Proteomic analysis on insulin signaling in human hematopoietic cells: identification of CLIC1 and SRp20 as novel downstream effectors of insulin

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Submitted 27 October 2004; accepted in final form 11 April 2005

The early protein kinase activation cascade is well documented. It has served as a scenario for early intracellular signal transduction including a wide variety of bioactivities, including hematopoietic cells. Although the early protein kinase activation cascade has been intensively studied, the whole picture of the intracellular signaling events has not yet been clarified. To identify novel downstream effectors of insulin-dependent signals in relatively early phases, we performed high-resolution two-dimensional electrophoresis (2-DE)-based proteomic analysis using human hematopoietic cells 1 h after insulin stimulation. We identified SRp20, a splicing factor, and CLIC1, an intracellular chloride ion channel, as novel downstream effectors besides previously reported effectors of Rho guanine nucleotide dissociation inhibitor 2 and glutathione S-transferase-pi. Reduction in SRp20 was confirmed by one-dimensional Western blotting. Moreover, MG-132, a proteasome inhibitor, prevented this reduction. By contrast, upregulation of CLIC1 was not observed in one-dimensional Western blotting, unlike the 2-DE results. As hydrophilic proteins were predominantly recovered in 2-DE, the discrepancy between the 1-DE and 2-DE results may indicate a certain qualitative change of the protein. Indeed, the nuclear localization pattern of CLIC1 was remarkably changed by insulin stimulation. Thus insulin induces the proteasome-dependent degradation of SRp20 as well as the subnuclear relocalization of CLIC1.

HL-60 cells; PDQuest; matrix-assisted laser desorption ionization coupled to time-of-flight mass spectrometry; Mascot

INSULIN AND INSULIN-LIKE GROWTH FACTOR I (IGF-I) are known as important regulators of a variety of biological effects, including growth, development, and metabolism. Moreover, insulin-dependent signals contribute to the regulation of azurophil granule-selective macroautophagy in human hematopoietic cells (16). The molecular mechanisms for the actions of insulin and IGF-I have been intensively studied by various approaches, including gene-targeting animal experiments (1, 8, 14, 22) and molecular cloning techniques (20, 21). Now, the scenario for early intracellular signal transduction including a protein kinase activation cascade is well documented. It has been revealed that common intracellular signaling pathways are working downstream of insulin and IGF-I, including insulin receptor substrates (IRSs) (12) and Shc (17). The IRSs phosphorylate phosphatidylinositols 3-kinase to activate Akt, which transmits signals for proliferation and survival as well as the hematopoietic macroautophagy regulation (16), and the mammalian target of rapamycin and S6 kinase, which transmit signals for growth and translation besides heaptic macroautophagy regulation (2). On the other hand, Shc transmits signals for differentiation in hematopoietic cells (25).

In contrast to the early signal transduction, the picture of the later signaling events remains rather obscure. A large number of still undetermined molecules may be working downstream of the insulin-dependent signals. To obtain the whole picture of the intracellular signaling events downstream of the insulin receptor, comprehensive studies such as transcriptome analysis and proteome analysis may be especially powerful. A transcriptome analysis can illuminate the intracellular signaling events if they require new transcriptions or altered message stabilities. However, changes in protein expression are not always associated with those of the message expression, and vice versa. Thus transcriptome analysis would occasionally bring about false positive and/or false negative results. In this sense, proteome analysis is thought to be a more practical tool. Moreover, proteome analysis has merit in demonstrating protein modification changes such as phosphorylation and acetylation besides the change in net expression amounts. Indeed, studies on proteome analysis have successfully identified the protein molecules associated with metabolic regulation in the liver (3, 7). However, proteome analysis on insulin signaling in hematopoietic cells has not been performed despite the significance of insulin-dependent signals in the hematopoietic system.

For the first time, we performed proteomic analysis using human hematopoietic cells with the high-resolution two-dimensional electrophoresis (2-DE) system. We show that SRp20, a splicing factor, and CLIC1, an intracellular chloride ion channel, are working as novel downstream effectors of insulin signaling. The biological relevance of these events is discussed.

MATERIALS AND METHODS

Cells, growth factors, and inhibitors. HL-60 cells were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, JRH Bioscience, Lenexa, KS). For insulin-stimulating experiments, cells were supplemented with insulin (10 ng/ml). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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01 April 2017
had been previously cultured in serum-free RPMI 1640 medium supplemented with 5 μg/ml human holo-transferrin (Sigma Chemical, St. Louis, MO) for 3 days, and then 5 μg/ml insulin (Sigma) were added. Transferrin was suspended in RPMI 1640 medium, and insulin was solubilized by 1 N hydrochloric. In some experiments, MG-132 (Calbiochem, La Jolla, CA) was added 30 min before insulin stimulation.

Two-dimensional gel electrophoresis with quantitative analyses. Insulin-depleted cells were stimulated by insulin. After a 1-h incubation, stimulated and nonstimulated cells were collected. After a washing with wash buffer (10 mM Tris-HCl buffer, pH 8.0, 5 mM magnesium acetate), 4 × 10^6 cells were suspended with 7 volumes of lysis buffer containing 2 M thiourea, 7 M urea, 4% (v/v) CHAPS, and 1 mM Pefabloc SC PLUS (Roche Diagnostics, Mannheim, Germany). The cell suspensions were kept for 10 min on ice, sonicated intermittently, and centrifuged at 12,000 g for 10 min at 4°C, and the supernatant fractions were collected. The protein concentration was determined in the lysis solution with a dye reagent from Amersham Biosciences (Piscataway, NJ), using BSA as a standard.

The lysate was alkylated with Ready Prep Reduction-Alkylation Kit (Bio-Rad Laboratories, Hercules, CA). The 120 μg of protein lysate per gel were subjected to two-dimensional gel electrophoresis (2-DE). One-dimensional isoelectric focusing was carried out using Immobiline dry strip (18 cm long, pH 3–10 nonlinear or pH 4–7 linear, Amersham Biosciences) in a horizontal electrophoresis system (Ettan IPGphor, Amersham Biosciences) according to the manufacturer’s instructions. After the one-dimensional electrofocusing, IGP gels were equilibrated with buffer containing 50 mM Tris·HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, and 0.5% diithiothreitol, followed by alkylolation with equilibration buffer containing 4.5% iodoacetamide instead of 0.5% diithiothreitol at room temperature for 15 min. The gels were subjected to two-dimensional SDS-PAGE (10% gel). Proteins were visualized in the gels by staining with SYPRO Ruby Protein Gel Stain (Bio-Rad Laboratories) for overnight. The fluorescence intensity of each protein spot was digitally recorded by FluorImager 595 (Amersham Biosciences) using ImageQuant software and the differential protein expression quantitatively analyzed by PDQuest software (Bio-Rad Laboratories). The density of each spot was normalized by that of the smallest β-actin spot. Initially, all of the spots were roughly matched by an automatic program in PDQuest software, which was followed by a more detailed manual matching process to correct inappropriate matching pairs. Three to six independent experiments were performed, and the results were statistically analyzed by Student’s r-test.

Mass spectrometric analysis. Mass spectrometric analysis was performed according to the method reported by Toda et al. (23), with slight modifications. Briefly, each protein spot in SYPRO Ruby-stained gels was picked by FluoroPhoreStar 3000 (Anatech, Tokyo, Japan). The pieces of gels were dehydrated in 50% acetonitrile and 50% ammonium bicarbonate, next in 100% acetonitrile, and dried. The proteins were digested with 5 μg/ml trypsin (sequencing grade modified trypsin; Promega, Madison, WI) at 30°C. After overnight protein digestion, peptide fragments in the digest were subjected to matrix-assisted laser desorption ionization (MALDI) coupled to a time-of-flight (TOF) (MALDI-TOF) mass spectrometer (AXIMA-CFR; Shimadzu, Kyoto, Japan) for peptide mass fingerprinting (PMF). Protein identification was performed with the Mascot server (Matrix Science, Boston, MA) and Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA). We selected the Homo sapiens database of SWISS-PROT and parameters: peptide tolerance ±0.4 Da and one missed cleavage. Carbamidomethyl modification of cysteine and acetylation of the NH2-terminal end or lysine and phosphorylation of serine, threonine, or tyrosine were considered.

Protein identification was repeated at least once with spots from different gels. Phosphorylated peptides were confirmed by MALDI-TOF-MS in a postsource decay (PSD) mode of AXIMA-CFR and AXIMA-CFRplus (Shimadzu). NH2-terminal acetylation was determined by MALDI-QIT-TOF-MS in an MS/MS mode (AXIMA-QIT, Shimadzu).

One-dimensional Western blotting. Cells (5 × 10^6) were lysed with 100 μl of 1× Laemmli’s sample buffer and boiled. Ten microliters of this lysate were subjected to SDS-PAGE with 15% gels. The electric transfer onto a polyvinylidene difluoride (PVDF) membrane was carried out with a semidry blotting apparatus (Bio-Rad Laboratories) at 50 mA/cm^2 for 45 min at room temperature using buffer containing 2.25% Tris, 10.8% glycine, and 20% methanol. The first antibody reaction was performed using anti-SRp20 antibody (7B4; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rho-guanine nucleotide dissociation inhibitor (Rho-GDI) antibody (A-20; Santa Cruz Biotechnology), anti-β-tubulin antibody (H-235; Santa Cruz Biotechnology), a sheep anti-CLIC1 antisera (25), anti-cyclin D3 antibody (C-16; Santa Cruz Biotechnology), anti-cyclin E antibody (M-20; Santa Cruz Biotechnology), and anti-cyclin A antibody (BF683; Upstate Biotechnology), Lake Placid, NY). The second antibody reaction and the final detection procedure were performed using ECL Western blotting detection reagents (Amersham Biosciences) or SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturers’ guidance. Information of the chemical luminescence was analogically developed onto Hyperfilm (Amersham Biosciences). After scanning of the developed film, the band intensities were calculated by ImageQuant software (Amersham Biosciences). Stripping of the first antibody was performed by incubating the PVDF membrane with Restore Western Blot Stripping Buffer (Pierce Biotechnology) at room temperature for 30 min.
Fig. 2. Peptide mass fingerprinting (PMF) of spots a–e. Spots a (B), b (C), c (D), d (E), e (F) were picked, and, after trypsin digestion, matrix-assisted laser desorption ionization coupled to a time-of-flight mass spectrometer (MALDI-TOF-MS) analysis was performed. As a positive control, a β-actin spot (∗ in Fig. 1) was picked and analyzed (A).
Two-dimensional Western blotting. SYPRO Ruby-stained proteins on gels were resublimated and transferred according to our previously reported method (23). Briefly, the stained gel was incubated in resolubilization buffer (0.2% wt/vol SDS, 0.3% wt/vol Tris, 0.7% wt/vol glycine) for 10 min and mounted onto a PVDF membrane in a semidry blotting apparatus (Bio-Rad Laboratories). Electrotransfer was carried out at 4 V/cm² for 1 h at room temperature using buffer containing 0.3% (wt/vol) Tris, 1.5% (wt/vol) glycine, 0.1% (wt/vol) SDS. The fluorescence images of the blotted PVDF membranes were scanned and recorded by FluorImager 595 (Amersham Biosciences). The PVDF membranes were further subjected to immunoblotting as in cases of 1-DE Western blotting.

Cell cycle analysis. Cells (5 × 10⁶) were collected, washed with PBS, and fixed with 70% ice-cold ethanol for 4 h. After treatment with RNase A (100 μg/ml, Sigma) for 30 min at 37°C, DNA was stained with 50 μg/ml propidium iodide (Sigma). Cell cycle analysis was performed by FACScalibur (Becton-Dickinson, Mountain View, CA) using CELL Quest software according to the manufacturer’s guidance.

Immunocytochemistry. Cells were fixed on slide glasses with a cytospin apparatus (Cytospin2, Shandon, Pittsburgh, PA) with further fixation with acetone-methanol solution (1:3). The immunostaining procedure was performed as described elsewhere (16) using anti-CLIC1 antibody (1:1,000 dilution) (24). The cells were observed by fluorescent microscopy with Normarsky differentiated interference contrast (Olympus Optical, Tokyo, Japan).

Statistical analysis. Student’s t-test was used to determine statistical significance. A P value of <0.05 was considered significant.

Results

2-DE protein expression profiles of human hematopoietic cells with or without insulin treatment. To identify novel downstream effectors in early phases of insulin-dependent signals in human hematopoietic cells, we performed the 2-DE-based differential protein expression analysis using human myeloblastic HL-60 cells. The cells which had been cultured in the absence of insulin for 3 days were treated with 5 μM insulin or water. After 1 h, cell lysates were prepared according to the standard isoelectric focusing electrophoresis method described in MATERIALS AND METHODS. In this procedure, highly hydrophobic, urea-insoluble proteins were eliminated during the centrifugation step as precipitants, and only the supernatant was used the immobilized pH gradient gel strip with a broad pH range (pH 3–10 nonlinear) for one-dimensional isoelectric focusing. Although more than 1,000 protein spots were visualized after SYPRO Ruby staining, PDQuest software-based analysis indicated that the spots having significant expression changes by insulin treatment were mainly located at pH 4–6 in the horizontal axis (data not shown). Thus we performed the following detailed analysis using the immobilized pH gradient gel strip with a narrower range (pH 4–7 linear) for finer resolution (Fig. 1). Over 600 protein spots were visualized by SYPRO Rub staining. From these spots, we selected the candidates for the subsequent mass spectrometric analysis according to the following criteria. The basal expression level was higher than 5% of that of the largest β-actin spot, and the increase or decrease in the expression after insulin stimulation was greater than twofold or less than one-half, respectively. After statistical analysis of the multiple experiments (n = 3–6), five candidates were determined (Fig. 1). These spots were picked from the gel and, after trypsin digestion, MALDI-TOF-MS analysis was performed. Figure 2 shows the PMF of each spot, with a PMF of β-actin as a positive control. These data were further analyzed, being sent to the Mascot search server, and it was suggested that spot a was CLIC1, spot b was Rho-GDI-2, spot c was glutathione S-transferase-π (GST-π), and spots d and e were SRp20 (Fig. 2 and Table 1). The Mascot score of each search result was 175 (spot a), 80 (spot b), 98 (spot c), 100 (spot d), and 64 (spot e), indicating that the protein identifications by PMF were highly reliable (the data are summarized in Table 1). The results of the statistical analysis for the expression amounts of these spots are summarized in Table 2. Among these spots, Rho-GDI-2 (spot b) and GST-π (spot c) have already been identified as downstream effectors of insulin. Rho-GDI-2 is reportedly released from the intracellular membrane fractions to the cytoplasm by insulin (19), and the expression of GST-π markedly increases after insulin stimulation (6). Thus we focused our research on the evaluation of spot a and spots d and e.

To confirm the Mascot search results, we performed 2-DE western blotting by transferring SYPRO Ruby-stained 2-DE protein spots to PVDF membrane. As shown in Fig. 3A, most of the proteins were properly transferred to the membrane with a SYPRO Ruby pattern similar to that of the original gel. As shown in Fig. 3B, spot a was indeed recognized by anti-CLIC1

Table 1. Protein identification by mass spectrometry analysis

<table>
<thead>
<tr>
<th>Spot</th>
<th>GenBank Acc. No.</th>
<th>Protein Name</th>
<th>Mr Theo, Da</th>
<th>Mr Obs, kDa</th>
<th>pI Theo</th>
<th>pI Obs</th>
<th>Mascot Score</th>
<th>Peptides</th>
<th>Sequence Coverage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>O00299</td>
<td>CLIC1</td>
<td>2.7248</td>
<td>29.0</td>
<td>5.09</td>
<td>5.16</td>
<td>175</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>b</td>
<td>P52566</td>
<td>Rho-GDI-2</td>
<td>2.3031</td>
<td>23.6</td>
<td>5.10</td>
<td>5.08</td>
<td>80</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>c</td>
<td>P09211</td>
<td>GST-π</td>
<td>2.3438</td>
<td>22.9</td>
<td>5.44</td>
<td>5.67</td>
<td>98</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>d</td>
<td>P23152</td>
<td>SRp20</td>
<td>1.9546</td>
<td>21.2</td>
<td>11.64</td>
<td>6.13</td>
<td>100</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>e</td>
<td>P23152</td>
<td>SRp20</td>
<td>1.9546</td>
<td>20.5</td>
<td>11.64</td>
<td>6.38</td>
<td>100</td>
<td>9</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 2. Ratios of spot intensities (insulin+/insulin−)

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Means ± SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>CLIC1</td>
<td>1.83±0.37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>b</td>
<td>Rho-GDI-2</td>
<td>2.03±0.43</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>c</td>
<td>GST-π</td>
<td>1.93±0.52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>d</td>
<td>SRp20</td>
<td>0.24±0.33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>e</td>
<td>SRp20</td>
<td>0.18±0.27</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Summarized results from 3–6 independent experiments are shown. Statistical analysis was performed by Student’s t-test.

Values of theoretical isoelectric points (pI) and molecular weights/masses (Mr) were obtained from Mascot search results. Theo, theoretical; Obs, observed; CLIC1, intracellular chloride ion channel; Rho-GDI-2, Rho-guanine nucleotide dissociation inhibitor 2; GST-π, glutathione-5-transferase-π; SRp20, a splicing factor. Calculations of experimental isoelectric point (pI) and molecular weight (Mr) were based on migration of the protein spot on 2-D gels using PDQuest
Fig. 3. 2-DE Western blotting. A: SYPRO Ruby-stained 2-DE gel, in which the lysate of buffer solution-treated cells were applied as in Fig. 1, left, was trimmed (top) and transferred onto a PVDF membrane (bottom). Transferred proteins were visualized by fluorescence image scanner. B: PVDF membrane was cut as indicated and blotted by anti-CLIC1 antiserum (bottom left) or anti-SRp20 antiserum (bottom right). Spots for CLIC1 and SRp20 are indicated with black arrows. Note that there is an extra spot on the anti-CLIC1-blotted membrane (indicated with white arrow).

Fig. 4. Amino acid sequencing of NH$_2$-terminal peptide fragment of CLIC1. Parent ion at m/z 1,458.74 in Fig. 2B in insulin-treated cells was subjected to subsequent analysis using MS/MS mode of MALDI-TOF-MS (AXIMA-QIT). The mass data of b-series of the product ions were analyzed by the PepSeq program in ProteinLynx software. NH$_2$-terminal acetylated peptides of N-acetyl-AEE (m/z 372.14, b$^3$ ion), N-acetyl-AEEQ (m/z 500.20, b$^4$ ion), N-acetyl-AEEQPV (m/z 725.30, b$^6$ ion), N-acetyl-AEEQPVQV (m/z 824.38, b$^7$ ion), N-acetyl-AEEQPVQVELF (m/z 1,066.50, b$^9$ ion), and N-acetyl-AEEQPVQVELFV (m/z 1,213.57, b$^{10}$ ion), and N-acetyl-AEEQPVQVELFV (m/z 1,312.64, b$^{11}$ ion) were detected. Peptide mass fingerprinting (PMF; A) and MS/MS data of b-series (B) are shown. Similar analysis concerning control cells also demonstrated NH$_2$-terminal acetylation of CLIC1 (data not shown).
antibody and spots d and e were recognized by anti-SRp20 antibody. We also studied the possible modifications on these two proteins. As shown in Fig. 1A, the observed isoelectric point (pI) of CLIC1 was 5.16, which is similar to the Mascot information (the calculated pI was 5.09). Interestingly, there was an extra small spot with a higher pI value (Fig. 3B, white arrow). Indeed, we detected a doublet band in one-dimensional Western blotting, where the lysate prepared for 2-DE was mixed with an isovolume of \( 2 \times \) Laemmli’s sample buffer and subjected to SDS-PAGE (data not shown and see Fig. 6B). Thus CLIC1 is expressed in at least two forms with different pI values in human hematopoietic cells, although the molecular basis for this difference was not elucidated.

This discrepancy may come from the modifications of SRp20. The MS digest analysis indicated that the SRp20 was phosphorylated at two sites, including Ser\(^{115}\) and Ser\(^{108}\), from the existence of the peptide fragment ions of \( m/z \) 751.31 and \( m/z \) 945.43 (Fig. 5A). Mass value of \( m/z \) 751.31 is speculated amino acid sequences as RRSPPRR\(^{113-117}\), RSPRR\(^{114-118}\), or SPRR\(^{115-119}\) (Fig. 5E). As concerns \( m/z \) 945.43, MS digest suggests the amino acid sequence as RRSPPP\(^{106-112}\), RSPPPRR\(^{107-113}\), or SPPPP\(^{108-114}\). Phosphorylated peptide was confirmed by MALDI-TOF-MS in a seamless PSD mode (AXIMA-CFR) that detected the neutral loss of phosphate group. As shown in Fig. 5, B and C, phosphorylation-dependent neutral loss (\(-80\) Da) and dehydration (\(-18\) Da) were detected in the fragments at \( m/z \) 751.31 and \( m/z \) 945.43. Conversely, the MALDI-TOF-MS PSD spectrum of the control peptide ion gated at \( m/z \) 1,043.57 showed no significant neutral loss (Fig. 5D). Next, the amino acid sequences of \( m/z \) 751.31, \( m/z \) 945.43, and \( m/z \) 1,043.57 were examined by the same method as described above by using AXIMA-CFR plus. The amino acid sequences of \( m/z \) 751.31 could not be determined because the fragment ion was low intensity (data not shown). However, it is presumable that the Ser\(^{115}\) is phosphorylated. From the mass spectra of gated ion at \( m/z \) 945.43, the amino acid sequence was determined as RRSPPPR\(^{106-112}\), and the position of phosphorylation was Ser\(^{108}\) (Fig. 6A). As a
negative control, m/z 1,043.57 was subjected to analysis using PSD mode of AXIMA-CFRplus (Fig. 6B). The amino acid sequence was decided as AFGYYGPLR(29–37), which was not phosphorylated. These results indicated that Ser115 and Ser108 were phosphorylated in human hematopoietic cells (Fig. 5E). Thus the two phosphorylations of SRp20 may be responsible for the acidic shift of SRp20 in 2-DE.

Thus the 2-DE-based differential protein expression analysis identified CLIC1 and SRp20 as novel downstream effectors of insulin in human myeloblastic HL-60 cells.

1-DE study of CLIC1 and SRp20 expressions after insulin stimulations. It is known that there are occasionally discrepancies between the results of 2-DE and 1-DE. The difference in the protein solubilization capacities between the two systems is thought to be one of the reasons. During cell lysate preparation in 2-DE, highly hydrophobic proteins are prone to make precipitations and thus be eliminated from the lysates after centrifugation. Thus the protein expression changes in 2-DE-based proteome analysis not only means that the net protein expression changes but also the changes in protein solubilization. So we studied the expressions of CLIC1 and SRp20 after insulin stimulation by 1-DE Western blotting.

In contrast to the results of 2-DE, there was no significant difference in CLIC1 expression between insulin-treated and nontreated samples in 1-DE (Fig. 7A). Moreover, CLIC1 was detected as a single band, unlike the 2-DE results, where CLIC1 was detected as two spots. Interestingly, CLIC1 was detected as a doublet band, and the expression amounts of CLIC1 were indeed upregulated by insulin stimulation when the 2-DE lysates were treated by an isovolume of 2x Laemmli’s buffer and subjected to 1-DE (Fig. 7B, lane 2). These findings strongly suggest that insulin treatment induced certain qualitative changes of CLIC1. Compared with 1-DE, the protein recovery rate in 2-DE was generally low: one-fourth the recovery as for CLIC1 (Fig. 7B, compare lanes 1 and 3) and one-eighth the recovery as for β-tubulin (Fig. 7B, compare lanes 1 and 3). However, the expression amounts of β-tubulin (Fig. 7B, compare lanes 1 and 2) and α-tubulin (data not shown) were not significantly changed by insulin treatment even in 2-DE lysates. By contrast, around a twofold increment in CLIC1 was reproducibly observed after insulin stimulation (Fig. 7B, C, and D, data not shown). We then examined the possibility that the insulin-mediated increments in CLIC1 in 2-DE lysate were associated with the changes in its subcellular
localization. As shown in Fig. 7, D and E, the nuclear localization pattern of CLIC1 was clearly changed by insulin treatment: CLIC1 was detected mainly as speckled forms in nuclear matrix in nontreated cells, whereas CLIC1 was located mainly at nucleoli in insulin-treated cells. Thus the changes in subnuclear localization may responsible for the expressional changes of CLIC1 in 2-DE.

Next, we studied the expression of SRp20 in 1-DE Western blotting. The SRp20 expression was actually reduced as in the case of 2-DE (Fig. 8A), indicating that the total amount of SRp20 was reduced by insulin treatment. To further investigate the molecular basis of insulin-mediated reduction in SRp20, the effects of the proteasome inhibitor MG-132 were examined. As shown in Fig. 8B, MG-132 inhibited the insulin-mediated reduction of SRp20 in a dose-dependent manner. MG-132 also blocked the degradation of cyclin D3 and enhanced the accumulation of cyclin D3 after insulin stimulation (Fig. 8B, lane 5). Interestingly, the recovery of SRp20 expres-
sion was observed 4 h after stimulation (Fig. 8A, lane 4). Because serum stimulation activates SRp20 transcription and increases the protein expression of SRp20 as the cells enter into S-phase (9), the recovery in SRp2 expression would be associated with the cell cycling progression. As shown in Fig. 9, the insulin treatment significantly increased the S-phased population. Thus the recovery in SRp20 in later phases is associated with an enhanced S-entry.

Thus insulin treatment causes qualitative changes of CLIC1 that are associated with its subnuclear localization and the proteasome-dependent degradations of SRp20 as early as 1 h.

**DISCUSSION**

We identified CLIC1 and SRp20 as novel downstream effectors of insulin-dependent signals in human hematopoietic cells by using a 2-DE-based proteome analytic system.

A 2-DE-based proteome analysis has merit in managing a wide spectrum of protein expressions at one time. Moreover, it can illustrate the change in modifications and subcellular localization of the proteins besides the change in net amounts. As in the case of CLIC1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions in 2-DE were upregulated by insulin stimulation, although no significant changes were detected in 1-DE (K. Saeki, unpublished observation). Because serum stimulation, which often induces similar protein expression changes as insulin stimulation, reportedly induces cytoplasmic transport of GAPDH (18), the upregulated expression of GAPDH in 2-DE may be associated with similar subcellular translocation.

Although CLIC1 functions as a chloride ion channel when localized to membranes (26), it is known that CLIC1 localizes principally to the cell nucleus in human hematopoietic cells (24). CLIC1 is structurally homologous to the GST superfamily of proteins with a redox-active site at the NH2 terminus (5). It is suggested that CLIC1 activity is under the control of redox-active signaling molecules in vivo (5). In this sense, it is interesting that GST-pi is also a downstream effector of insulin as we showed (Fig. 1A, spot c) and reported elsewhere (6). It is known that hyperglycemia and, to a lesser extent, insulin resistance cause oxidative stress (15, 13). Insulin signaling might possibly contribute to the reduction of oxidative stresses by changing the expression patterns of CLIC1 and GST-pi. Further investigations are required to understand the molecular basis and biological relevance of insulin-induced changes in CLIC1 in the 2-DE system.

Fig. 9. Cell cycle analysis and expressions of cyclins. A: cells cultured with transferrin-supplemented serum-free medium for 3 days were stimulated with buffer solution (bottom left) or insulin (bottom right). After another 10-h incubation at 37°C, cells were fixed with 70% ethanol and subjected to DNA content assessment by fluorescence-activated cell sorting. Cells cultured in the presence of serum were also subjected to flow cytometric analysis (top left). B: cells cultured with transferrin-supplemented serum-free medium for 3 days were stimulated with buffer solution (lane 2) or insulin (lane 3). Cell lysates were prepared after another 4-h incubation. Western blotting was performed using anti-cyclin D3, followed by anti-cyclin E and anti-cyclin A reblotting.
As for spots d and e of SRp20, we could not find any differences in PMFs. One interpretation is that distinct phosphorylations took place at their COOH-terminal SR domains. Because the SR domain is extremely rich in arginine residues, this domain should be degraded into pieces after trypsin digestion, and, as a result, the peptide fragment ions might be hardly detectable. In any case, the expressions of spots d and e were both decreased by insulin stimulation, and thus the precise determination of structural differences between the two spots would be a less important subject for an understanding of biological effects of insulin. As we showed, the insulin-induced reduction in SRp20 was inhibited by pretreatment of the cells by MG-132, a reversible proteasome inhibitor (Fig. B). Quite unexpectedly, lactacystin, an irreversible proteasome inhibitor, could not inhibit the reduction of SRp20, although it effectively enhanced an insulin-dependent accumulation of cyclin D3 (K. Saeki, unpublished observation), suggesting that there might be at least two different proteasome-dependent protein degradation systems with distinct lactacystin susceptibilities.

What is the impact of SRp20 reduction by insulin? SRp20 is a splicing factor involved in the regulation of alternative splicing of certain precursor RNA, including SRp20 itself. Its roles for embryogenesis have been shown: an inactivation of SRp20 gene in mice resulted in a failure to form blastocysts, and embryos died at morula stage (11). Although complete loss of SRp20 functions is toxic, its mild reduction may play roles in particular situations. It is reported that overexpression of ASF/SF2, an alternative splicing regulator that antagonizes the function of SRp20 (10), was detected in malignant ovarian tissues (4). A transient reduction of SRp20 by insulin might upregulate the activity of ASF/SF2 and thus trigger signals for cell proliferation. Further investigations are required to determine the in vivo significance of a transient reduction of SRp20 after insulin stimulation.

ACKNOWLEDGMENTS

We greatly thank Masaki Yamada and Tsutomu Nishine of Shimadzu Corporation for technical assistance with the amino acid sequence analysis performed using an MS/MS and PSD mode of MALDI-TOF-MS (AXIMA-QFT and AXIMA-CFRplus).

GRANTS

This work was supported, in part, by a grant for diabetes research (MF-4) from the Organization for Pharmaceutical Safety and Research (to Y. Kuburagi).

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