

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

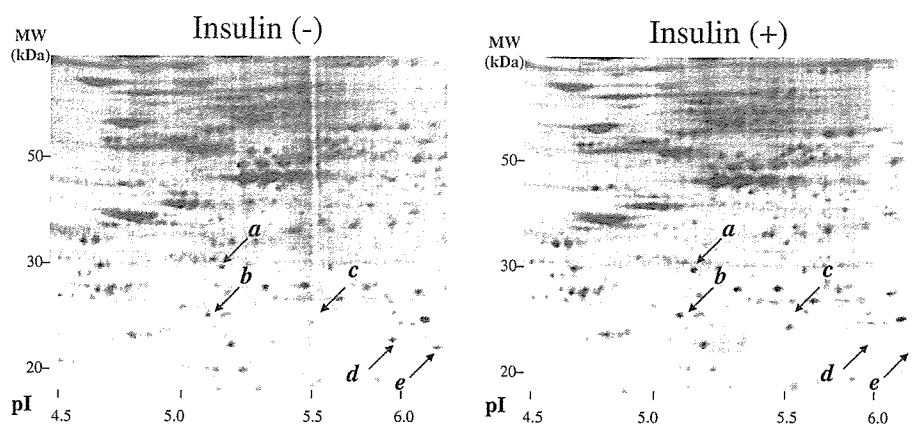
**Two-dimensional gel electrophoresis with quantitative analyses.** Insulin-depleted cells were stimulated by insulin. After a 1-h incubation, stimulated and nonstimulated cells were collected. After a washing with wash buffer (10 mM Tris·HCl buffer, pH 8.0, 5 mM magnesium acetate),  $4 \times 10^7$  cells were suspended with 7 volumes of lysis buffer containing 2 M thiourea, 7 M urea, 4% (wt/vol) 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  $\mu\text{g}$  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, IPG 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% dithiothreitol, followed by alkylation with equilibration buffer containing 4.5% iodoacetamide instead of 0.5% dithiothreitol 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 Fluor-Imager 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  $\beta$ -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 *t*-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  $\mu\text{g/ml}$  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  $\pm 0.4$  Da and one missed cleavage. Carbamidomethyl modification of cysteine and acetylation of the  $\text{NH}_2$ -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).  $\text{NH}_2$ -terminal acetylation was determined by MALDI-QIT-TOF-MS in an MS/MS mode (AXIMA-QIT, Shimadzu).

**One-dimensional Western blotting.** Cells ( $5 \times 10^5$ ) were lysed with 100  $\mu\text{l}$  of 1 $\times$  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<sup>2</sup> 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- $\beta$ -tubulin antibody (H-235; Santa Cruz Biotechnology), a sheep anti-CLIC1 antiserum (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. 1. Two-dimensional electrophoresis (2-DE) profile of human hematopoietic HL-60 cells with or without insulin treatment. HL-60 cells were cultured with transferrin-supplemented serum-free medium for 3 days. Then buffer solution (left) or 5  $\mu\text{g/ml}$  insulin (right) was added, and cells were cultured for another 1 h at 37°C. Cell lysates were prepared as described in Experimental Procedures, and 2-DE was carried out. PDQuest software-based analysis demonstrated that the 5 spots (indicated by arrows) showed significant differences in their expressions with or without insulin treatment. *Spot a*, CLIC1, an intracellular chloride ion channel; *spot b*, Rho-guanine nucleotide dissociation inhibitor 2 (Rho-GDI-1); *spot c*, and glutathione *S*-transferase-pi (GST-pi); *spots d* and *e*, SRp-20, a splicing factor; \*isoform of  $\beta$ -actin.



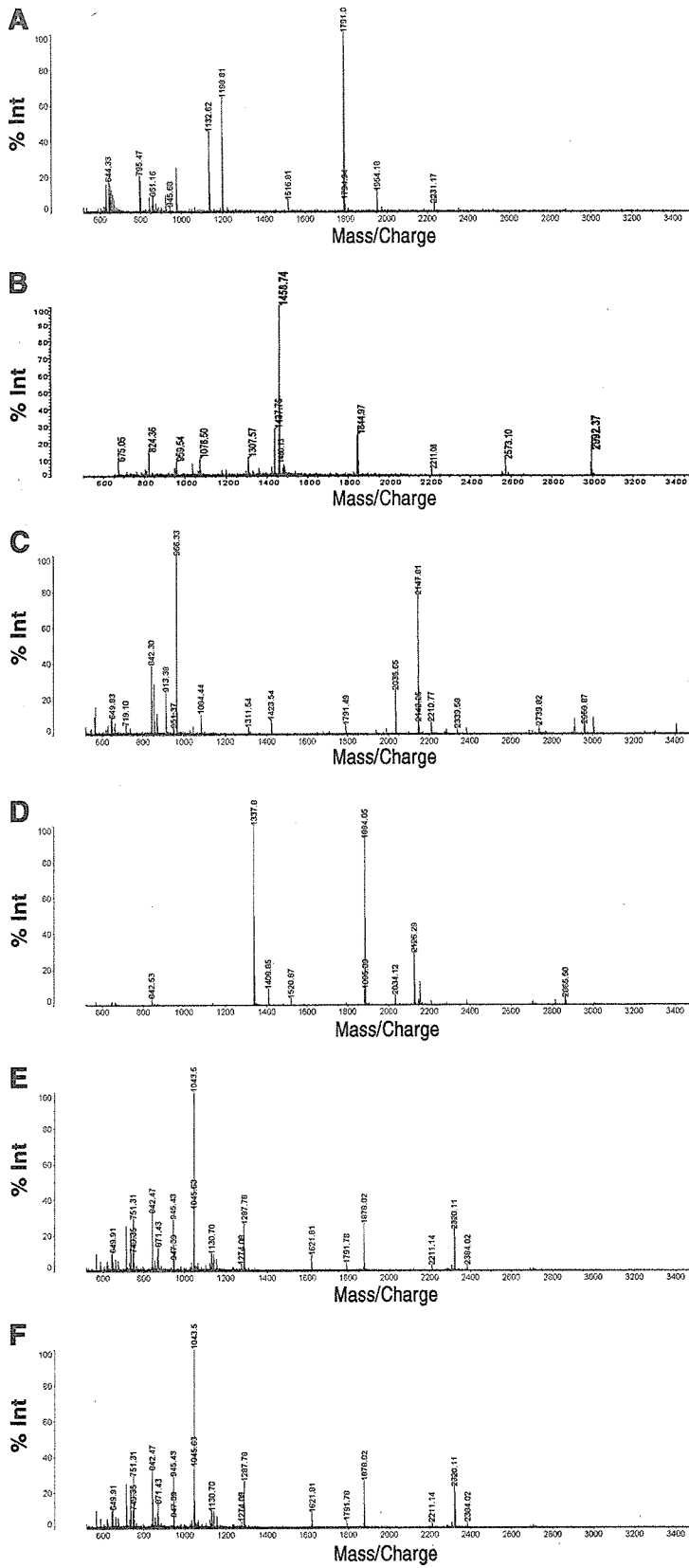


Fig. 2. Peptide mass fingerprinting (PMF) of spots *a*–*e*. Spots *a* (*B*), *b* (*C*), *c* (*D*), *d* (*E*), *e* (*F*) were picked, and, after trypsin digestion, matrix-assisted laser desorption ionization coupled to a time-of-flight mass spectrometer (MALDI-TOF-MS) analysis was performed. As a positive control, a  $\beta$ -actin spot (\* in Fig. 1) was picked and analyzed (*A*).

**Two-dimensional Western blotting.** SYPRO Ruby-stained proteins on gels were resolubilized 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/cm<sup>2</sup> 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 ( $5 \times 10^5$ ) were collected, washed with PBS, and fixed with 70% ice-cold ethanol for 4 h. After treatment with RNase A (100  $\mu$ g/ml, Sigma) for 30 min at 37°C, DNA was stained with 50  $\mu$ g/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  $\mu$ g/ml 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 fractions were used for 2-DE. In preliminary experiments, we 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

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

Spot	Protein	Means $\pm$ SD		<i>P</i> Value
		Mean	SD	
<i>a</i>	CLIC1	1.83	0.37	<0.01
<i>b</i>	Rho-GDI-2	2.03	0.43	<0.02
<i>c</i>	GST-pi	1.93	0.52	<0.05
<i>d</i>	SRp20	0.24	0.33	<0.01
<i>e</i>	SRp20	0.18	0.27	<0.005

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

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 Ruby 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  $\beta$ -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 digestions, MALDI-TOF-MS analysis was performed. Figure 2 shows the PMF of each spot, with a PMF of  $\beta$ -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-pi (GST-pi), 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-pi (*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-pi 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

Spot	GenBank Acc. No.	Protein Name	Mr		pI		Mascot Score	Peptides		Sequence Coverage, %
			Theo, Da	Obs, kDa	Theo	Obs		Match	Total	
<i>a</i>	O00299	CLIC1	2,7248	29.0	5.09	5.16	175	10	11	47
<i>b</i>	P52566	Rho-GDI-2	2,3031	23.6	5.10	5.08	80	6	9	24
<i>c</i>	P09211	GST-pi	2,3438	22.9	5.44	5.67	98	6	8	48
<i>d</i>	P23152	SRp20	1,9546	21.2	11.64	6.13	100	9	11	43
<i>e</i>	P23152	SRp20	1,9546	20.5	11.64	6.38	100	9	11	43

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-pi, glutathione-*S*-transferase-pi; 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.

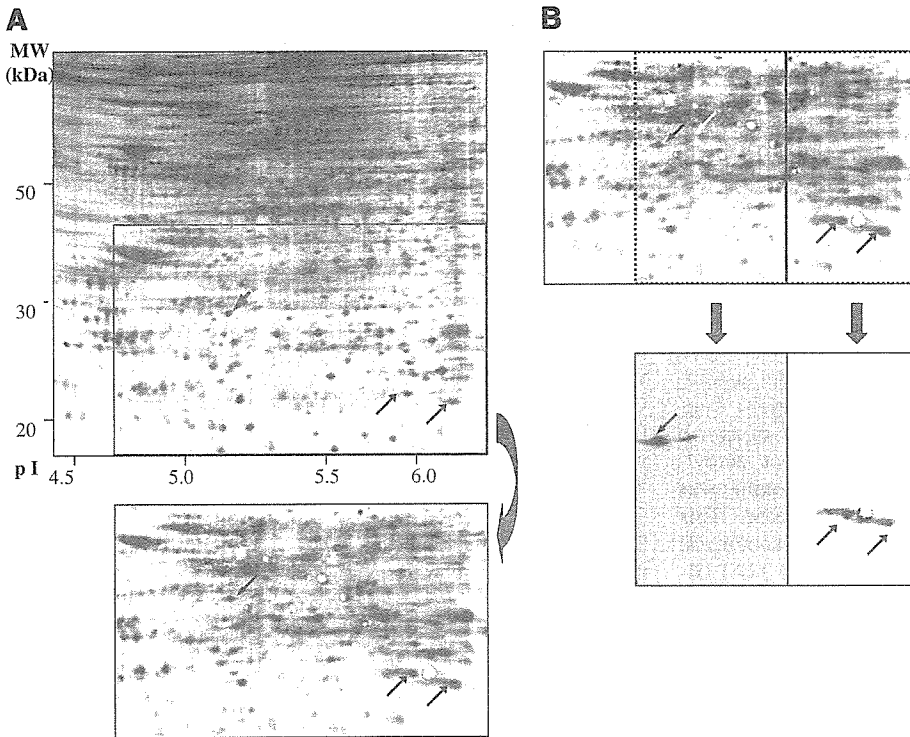
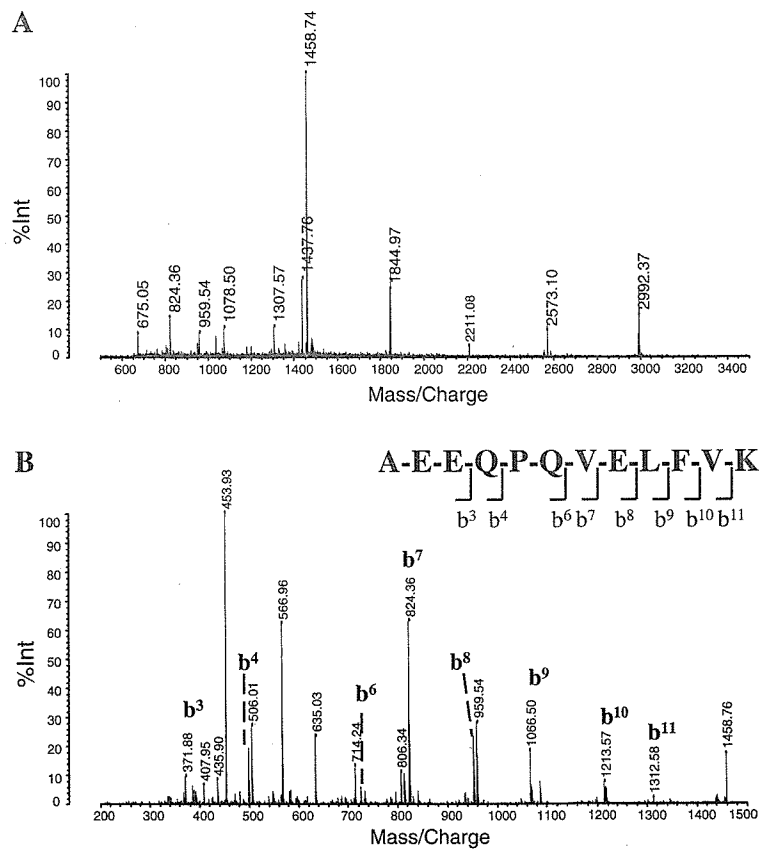


Fig. 3. 2-DE Western blotting. A: SYPRO Ruby-stained 2-DE gel, in which the lysate of buffer solution-treated cells were applied as in Fig. 1, left, was trimmed (top) and transferred onto a PVDF membrane (bottom). Transferred proteins were visualized by fluorescence image scanner. B: PVDF membrane was 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<sub>2</sub>-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<sub>2</sub>-terminal acetylated peptides of *N*-acetyl-AEE (*m/z* 372.14, b<sup>3</sup> ion), *N*-acetyl-AEEQ (*m/z* 500.20, b<sup>4</sup> ion), *N*-acetyl-AEEQPQ (*m/z* 725.30, b<sup>6</sup> ion), *N*-acetyl-AEEQPQV (*m/z* 824.38, b<sup>7</sup> ion), *N*-acetyl-AEEQPQVE (*m/z* 953.42, b<sup>8</sup> ion), *N*-acetyl-AEEQPQVEL (*m/z* 1,066.50, b<sup>9</sup> ion), *N*-acetyl-AEEQPQVELF (*m/z* 1,213.57, b<sup>10</sup> ion), and *N*-acetyl-AEEQPQVELFV (*m/z* 1,312.64, b<sup>11</sup> 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<sub>2</sub>-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× 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. The MS digest analysis of *spot a* in the 2-DE profiles of both the insulin-treated and control cells demonstrated that the peptide fragment at mass-to-charge ratio (*m/z*) 1,458.74 in PMF of *spot a* represented the NH<sub>2</sub> terminus acetylated fragment acetyl-AEEQPQVELFVK, indicating that the first methionine was eliminated and the second alanine was N-acetylated. This finding was indeed confirmed by the amino acid sequencing at the *m/z* 1,458.74 fragment by the MS/MS mode of MALDI-TOF-MS, as shown in Fig. 4.

As for SRp20, the observed pI values of *spots d* and *e* were 6.13 and 6.38, respectively (Fig. 1 and Table 1), in contrast to the Mascot software information (the calculated pI was 11.64).

This discrepancy may come from the modifications of SRp20. The MS digest analysis indicated that the SRp20 was phosphorylated at two sites, including Ser<sup>115</sup> and Ser<sup>108</sup>, 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 RRSPR<sub>(113-117)</sub>, RSPRR<sub>(114-118)</sub>, or SPRRR<sub>(115-119)</sub> (Fig. 5E). As concerns *m/z* 945.43, MS digest suggests the amino acid sequence as RRSPPPR<sub>(106-112)</sub>, RSPPPRR<sub>(107-113)</sub>, or SPPPPRR<sub>(108-114)</sub>. 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-CFRplus. 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<sup>115</sup> is phosphorylated. From the mass spectra of gated ion at *m/z* 945.43, the amino acid sequence was determined as RRSPPPR<sub>(106-112)</sub>, and the position of phosphorylation was Ser<sup>108</sup> (Fig. 6A). As a

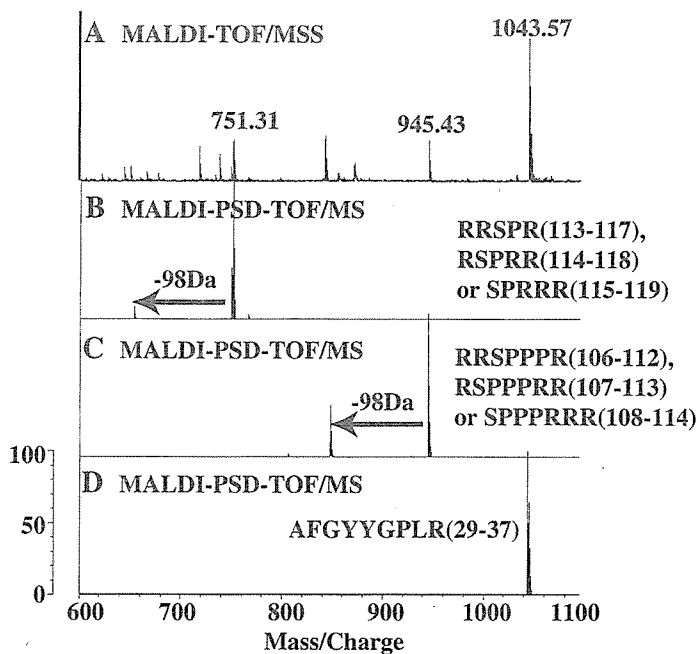
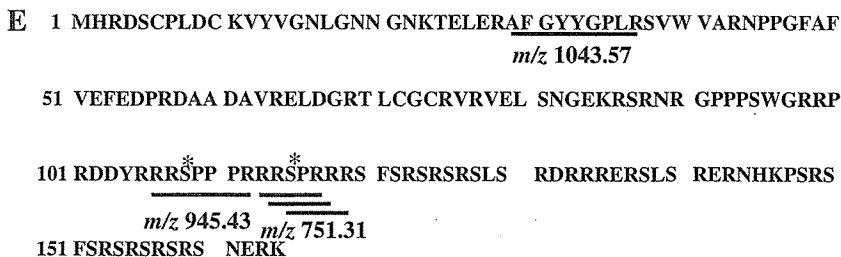


Fig. 5. Confirmation of SRp20 phosphorylation at specific serine residues. A: gated ions at *m/z* 751.31, 945.43, and 1,043.57 in Fig. 2E were subjected to analysis of neutral loss by MALDI-TOF-MS (AXIMA-CFR) in post-source decay (PSD) mode. The 98-Da loss of mass values was detected in gated ion at *m/z* 751.31 (B) and *m/z* 945.43 (C), but not in gated ion at *m/z* 1,043.57 (D) as a negative control. E: primary sequence of SRp20. \*Phosphorylated amino acid.



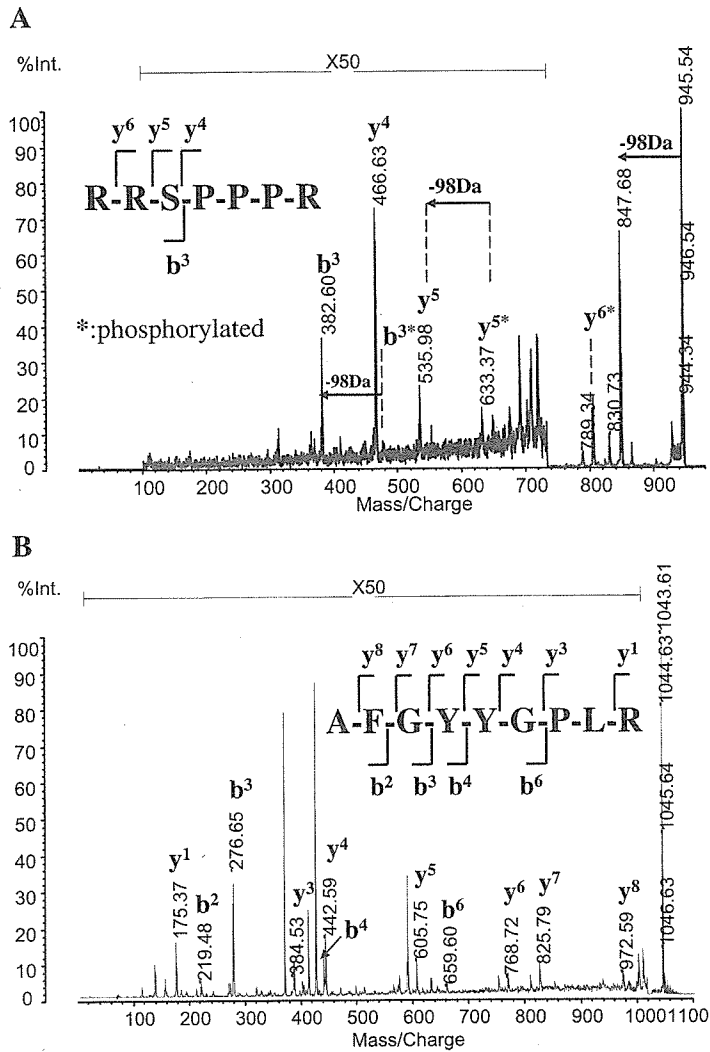


Fig. 6. Sequence analysis of gated ions at  $m/z$  945.43 and 1,043.57 by MALDI-TOF-MS in PSD mode. A: gated ion at  $m/z$  945.29 in Fig. 5C was subjected to subsequent analysis using PSD mode of MALDI-TOF-MS (AXIMA-CFRplus) B: three neutral loss ions ( $-98$  Da) of  $m/z$  480, 633, and 945 were detected. Gated ion at  $m/z$  1,043.57 in Fig. 5D was subjected to the same analysis. Neutral loss ion ( $-98$  Da) was not detected.

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<sub>(29-37)</sub>, which was not phosphorylated. These results indicated that Ser<sup>115</sup> and Ser<sup>108</sup> 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  $2\times$  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  $\beta$ -tubulin (Fig. 7B, compare lanes 1 and 3). However, the expression amounts of  $\beta$ -tubulin (Fig. 7B, compare lanes 1 and 2) and  $\alpha$ -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. 7, B and C, and 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

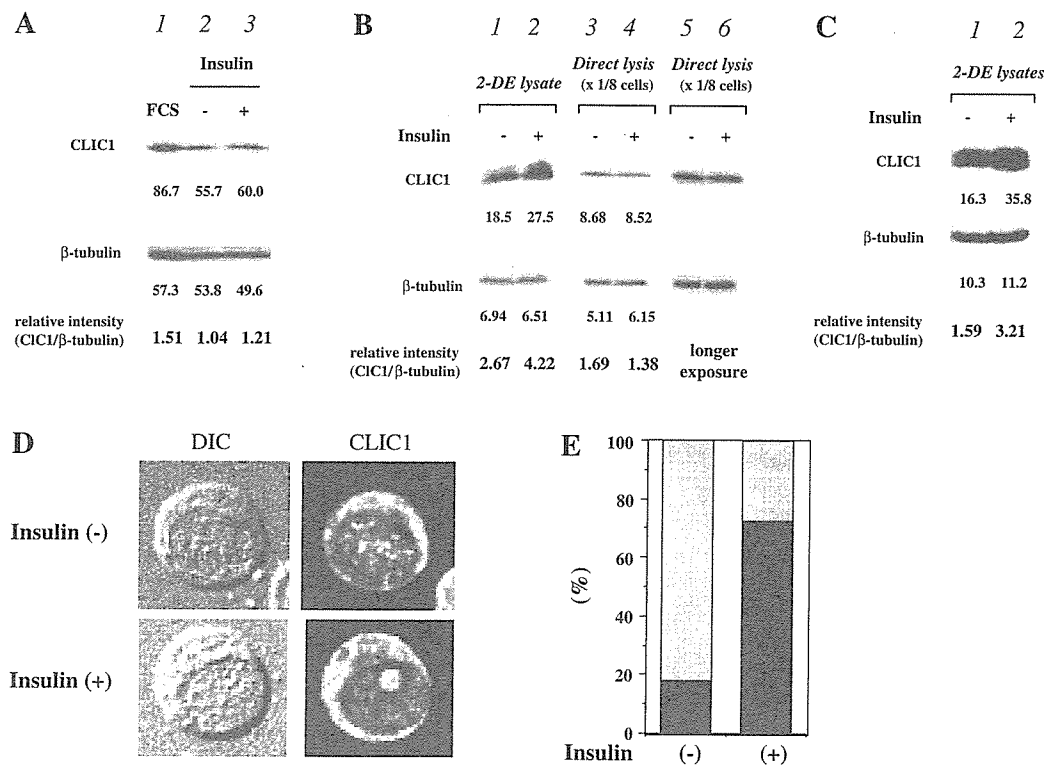


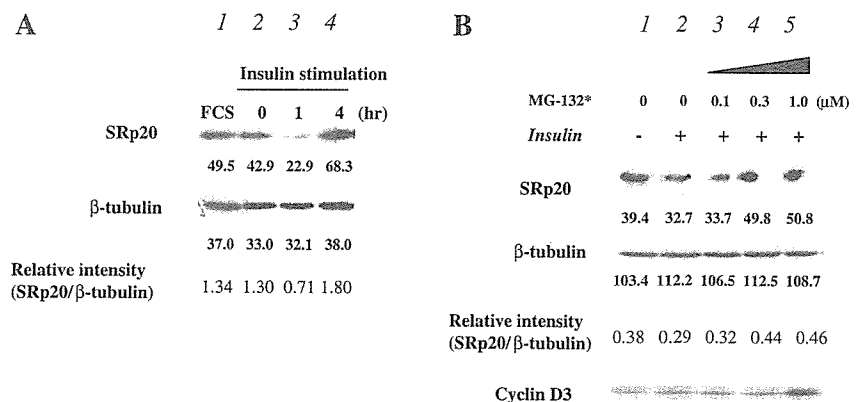
Fig. 7. 1-DE Western blotting of CLIC1. Cells that had been cultured with transferrin-supplemented serum-free medium for 3 days were stimulated by buffer solution (lane 2) or insulin (lane 3) and incubated for another 1 h at 37°C. **A**: cells were directly lysed with 1× Laemmli's sample buffer and subjected to 1-DE. Western blotting was performed using anti-CLIC1 antiserum. The lysate of the cells that had been cultured in the presence of FCS was also subjected to 1-DE (lane 1). Numerals under each band indicates intensity of the protein band. After the first antibody was stripped, the PVDF membrane was reblotted with anti- $\beta$ -tubulin antibody. **B**: cell lysates prepared according to 2-DE protocol were mixed with isovolume of 2× Laemmli's sample buffer (lanes 1 and 2). One-eighth of the cells were directly lysed with 1× Laemmli's sample buffer and subjected to 1-DE (lanes 3 and 4). Longer exposure results of lanes 3 and 4 are shown in lanes 5 and 6, respectively. **C**: results of independently performed experiment from **B** are shown. **D** and **E**: subcellular localization of CLIC1. **D**: insulin-treated or nontreated HL-60 cells were stained with anti-CLIC1 antibody. DIC, photograph with Normarsky differentiated interference contrast. **E**: percentages of cells with nuclear speckled staining pattern (filled bars) and cells with nucleoli-staining pattern (gray bars) are shown.

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 be 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-

Fig. 8. 1-DE Western blotting of SRp20. Cells were cultured with transferrin-supplemented serum-free medium for 3 days. **A**: cells were then stimulated with insulin, and cell lysates were prepared at indicated times (lanes 2–4). Western blotting was performed using anti-SRp20 antiserum. The lysate of cells cultured in the presence of FCS was also subjected to 1-DE (lane 1). Numerals under each band indicates intensity of protein band. After the first antibody was stripped, PVDF membrane was reblotted with anti- $\beta$ -tubulin antibody. **B**: DMSO or increasing doses of MG-132 were added 30 min before insulin stimulation. Cell lysates were prepared 1 h after stimulation and subjected to 1-DE. Western blotting was performed using anti-SRp20 antiserum. PVDF membrane was reblotted with anti- $\beta$ -tubulin antibody and further with cyclin D3 antibody.



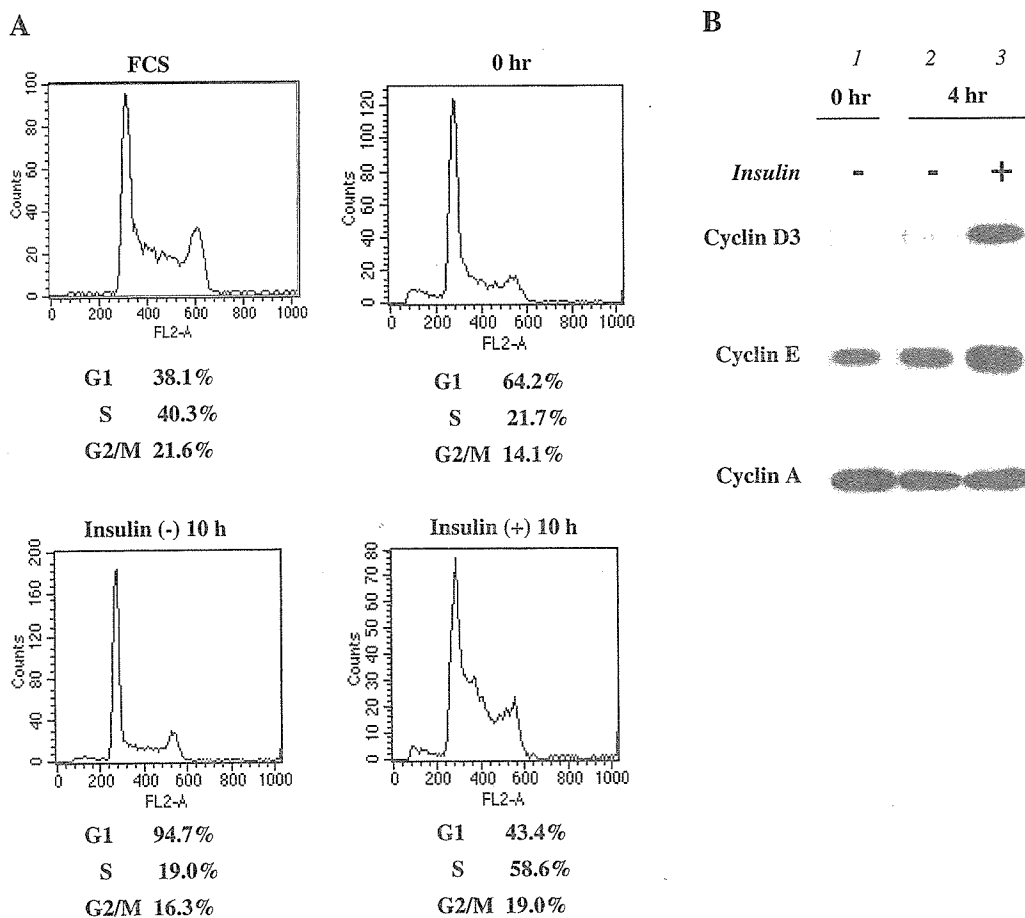


Fig. 9. Cell cycle analysis and expressions of cyclins. A: cells cultured with transferrin-supplemented serum-free medium for 3 days were stimulated with buffer solution (*bottom left*) or insulin (*bottom right*). After another 10-h incubation at 37°C, cells were fixed with 70% ethanol and subjected 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.

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 SRp20 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 NH<sub>2</sub> 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.



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. 8B). 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.

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## Synergistic Effects of Dehydroepiandrosterone and Retinoic Acid on Granulocytic Differentiation of Human Promyelocytic NB4 Cells

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### Abstract

We report a novel effect of dehydroepiandrosterone (DHEA) on human granulocyte differentiation: DHEA enhances the all-*trans*-retinoic acid (ATRA)-induced differentiation of promyelocytic NB4 cells. DHEA (100  $\mu$ M) significantly augmented the respiratory burst activity of NB4 cells treated with 1 nM ATRA, whereas DHEA alone did not induce respiratory burst activity. The protein and message expressions of p67<sup>phox</sup>, the gene for the dose-limiting component of phagocyte NADPH oxidase, were significantly enhanced by the coexistence of DHEA and ATRA. The protein expression of p47<sup>phox</sup>, another component of phagocyte NADPH oxidase, was also up-regulated by DHEA and ATRA. Moreover, the ATRA-induced increment of CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) and the reciprocal reduction in C/EBP $\alpha$  expression were also potentiated by DHEA. In contrast, the expression of PU.1, a transcription factor reportedly involved in the basal expression of p67<sup>phox</sup> in monocytic cells, was only slightly up-regulated by DHEA and ATRA. Interestingly, DHEA sulfate (DHEAS), the sulfate ester of DHEA that exists in peripheral blood at a concentration approximately 3 orders of magnitude larger than that of DHEA, did not stimulate the ATRA-induced differentiation of NB4 cells. Thus, DHEA, but not DHEAS, plays important roles in synergy with ATRA during granulocyte differentiation of human promyelocytic NB4 cells.

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**Key words:** DHEA; Granulocytic differentiation; NB4 cells; All-*trans*-retinoic acid; Respiratory burst activity

### 1. Introduction

Among the various steroid hormones, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are unique in that only the adrenal glands of humans and a few higher primates can synthesize and secrete these hormones in large amounts (reviewed in [1]). Because the plasma concentrations of DHEA and DHEAS steadily decrease with age, DHEA/DHEAS have been considered antiaging hormones (reviewed in [2]). Actually, supplementation with DHEA or DHEAS shows the benefit of preventing lifestyle-related diseases, including obesity, diabetes, and coronary heart disease (reviewed in [3]).

Although free steroids are highly insoluble in water, they become water soluble when conjugated with sulfate. The sul-

fate ester forms of steroid hormones, however, cannot bind to their nuclear receptors. They become activated only after they are desulfated by steroid sulfatase [4]. Because steroid sulfatase is ubiquitously present in mammalian tissues, it is sometimes difficult to distinguish the *in vivo* effects of DHEA and DHEAS. Nevertheless, *in vitro* experiments have shown a clear discrepancy between the effects of DHEA and DHEAS. DHEA, but not DHEAS, inhibits preadipocyte growth and differentiation [5]. In contrast, DHEAS, but not DHEA, stimulates peroxisome proliferator-activated receptor  $\alpha$ -induced gene expression [6]. These steroid hormones sometimes show opposite effects. For example, DHEA decreases, but DHEAS increases, the apoptosis of neural precursors from the rat embryonic brain [7]. On the other hand, both DHEA and DHEAS show similar anti-inflammatory effects [8].

Despite the variety of beneficial effects of DHEA/DHEAS, the molecular bases of their effects are largely unknown. Although DHEA reportedly acts as a weak agonist of the pregnane X receptor [9], whether another high-affinity receptor exists remains elusive. Moreover, DHEA and DHEAS can also function as modulators of

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intracellular signal transduction. For example, these hormones can scavenge reactive oxygen species and inhibit nuclear factor  $\kappa$ B (NF- $\kappa$ B) activity, resulting in a suppression of inflammation [8].

Although rodent steroidogenic glands, including the adrenals, do not secrete significant amounts of DHEA, possible de novo synthesis of DHEA/DHEAS in the rat brain has been reported [10,11]. DHEA and DHEAS have also been reported to act as neuroactive neurosteroids in the brain by promoting the proliferation and differentiation of neurons [10], protecting apoptosis [12], and even stimulating apoptosis [7]. However, the roles of DHEA/DHEAS in the hematopoietic system are poorly understood. DHEA reportedly ameliorated granulopenia after whole-body  $\gamma$  irradiation in mice [13]. On the other hand, DHEA was shown to inhibit natural killer cell generation and lymphopoiesis but not myelopoiesis in lethally irradiated mice [14]. Apart from the inferences from the studies with these animal models, however, the roles of DHEA/DHEAS in human myelopoiesis have not been studied so far.

We report our study of the effects of DHEA and DHEAS on NB4 cells, undertaken to obtain some insights into the effects of these steroid hormones on human myeloid differentiation. Our results show that DHEA, but not DHEAS, enhances the differentiation of human promyelocytic NB4 cells in combination with a low dose of all-*trans*-retinoic acid (ATRA). The molecular mechanism of DHEA action is also discussed.

## 2. Materials and Methods

### 2.1. Cells and Reagents

Human promyelocytic NB4 cells were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS, USA). DHEA, DHEAS, and ATRA (all from Sigma Chemical Company, St. Louis, MO, USA) were dissolved in solvent solution (90% ethanol and 10% dimethyl sulfoxide [DMSO]), water, and 100% ethanol, respectively. *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbolmyristate acetate (PMA) (Sigma Chemical Company) were dissolved in DMSO.

### 2.2. DNA Fragmentation Assay for Apoptosis Detection

The DNA fragmentation assay was performed as described [15]. Cells ( $3 \times 10^6$ ) were lysed with 350  $\mu$ L lysis buffer (5 mM Tris, pH 8.0, 0.5% Triton X-100, and 10 mM EDTA). Cell lysates were centrifuged at 15,000 rpm at room temperature for 20 minutes, and the supernatant was treated with 1 mg/mL of heat-inactivated ribonuclease A (Sigma Chemical Company) at 42°C for 60 minutes and with 200  $\mu$ g/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) for another 60 minutes. After phenol extraction, phenol/chloroform extraction, and ethanol precipitation, the DNA sample was applied to a 1.5% agarose gel, separated by horizontal electrophoresis, and visualized by staining with 500 ng/mL ethidium bromide. A

100-base pair (bp) ladder marker (Toyobo, Osaka, Japan) was used to evaluate the molecular weights of the fragmented DNA.

### 2.3. Assessment of Respiratory Burst Activity

Respiratory burst activity was evaluated by quantifying superoxide ( $O_2^-$ ) release. Superoxide was assayed by the superoxide dismutase-inhibitable reduction of ferricytochrome *c*, and the continuous assay was performed in a Hitachi 556 double-wavelength spectrophotometer (Hitachi, Tokyo, Japan) equipped with a thermostated cuvette holder (37°C), as described previously [16]. The final cell concentration was  $1 \times 10^6$  cells/mL. The reduction of cytochrome *c* was measured at 550 nm with a reference wavelength of 540 nm, and the time course of cytochrome *c* reduction (absorbance change at 550-540 nm) was followed on the recorder. The amount of superoxide released was calculated from the amount of cytochrome *c* reduced in 5 minutes after the addition of FMLP and from the linear portion of the cytochrome *c*-reduction curve for PMA.

### 2.4. Western Blotting

Cells ( $5 \times 10^5$ ) were lysed with 100  $\mu$ L of  $1 \times$  Laemmli sample buffer and boiled. Cell lysates were then subjected to electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate (SDS). After electrophoresis, proteins were transferred from the gel onto a nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm<sup>2</sup> for 2 hours at 4°C. Residual binding sites on the membrane were blocked by incubating the membrane for 1 hour at 25°C in Tris buffer (150 mM Tris-HCl, pH 7.6) containing 3% bovine serum albumin. After washing, the membranes were incubated with antibodies to p67<sup>phox</sup>, p47<sup>phox</sup>, CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ), C/EBP $\beta$ , PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and tubulin- $\alpha$  (Lab Vision, Fremont, CA, USA) for 4 hours at 25°C. The secondary antibody reactions were performed with horseradish peroxidase-conjugated antimouse and antirabbit monoclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) or horseradish peroxidase-conjugated antish sheep monoclonal antibody (Chemicon, Temecula, CA, USA). The final detection procedure was performed by using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and exposing Hyperfilm (Amersham Biosciences, Buckinghamshire, UK) as described previously [17].

### 2.5. Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from  $5 \times 10^6$  cells with an RNeasy Mini kit (Qiagen, Tokyo, Japan), and complementary DNA was synthesized with a Superscript II kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The reverse transcriptase-polymerase chain reaction (RT-PCR) procedure was performed with Ex Taq (TaKaRa Shuzo, Shiga, Japan) and a DNA thermal cycler (PJ2000; Perkin-Elmer, Foster City, CA, USA) with 30 cycles of 95°C

for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The primers used were 5'-TGGAGTGTGTCTGGAAGCAG-3' and 5'-ATCTCTGGGGTTTTCCGGTCT-3' for p67<sup>phox</sup> and 5'-CTCTTCCAGCCTTCCTTCCT-3' and 5'-AGCACTGTGTTGGCGTACAG-3' for  $\beta$ -actin. The PCR products were separated by agarose gel electrophoresis, and the DNA was visualized by ethidium bromide staining.

## 2.6. Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) was performed with histone H3 ChIP assay kits (Upstate Biotechnology, Lake Placid, NY, USA). Cells ( $1 \times 10^6$ ) were fixed with 1% paraformaldehyde at 37°C for 10 minutes. The cells were sedimented, washed, and lysed with SDS lysis buffer (50 mM Tris-HCl, 1% SDS, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/mL aprotinin, and 1  $\mu$ g/mL pepstatin A). The lysates were sonicated to reduce DNA lengths to between 200 bp and 1000 bp. The soluble fraction was diluted, precleared with salmon sperm DNA/protein A-agarose, and then incubated with 6  $\mu$ L of antiserum specific for the acetylated forms of histone H3. Then, immune complexes were precipitated with protein A-agarose. The precipitated DNA was eluted with an elution buffer (0.1 M NaHCO<sub>3</sub> containing 1% SDS). The eluted material was incubated at 65°C for 6 hours to reverse the formaldehyde cross-links, and DNA was extracted with phenol and chloroform. Ethanol-precipitated DNA was solubilized in water (equivalent to  $1 \times 10^6$  cells per 100  $\mu$ L). Semiquantitative PCR was performed with 3, 1, and 0.3  $\mu$ L of DNA samples (a 3-fold dilution) with 29 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide staining. The primers used were 5'-CTTTCAGTGGAGAGGGGATG-3' and 5'-AAGCCAAAACAGCCTGAAG-3' for the p67<sup>phox</sup> promoter region, 5'-CTTCAGGCTGTTTTTGCTT-3' and 5'-CCA

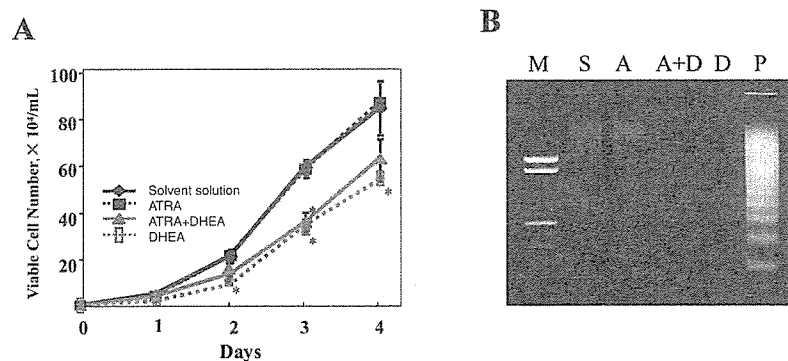
TGAGAGAGAAAAGGAAAGAAG-3' for its 3' downstream region, and 5'-TAAGACTTGCATGCACATACATTG-3' and 5'-ACTGCCAAGACTTTTTCTAGCCTTA-3' for its 5' upstream region. For the lamin A gene ChIP assay, 5'-GGACCTGCAGGAGCTCAATGATCG-3' and 5'-CCTTAAACTCCTCACGCACTTTGC-3' were used as primers.

## 3. Results

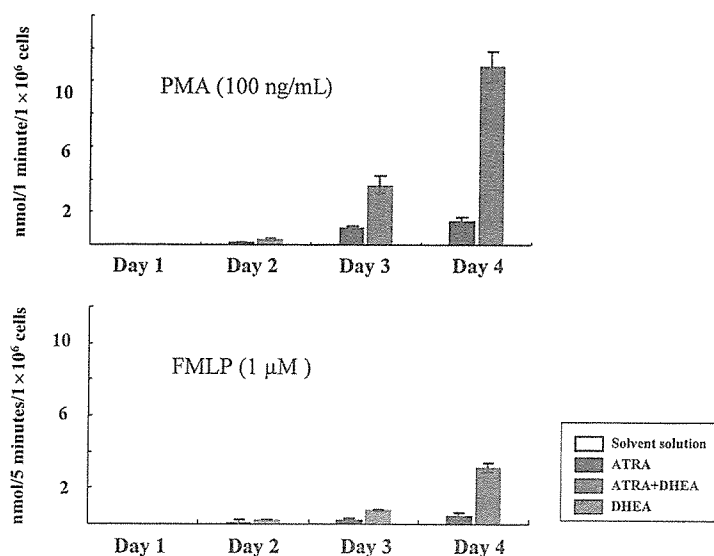
### 3.1. DHEA Enhances NB4 Differentiation in Cooperation with ATRA

In this study, we used a relatively high dose of DHEA (100  $\mu$ M) in combination with a low dose of ATRA (1 nM) because the lower concentration of DHEA did not exert any effects on NB4 cells (data not shown). One hundred micromolar DHEA alone induced a slight but significant growth suppression (Figure 1A), as has been described in studies of mouse 3T3-L1 cells (median effective dose, 30  $\mu$ M) [5] and rat and pig primary stromal-vascular cells [18], suggesting that DHEA exerts a particular effect on NB4 cells. Although 100  $\mu$ M DHEA has also been reported to induce the death of murine preadipocytic 3T3-L1 cells [5], it did not induce the death of human promyelocytic NB4 cells, as assessed by trypan blue staining (data not shown) and apoptotic DNA-ladder formation (Figure 1B).

In the initial experiment of cellular differentiation, we studied the respiratory burst activity controlled by phagocyte NADPH oxidase, a highly sensitive and specific differentiation marker for granulocyte and monocyte lineages. As shown in Figure 2, DHEA up-regulated respiratory burst activity induced by 1 nM ATRA under both PMA-triggered and FMLP-triggered conditions ( $P < .01$ ), although DHEA alone did not induce respiratory burst activity. Thus, DHEA enhances the differentiation-dependent acquisition of respiratory burst activity in synergy with a low dose of ATRA.



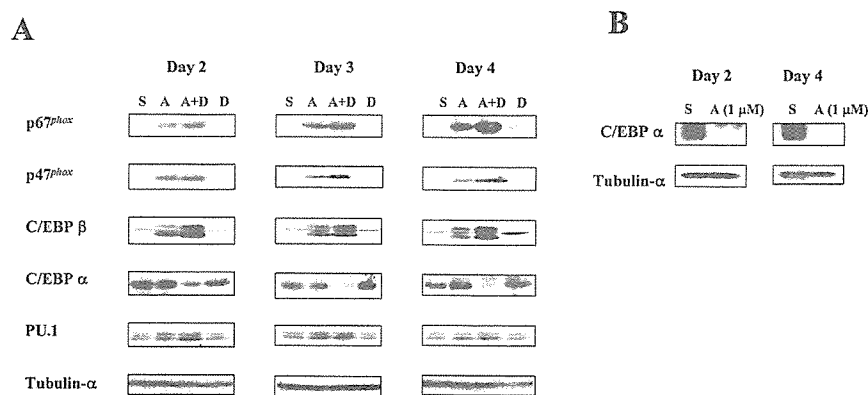
**Figure 1.** Dehydroepiandrosterone (DHEA) suppresses the growth but does not reduce the viability of NB4 cells. A, Proliferation curve. NB4 cells were treated with solvent solution (final concentrations, 0.09% ethanol and 0.01% dimethyl sulfoxide), 1 nM *all-trans*-retinoic acid (ATRA), 1 nM ATRA plus 100  $\mu$ M DHEA, or 100  $\mu$ M DHEA as indicated, and the numbers of viable cells were counted over time. Representative data from 3 independent experiments are shown, and the data are expressed as the mean  $\pm$  SD of triplicate cultures. B, NB4 cells were treated with solvent solution (S), ATRA (A), ATRA plus DHEA (A+D), or DHEA (D) as indicated. At day 3, low molecular weight DNA was extracted and separated by 1.5% agarose gel electrophoresis. Also indicated as a positive control is the DNA sample from overgrown HL-60 cells (P) [15]. Representative data from 3 independent experiments are shown.



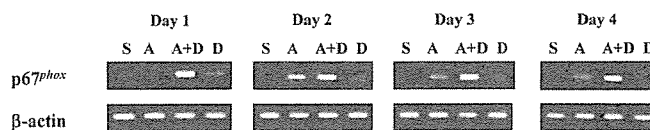
**Figure 2.** Dehydroepiandrosterone (DHEA) enhances all-*trans*-retinoic acid (ATRA)-induced acquisition of respiratory burst activity. NB4 cells were treated with solvent solution (final concentrations, 0.09% ethanol and 0.01% dimethyl sulfoxide), 1 nM ATRA, 1 nM ATRA plus 100  $\mu$ M DHEA, or 100  $\mu$ M DHEA as indicated. Cells were collected over time, and respiratory burst activity was evaluated. Superoxide ( $O_2^-$ ) release stimulated by 100 ng/mL phorbolmyristate acetate (PMA) (upper panel) and by 1  $\mu$ M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (lower panel) was determined by the reduction of cytochrome *c* and is expressed in nmol/min per  $1 \times 10^6$  cells for PMA and nmol/5 min per  $1 \times 10^6$  cells for FMLP. Representative data from 3 independent experiments are shown, and the data are expressed as the mean  $\pm$  SD of triplicate cultures.

We then used Western blotting to investigate the expression of the proteins that are involved in or associated with granulocytic differentiation. As shown in Figure 3A, the expression level of p67<sup>phox</sup>, the gene for a dose-limiting component of phagocyte NADPH oxidase [19], was synergistically augmented by DHEA and ATRA, although DHEA alone did not induce p67<sup>phox</sup> expression. The expression of

p47<sup>phox</sup>, the gene for another inducible component of phagocyte NADPH oxidase, was also up-regulated by the presence of both DHEA and ATRA (Figure 3A). The expression of C/EBP $\beta$ , a transcription factor that reportedly increases during myeloid differentiation, was indeed enhanced by DHEA and ATRA (Figure 3A). The expression of C/EBP $\alpha$ , which decreases when NB4 cells become differentiated into more



**Figure 3.** Dehydroepiandrosterone (DHEA) augments all-*trans*-retinoic acid (ATRA)-induced alteration in the expression of differentiation-associated proteins. A, NB4 cells were treated with solvent solution (S) (final concentrations, 0.09% ethanol and 0.01% dimethyl sulfoxide), 1 nM ATRA (A), 1 nM ATRA plus 100  $\mu$ M DHEA (A+D), or 100  $\mu$ M DHEA (D) as indicated. Total cell lysates were prepared over time, and Western blotting was performed by using anti-p67<sup>phox</sup>, anti-p47<sup>phox</sup>, anti-C/EBP $\beta$ , anti-C/EBP $\alpha$ , anti-PU.1, and anti-tubulin- $\alpha$  antibodies. Representative data from 3 independent experiments are shown. B, NB4 cells were treated with solvent solution (S) (final concentration, 0.1% ethanol) or a high dose of ATRA (A) (1  $\mu$ M) as indicated. Total cell lysates were prepared at day 2 and day 4, and Western blotting was performed with anti-C/EBP $\alpha$  and anti-tubulin- $\alpha$  antibodies. Representative data from 3 independent experiments are shown.



**Figure 4.** Dehydroepiandrosterone (DHEA) and all-*trans*-retinoic acid (ATRA) synergistically up-regulate p67<sup>phox</sup> message expression. NB4 cells were treated with solvent solution (S) (final concentrations, 0.09% ethanol and 0.01% dimethyl sulfoxide), 1 nM ATRA (A), 1 nM ATRA plus 100 μM DHEA (A+D), or 100 μM DHEA (D) as indicated. Total RNA was extracted over time, and reverse transcriptase–polymerase chain reaction analyses were performed with p67<sup>phox</sup>-specific primer pairs (upper panels) or β-actin–specific primer pairs (lower panels). Representative data from 3 independent experiments are shown.

mature stages by a high ATRA dose (Figure 3B), was down-regulated by DHEA and ATRA (Figure 3A). On the other hand, the expression of PU.1, a transcription factor involved in basal p67<sup>phox</sup> expression in monocytic cells, was only slightly and transiently (only at day 2) up-regulated by the presence of both DHEA and ATRA (Figure 3A).

Thus, a relatively high dose of DHEA and a low dose of ATRA synergistically enhance the differentiation of NB4 cells by augmenting respiratory burst activity, differentiation-dependent p67<sup>phox</sup> and p47<sup>phox</sup> induction, differentiation-associated up-regulation of C/EBPβ, and the reciprocal differentiation-associated down-regulation of C/EBPα, without significantly affecting PU.1 expression.

### 3.2. DHEA Up-regulates p67<sup>phox</sup> Message Expression without Affecting Histone H3 Acetylation

Because p67<sup>phox</sup> is the most critical component of phagocyte NADPH oxidase for the acquisition of respiratory burst activity during myeloid differentiation, we focused our study on the molecular mechanism of p67<sup>phox</sup> induction. First, we used RT-PCR to examine p67<sup>phox</sup> message expression in the time course after ATRA and DHEA treatment. As shown in Figure 4, the expression of p67<sup>phox</sup> message was synergistically up-regulated by DHEA and ATRA. Two independent experiments gave similar results (data not shown).

Because DHEA is a steroid hormone, it may possibly act as a ligand of a nuclear receptor to enhance transcription. It may also work in the cytoplasm, because DHEA and DHEAS, unlike other steroid hormones, function as cytoplasmic signal transduction modulators. For example, they are reportedly involved in anti-inflammation via the scavenging of reactive oxygen species, thereby inhibiting NF-κB activities [8]. Because sulfate ester forms of steroid hormones cannot bind to nuclear receptors, DHEAS cannot affect NB4 differentiation if DHEA works as a ligand of a nuclear receptor. On the other hand, DHEAS can show an effect similar to that of DHEA if DHEA works in the cytoplasm as a signal transduction modulator.

To address this point, we added DHEAS to NB4 cells along with a low dose of ATRA and evaluated the differentiation-dependent respiratory burst activity. As shown in Figure 5, DHEAS completely failed to enhance the ATRA-induced respiratory burst activity of NB4 cells under the same conditions in which DHEA potently enhanced this activity (Figure 2). Thus, it is highly possible that DHEA functions as a ligand of a particular nuclear receptor.

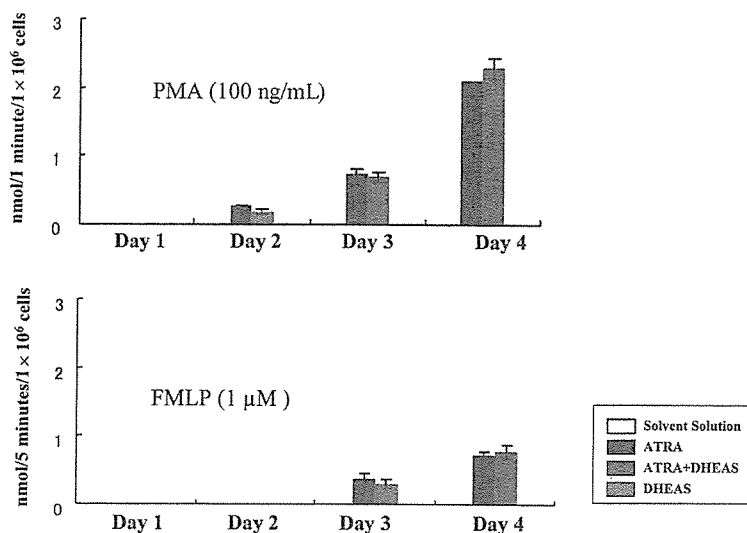
Steroid hormones play important roles in chromatin remodeling by regulating the corepressor/coactivator-complex exchanges [20]. On binding of steroid hormones to their nuclear receptors, the histone deacetylase-containing corepressor complex is eliminated, and the histone acetyltransferase-containing coactivator complex is recruited to the chromatin, inducing the hyperacetylation of histone H3. Therefore, we used the ChIP assay to examine the histone H3 acetylation state at the p67<sup>phox</sup> promoter region (corresponding to bp –246 to bp +14 relative to the transcription start site), a 5' upstream region (corresponding to bp –2261 to bp –1928 relative to the transcription start site), and a 3' downstream region (corresponding to bp +14 to bp +329 relative to the transcription start site).

As shown in Figure 6, histone H3 acetylation was up-regulated by ATRA treatment by approximately 3-fold in all of these regions. ChIP assays targeting broader regions in the p67<sup>phox</sup> gene (covering bp –2261 to bp +36,922 relative to the transcription start site) gave similar results (data not shown). Again, DHEA alone did not influence the histone H3 acetylation states. Unexpectedly, DHEA did not enhance ATRA-induced histone H3 acetylation (Figure 6), although it clearly augmented ATRA-induced p67<sup>phox</sup> message expression (Figure 4). Thus, DHEA up-regulates p67<sup>phox</sup> message expression without affecting histone H3 acetylation.

## 4. Discussion

We have shown that DHEA enhances the ATRA-induced differentiation of human promyelocytic NB4 cells. We have demonstrated that DHEA potentiates the ATRA-induced respiratory burst activity via a synergistic up-regulation of p67<sup>phox</sup> message expression. It also intensifies the ATRA-induced alteration in the expression of proteins such as C/EBPα and C/EBPβ.

The *in vivo* significance of retinoic acid signaling in myeloid differentiation is rather controversial. In the *in vitro* colony-formation assay, bone marrow populations of retinoic acid receptor α1/retinoic acid receptor γ double-null mice were blocked at the myelocyte stage and, to a lesser extent, at the metamyelocyte stage. However, the bone marrow cells appear to differentiate normally *in vivo*, suggesting an effective compensation mechanism in these mice, at least *in vivo* [21]. An argument against the *in vivo* significance of ATRA in granulocyte differentiation derives from the fact that the plasma concentration of ATRA is quite low. The ATRA con-



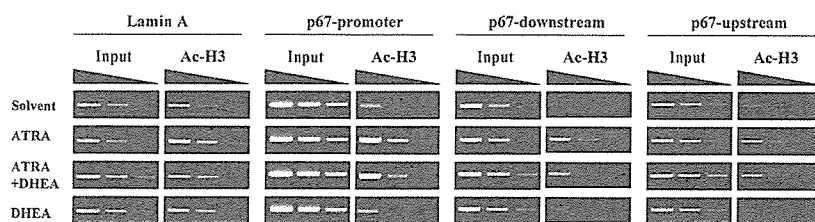
**Figure 5.** Dehydroepiandrosterone sulfate (DHEAS) does not enhance all-*trans*-retinoic acid (ATRA)-induced respiratory burst activity. NB4 cells were treated with solvent solution (final concentration, 0.09% ethanol), 1 nM ATRA, 1 nM ATRA plus 100  $\mu$ M DHEAS, or 100  $\mu$ M DHEAS as indicated. Cells were collected over time, and the respiratory burst activity was evaluated. Superoxide ( $O_2^-$ ) release stimulated by 100 ng/mL phorbolmyristate acetate (PMA) (upper panel) and by 1  $\mu$ M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (lower panel) was determined by the reduction of cytochrome *c*, and is expressed in nmol/min per  $1 \times 10^6$  cells for PMA and nmol/5 min per  $1 \times 10^6$  cells for FMLP. Representative data from 3 independent experiments are shown, and the data are expressed as the mean  $\pm$  SD of triplicate cultures.

concentration in peripheral blood is approximately 15 nM in healthy individuals, whereas 1  $\mu$ M ATRA has been used in *in vitro* differentiation experiments. Certain factors that exist in human plasma but not in fetal bovine serum may work in cooperation with ATRA to make it possible for a low dose of ATRA to induce myeloid differentiation *in vivo*. Our findings strongly suggest that DHEA, which potentiates the action of a low dose of ATRA (1 nM) and cooperatively induces myeloid differentiation, is one candidate for such a cooperative factor of ATRA. Apart from the *in vivo* significance of DHEA, it can also provide therapeutic benefits for the treatment of ATRA-resistant promyelocytic leukemia by potentiating the weakened ATRA signaling.

The molecular mechanism of cooperation between DHEA and ATRA during granulocytic differentiation of NB4 cells remains elusive. DHEA has been reported to act as an inhibitor of glucose-6-phosphate dehydrogenase and thus inhibit the PMA-triggered respiratory burst in human neu-

trophils [22]. In our study, however, DHEA did not inhibit, but rather augmented, PMA-triggered respiratory burst activity in ATRA-treated differentiated NB4 cells. Thus, a completely different mechanism must have been working in our case. DHEA and retinoic acid have also been reported to synergistically promote the differentiation of human neuroblastoma cells [23]. In that case, however, DHEA enhanced neuroblastoma differentiation via signaling that was not mediated by retinoic acid receptor: retinoic acid enhances the activity of matrix metalloproteinase 2, and DHEA enhances the activity of matrix metalloproteinase 9 [23].

In the present study, the effects of ATRA and ATRA plus DHEA were highly analogous, with the ATRA effect becoming amplified under conditions of ATRA plus DHEA with respect to p67<sup>phox</sup> message expression and protein expression of p67<sup>phox</sup>, p47<sup>phox</sup>, C/EBP $\alpha$ , and C/EBP $\beta$ . Thus, DHEA signaling seems likely to have certain interactions with ATRA signaling, at least in NB4 cells. As we have



**Figure 6.** Histone H3 acetylation (Ac-H3) of the p67<sup>phox</sup> gene during NB4 differentiation. NB4 cells were treated with solvent solution (final concentrations, 0.09% ethanol and 0.01% DMSO), 1 nM all-*trans*-retinoic acid (ATRA), 1 nM ATRA plus 100  $\mu$ M dehydroepiandrosterone (DHEA), or 100  $\mu$ M DHEA as indicated. At day 4, cells were collected and the chromatin immunoprecipitation assay was performed with specific primer pairs for the lamin A gene, the p67<sup>phox</sup> gene promoter region, and its upstream and downstream regions. Representative data from 3 independent experiments are shown.



shown, DHEAS did not show synergy with ATRA during NB4 differentiation, suggesting that DHEA acts as a ligand of a nuclear receptor. Because a synergistic effect of DHEA and ATRA was detected only when ATRA was used at a low concentration, the downstream signaling of DHEA may converge to ATRA signaling and thus amplify the final common pathway of NB4 cell differentiation. That DHEA alone had no effects on NB4 cell differentiation indicates that a low dose of ATRA is required, not only for the differentiation trigger but also for an elicitation of DHEA's actions. ATRA possibly induces the expression of a still-unknown DHEA receptor.

Even at a low concentration, ATRA induced the histone H3 acetylation of the p67<sup>phox</sup> gene, and this mechanism may be responsible for the observed enhanced transcription of this gene by ATRA (Figure 4). DHEA alone, however, did not induce histone H3 acetylation of p67<sup>phox</sup> and had no enhancing effect on ATRA-induced histone H3 acetylation, in spite of its enhancing effect on the transcription of p67<sup>phox</sup> in the presence of ATRA. Thus, DHEA conceivably affected p67<sup>phox</sup> transcription via a mechanism independent of histone H3 acetylation. Several possible mechanisms could explain these phenomena. For example, DHEA might directly activate the transcription factors for the p67<sup>phox</sup> gene, such as Sp1, PU.1, AP-1, and C/EBP $\beta$  [24]. Alternatively, DHEA might stabilize p67<sup>phox</sup> messenger RNA, resulting in its accumulation.

There are no major qualitative differences in the actions of ATRA and DHEA, both of which have been hypothesized to use nuclear receptors to initiate the transcription of target genes during cellular differentiation. In contrast, DHEA did not induce histone H3 acetylation of the p67<sup>phox</sup> gene under the conditions of ATRA-induced p67<sup>phox</sup> gene acetylation. Thus, the 2 agonists seem to act on cells via similar but not identical pathways. Similar parts of the signaling pathways cross-talk with each other, and an independent pathway may induce additive or synergistic effects. Further study is required for a comprehensive understanding of the complex cross-talk between ATRA and DHEA. In particular, identification of a possible nuclear receptor for DHEA is the most important problem to be resolved.

## Acknowledgments

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## Clonal T Cells of Pure Red-Cell Aplasia

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This study detected clonal T cells in patients with acquired pure red-cell aplasia (PRCA) by Southern blotting and polymerase chain reaction (PCR). Twenty-nine adult patients with acquired PRCA were enrolled in this study. Seventeen patients had primary acquired PRCA, while 12 patients had the secondary form. Twenty-two of 29 (76%) patients demonstrated TCR rearrangement by at least one method. We divided the patients into three groups depending on T-cell clonality. The CD4/8 ratio of patients who were positive on Southern blotting was significantly lower than that of other groups. Except for the CD4/8 ratio, other laboratory findings did not significantly differ among the three groups. The CD4/8 ratio should be a useful surrogate marker to detect T-cell clonality. *Am. J. Hematol.* 79:332–333, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** pure red-cell aplasia; T-cell receptor

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### INTRODUCTION

Acquired primary pure red-cell aplasia (PRCA) has been associated with T cells that inhibit marrow erythropoiesis. Whereas LGL leukemia is a typical disorder involving clonal T cells [1], there have been some reports of clonal T-cell existing in other disorders [2]. We investigated clonal T cells in patients with acquired PRCA.

### PATIENTS AND METHODS

Patients with acquired PRCA treated at 12 institutes affiliated with the Study Group for Intractable Hematopoietic Disease supported by the Ministry of Health and Welfare of Japan were studied between 1996 and 2000. All patients fulfilled standard criteria for PRCA and had anemia with reticulocyte count <1%. To detect T-cell clonality, Southern blot analysis and stepdown polymerase chain reaction (PCR) were performed. Amplification of the TCR- $\beta$  and - $\gamma$  chain genes was carried out using the technique described by McCarthy et al. [3]. Sequences and sources of primer were described by Diss et al. [4].

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### RESULTS

Twenty-nine adult patients with acquired PRCA were enrolled. Seventeen patients had primary acquired PRCA, while 12 patients had the secondary form. Two patients' cases were associated with LGL leukemia. Two patients were associated with thymoma. Two patients' cases were associated with autoimmune disease (rheumatoid arthritis). Six patients' cases were associated with myelodysplastic syndrome (MDS). Nine of 29 (31%) patients showed TCR rearrangement on Southern blotting and PCR. Thirteen of 29 (45%) showed such an rearrangement on PCR. Seven of 29 (24%) patients did not show any TCR rearrangement.

The patients with acquired PRCA were divided into three groups depending on T-cell clonality

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TABLE I. Comparison Between Three Groups Classified by T Cell Clonality

	Positive on Southern blotting	Positive on PCR	No clonal T-cell	P
N	9	13	7	
Hemoglobin (g/dl)	8.9 ± 2.80	7.5 ± 2.9	8.9 ± 3.0	NS
Mean corpuscular volume (fL)	103 ± 14	101 ± 11	100 ± 10	NS
White cell count (×10 <sup>9</sup> )	5840 ± 4280	3540 ± 850	4230 ± 1830	NS
Absolute neutrophil count (×10 <sup>9</sup> )	2890 ± 3070	1900 ± 1190	2630 ± 1750	NS
Absolute granular lymphocyte count (×10 <sup>9</sup> )	1630 ± 1800	400 ± 250	440 ± 340	NS
CD 4/8 ratio	0.24 ± 0.17	0.84 ± 0.63	1.35 ± 0.24	0.02

(Table I). Nine patients were positive on Southern blotting. Among the patients who were PCR positive, 13 were positive only on PCR rearrangement. There were 7 patients with no clonal T cells. The hematologic parameters did not differ between the groups, but the CD 4/8 ratio of patients who were positive on Southern blotting was significantly lower than those of other groups.

## DISCUSSIONS

Semenzato et al. reported that the clinical status of patients with a relatively low absolute number of granular lymphocytes resembled that of those with greater than 2,000 granular lymphocytes/ $\mu$ L [5]. In this report, only two patients (LGL leukemia) showed an absolute granular lymphocyte count greater than 2,000. Laboratory findings in these patients did not differ from those of other patients. Go et al. proposed criteria for lymphoproliferative disease of granular T lymphocytes (T-LDGL) with unexplained abnormal blood counts (cytopenia macrocytosis and/or lymphocytosis) and expression of a clonal T-cell receptor rearrangement [6]. According to these criteria, 22 of 29 (76%) patients in this report (patients who were

positive on Southern blotting and PCR) were diagnosed as having T-LDGL.

Except for the CD4/8 ratio, laboratory findings did not significantly differ among the three groups. In acquired PRCA, an inverted CD4/8 ratio may indicate the existence of a clonal T-cell population.

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## DNA microarray analysis of dysplastic morphology associated with acute myeloid leukemia

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**Objective.** Acute myeloid leukemia (AML) develops de novo or secondarily to either myelodysplastic syndrome (MDS) or anticancer treatment (therapy-related leukemia, TRL). Prominent dysplasia of blood cells is apparent in individuals with MDS-related AML as well as in some patients with TRL or even with de novo AML. The clinical entity of AML with multilineage dysplasia (AML-MLD) is likely to be an amalgamation of MDS-related AML and de novo AML-MLD. The aim of this study was to clarify, by the use of high-density oligonucleotide microarrays, whether these subcategories of AML are intrinsically distinct from each other.

**Materials and Methods.** The AC133<sup>+</sup> hematopoietic stem cell-like fractions were purified from the bone marrow of individuals with de novo AML without dysplasia (n = 15), AML-MLD (n = 11), MDS-related AML (n = 11), or TRL (n = 2), and were subjected to the synthesis of cRNA which was subsequently hybridized to microarray harboring oligonucleotide corresponding to more than 12,000 probe sets.

**Results.** We could identify many genes whose expression was specific to these various subcategories of AML. Furthermore, with the correspondence analysis/three-dimensional projection strategy, we were able to visualize the independent, yet partially overlapping, nature of current AML subcategories on the basis of their transcriptomes.

**Conclusion.** Our data indicate the possibility of subclassification of AML based on gene expression profiles of leukemic blasts. © 2004 International Society for Experimental Hematology. Published by Elsevier Inc.

Acute myeloid leukemia (AML) may develop de novo or as a result of either myelodysplastic syndrome (MDS) or anticancer treatment [1]. Given that MDS is characterized by dysplastic changes in differentiated blood cells, individuals with MDS-related leukemia often manifest prominent dysplasia in their blood cells. Therapy-related acute leukemia (TRL) may develop after the administration of alkylating agents, topoisomerase inhibitors, or radiotherapy. The clinical outcome of TRL is generally worse than that of de novo AML [2], and a subset of individuals with TRL also exhibit multilineage dysplasia of blood cells.

A clinical record of a preceding MDS phase is also an indicator of poor prognosis for the individuals with AML.

Therefore, to predict the outcome of, and to optimize the treatment for, each AML patient, it would be important to differentiate de novo AML from MDS-related AML and TRL. However, even in the bone marrow (BM) of healthy elderly people, it is not rare to find dysplastic changes (in particular, dyserythropoiesis) in differentiated blood cells [3]. Therefore, the differential diagnosis among such AML-related disorders is not always an easy task in the clinical settings, especially if a prior record of hematopoietic parameters is not available.

Making issues further complicated, prominent dysplasia in blood cells may be found among certain cases of de novo AML, with which prior MDS phases can be excluded [4,5]. It is known that such de novo AML with dysplasia has a poor outcome with conventional chemotherapy, as does MDS-related leukemia [6]. However, Taguchi et al. have argued that the former may be a distinct clinical entity from the

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latter based on the finding that the former cases respond far better to allogeneic bone marrow transplantation than the latter one [7]. In the revised classification of AML by the World Health Organization (WHO) [1], an entity of AML with multilineage dysplasia (AML-MLD) has been proposed, which includes both de novo AML with dysplasia and secondary AML from MDS. Whether such amalgamation holds a clinical relevance awaits further studies on this issue.

DNA microarray has made it possible to measure the expression levels in tens of thousands of genes simultaneously, and thus should be a promising tool to discover useful and reliable molecular markers for these AML-related disorders. However, a simple comparison of BM mononuclear cells (MNCs) with DNA microarray is likely to generate a large body of pseudopositive and pseudonegative data, which may only reflect different proportions of blastic cells within BM or the different lineage commitment of leukemic cells [8]. To minimize such “population-shift effect,” it should be effective to isolate and compare leukemic blasts at the same differentiation level from AML-related disorders.

Toward this goal, we started the Blast Bank project to purify and store AC133 surface marker [9]–positive hematopoietic stem cell (HSC)–like fractions from patients with a wide range of hematological disorders. Microarray analysis of these Blast Bank specimens has been highly successful in the isolation of molecular markers to differentiate de novo AML from MDS-related leukemia [8,10], and in the identification of genes that may be involved in the stage progression mechanism in chronic myeloid leukemia (CML) [11] or MDS [12]. Further, a proteomics approach with these Bank cells could identify a protein that may be associated with chromosome instability in leukemic cells [13].

We have now determined the expression intensities for more than 12,000 human probe sets in a total of 39 Blast Bank specimens, including those from 15 cases of de novo AML without dysplasia, 11 cases of MDS-related leukemia, 11 cases of AML-MLD, and 2 cases of TRL. The resulting large data set was analyzed to address whether these clinical entities are actually distinct from each other or whether they partially overlap.

## Patients and methods

### *Purification of AC133<sup>+</sup> cells*

BM aspirates were obtained from the study subjects with written informed consent. From each specimen, MNCs were isolated by Ficoll-Hypaque density gradient centrifugation, and were labeled with magnetic bead–conjugated anti-AC133 monoclonal antibody (AC133 MicroBead; Miltenyi Biotec, Auburn, CA, USA). AC133<sup>+</sup> HSC-like fractions were then purified through a miniMACS magnetic cell separation column (Miltenyi Biotec), and enrichment of the HSC-like fraction was evaluated by subjecting portions of the MNC and AC133<sup>+</sup> cell preparations either to staining with Wright-Giemsa solution or to the analysis of the expression of CD34,

CD38, and AC133 by flow cytometry (FACScan; Becton-Dickinson, Mountain View, CA, USA). In most instances, the CD34<sup>high</sup>CD38<sup>low</sup> fraction constituted greater than 90% of the eluate of the affinity column.

### *DNA microarray analysis*

Total RNA was extracted from the AC133<sup>+</sup> cell preparations by an RNeasy Mini column with RNase-free DNase (both from Qiagen Inc., Valencia, CA, USA), and was subjected to two rounds of amplification of mRNA fractions by T7 RNA polymerase [14]. The high fidelity of the amplification step was confirmed previously [10]. One microgram of the amplified complementary RNA (cRNA) was then converted to double-stranded cDNA by PowerScript reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA, USA), which was used to prepare biotin-labeled cRNA with ENZO BioArray transcript labeling kit (Affymetrix, Santa Clara, CA, USA). Hybridization of the samples with GeneChip HGU95Av2 microarrays was conducted by the GeneChip system (Affymetrix), revealing the expression intensities of 12,625 probe sets in each sample.

The transcriptome of 10 cases each with de novo AML and MDS-related AML has been already reported separately [10], aiming at the comparison between these two clinical conditions with the same differentiation background; the M2 subtype according to the classification of the French-American-British (FAB) Cooperative Group [15].

### *Statistical analysis*

The fluorescence intensity for each gene was normalized relative to the median fluorescence value for all human genes with a “Present” or “Marginal” call (Microarray Suite; Affymetrix) in each hybridization. Hierarchical clustering of the data set and analysis of variance (ANOVA) were performed with GeneSpring 6.0 software (Silicon Genetics, Redwood, CA, USA). Correspondence analysis [16] was performed with the ViSta software (<http://www.visual-stats.org>) for all genes showing a significant difference. Each sample was plotted in three dimensions based on the coordinates obtained from the correspondence analysis. All array data as well as details of the genes shown in the figures are available as supplementary information at the *Experimental Hematology* web site.

## Results

### *Comparison of AML-MLD and MDS-related AML*

Summarized in Table 1 are the clinical characteristics of 39 patients enrolled in this study, including 15 cases with de novo AML without dysplasia, 11 cases with AML-MLD, 11 cases with MDS-related AML, and 2 cases with TRL. The presence of “MLD” was determined according to the definition in the WHO classification [1], by a central review at the Department of Hematology and Molecular Medicine Unit, Nagasaki University, which is also a “central review institute” for the Japan Adult Leukaemia Study Group. It should be noted that favorable karyotypes, t(8;21) and inv(16), were found only in the cases with AML without dysplasia.

According to the WHO proposal of classification, AML-MLD is likely to be an amalgamation of bona fide de novo