp, 21; Phe, 7; Leu, 3; Met, 7; Asn, 3; Pro, 7; Arg, 7; Ser, 7; d Met, 7; Asn, 3; Pro, 3; Arg, 10; Ser, 3; e Asp, 10; Leu, 7; Met, 7; Pro, 24; Arg, 24.

The previously reported localization of EWS protein in the nucleus (20) and the cytosol (14), both of which we confirmed (data not shown), together with the present finding of the EWS protein to be accessible on the cell surface means that this protein shuttles between the nucleus, cytosol, and the cell surface. A similar behavior was reported for nucleolin. This major nucleolar protein shuttles between the cytosol and the nucleus and has also been detected on the cell surface of different cells. The C-terminal domain of nucleolin is, as in the EWS protein, rich in glycine residues and interspersed with dimethylarginines. It was suggested as a potential receptor in the human immunodeficiency virus binding processes by interaction with the V3 loop of gp120 (21). So far we found cell-surface-exposed EWS protein in all investigated cells, i.e. Jurkat, H9, C816645 T cell lines, and PBMC cells, but also NIH3T3 fibroblasts (not shown). Remarkably, tumor cell lines showed a higher level of EWS protein expression on the cell surface (~4-fold) than PBMC cells and fibroblasts.

**Recognition Sequence of Asymmetric Methylation Sites**—Arginine methylation is a post-translational modification found mainly in nuclear proteins that interact with RNA (22). This modification is catalyzed by protein-arginine N-methyltransferases (PRMTs), utilizing S-adenosyl-l-methionine as the donor of methyl groups (23). Type I protein-arginine N-methyltransferases (EC 2.1.1.23) catalyze the formation of N\(^{\text{c}}\)-monomethylarginine and asymmetric N\(^{\text{c}}\)-N\(^{\text{e}}\)-dimethylarginine residues, whereas Type II enzymes catalyze the formation of N\(^{\text{c}}\)-monomethylarginine and symmetric N\(^{\text{c}}\)-N\(^{\text{e}}\)-dimethylarginine residues (22). The Type III enzyme found in yeast catalyzes the monomethylation of the internal \(\delta\)-guanidino nitrogen atom of arginine residues (24). Three Type I PRMTs from mammalian cells PRMT1 (25), PRMT3 (26), and coactivator-associated arginine methyltransferase 1 (CARM1) (27) have been reported. PRMT1, a predominantly nuclear protein, methylates arginine residues of many proteins, among them RNA-binding proteins (22, 28). PRMT3 is a predominantly cytoplasmic protein whose activity overlaps with that of PRMT1 (26). A number of in vivo substrates for Type I PRMTs have been identified, including the heterogeneous nuclear ribonucleoprotein A1 (29), fibrillarin, nucleolin (22), high molecular weight fibroblast growth factor-2 (30), and poly(A)-binding protein II (28).

The sites of methylated or dimethylated arginine residues had been directly determined by Edman sequencing or by a combination of Edman sequencing and mass spectrometric approaches. In this study, the arginine dimethylation sites of the EWS protein from the cell surface of Jurkat and H9 cell lines were elucidated by mass spectrometric peptide mapping and sequencing. We found up to the present the EWS protein to be the most extensively methylated protein in vivo in higher eukaryotes. From sequences of known sites of asymmetric arginine methylation, a preferred recognition motif (G/F)-GRRGG(G/P) had been proposed (Refs. 22 and 29; Table II). The shorter sequence GRG had also been suggested as a specificity determinant for arginine methylation by a Type I enzyme, although the Gly at position ~1 could be replaced by a few amino acids other than glycine (31). Only the Gly +1 C-terminal of the arginine was found to be completely conserved. In the present study, all 29 identified dimethylarginines are indeed followed by a glycine (Table II). Conversely, all arginines in Arg-Gly sequences mapped by mass spectrometry (29 out of a total of 30 Arg-Gly sequences) were at least partially methylated. The sequence Arg-Gly-Gly is present in 21 of the 29 methylation sites; however, only 11 (38%) methylated arginine residues were found in a Gly-Arg-Gly sequence. The prevalence of Gly at positions ~2 and ~3 in EWS protein is even higher than at position ~1. Obvious deviations from the suggested consensus sequence are the small number of phenylalanines at position ~2 and ~3 and of glycines at position ~3. Thus, the suggested recognition sequence for asymmetric arginine dimethylation is only partially consistent with the sequences identified in this study. Only the presence of the Gly +1 C-terminal of the arginine residue is a strict requirement for asymmetric dimethylation but not the Gly N-terminal of the arginine. The found differences may be attributed to an insufficiency of defined consensus sequence based on only 29 in vivo arginine methylation sites or to different Type I PRMTs with similar specificity that are involved in methylation of the arginine residues in EWS protein. Arginine methylation sites very distinct from those found in EWS protein and the other sites listed in Table II were observed in poly(A)-binding protein II. In this protein, almost all of the asymmetrically dimethylated and monomethylated arginines were found in Arg-Xaa-Arg cluster (28).

**Role of Methylation of EWS Protein**—EWS protein belongs to a large family of RNA-binding proteins that includes heterogeneous nuclear ribonucleoproteins, mRNA poly(A)-binding proteins (32), and alternative splicing factors (33, 34). The methylation state of RNA-binding proteins (Table II) is likely to have an important effect on their function. One type of methylated RNA binding domain consists of RGG boxes, defined as closely repeated RGG tripeptides interrupted by other amino acids. The RNA binding of EWS protein is localized in the C-terminal domain, which contains RGG boxes (Ref. 10; Fig. 2, residues 288–656). Considering the RNA binding properties of these proteins, arginine methylation in the RNA binding domain may regulate the RNA-EWS protein interaction most likely by preventing hydrogen bonding, by short range ionic interactions, and/or by introducing steric constraints and therefore diminishes otherwise strong interactions between unmodified arginine residues and RNA (35). The methylated form of one of the well studied shutting heterogeneous nuclear
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ribonucleoproteins, A1, showed decreased affinity for single-stranded DNA compared with the unmethylated form (36).

The changes in the physico-chemical properties, e.g. increased hydrophobicity, loss of hydrogen bonding, and ionic interactions resulting from arginine methylation of nuclear as well as of cell-surface-localized EWS protein, might favor crossing the various cellular membranes or its embedding in the plasma membrane. Arginine methylation was found to facilitate export of certain heterogeneous nuclear ribonucleoproteins out of the nucleus, especially of proteins that bind mRNA (37). These observations suggest that methylation of EWS protein may play a role in its nucleocytoplasmic shuttling and that it acts as a carrier for export of RNA constituents to the cytosol due to its RNA binding properties.

Asymmetric methylation might also play a role in signal transduction. Stimulation of pre-B cells with lipopolysaccharide resulted in increased methylation of membrane proteins (22). EWS protein, which is as the present results show extensively methylated on arginine residues, was found to interact with cellular kinases, suggesting its participation in cell signaling and proliferation. Under conditions leading to Bruton’s tyrosine kinase activation, EWS protein was phosphorylated on tyrosines in mitotically arrested B cells (13) and interacted with Pyk2 in an activation-dependent manner (14).

The presence of asymmetrically arginine-dimethylated EWS protein as well as of nucleolin on the cell surface raises the question as to the significance of this localization. The present findings, i.e., the shuttling properties, together with the discussed properties of the EWS protein allow one to speculate that EWS protein or putative splicing products thereof can function as binding proteins or receptors for various ligands on the cell surface, e.g. nucleic acids, be it alone or in a complex with other proteins and, thus, might mediate, in an alternative regulation mechanism, between extracellular and nuclear events.

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