Expression and Localization of the CDC34 Ubiquitin-conjugating Enzyme in Pediatric Acute Lymphoblastic Leukemia

Elena Eliseeva, Debananda Pati, Mitchell B. Diccinanni, Alice L. Yu, Syed K. Mohsin, Judith F. Margolin, and Sharon E. Plon

Department of Pediatrics, Texas Children’s Cancer Center, Baylor College of Medicine, Houston, Texas 77030 [E. E., D. P., J. F. M., S. E. P.]; The Breast Center, Baylor College of Medicine, Houston, Texas 77030 [S. K. M.]; and Department of Pediatrics, University of California at San Diego Medical Center, San Diego, California 92103 [M. B. D., A. L. Y.]

Abstract
Ubiquitin-dependent protein degradation impacts many cellular processes. However, the regulation of ubiquitin-conjugating enzymes (UBCs) in cancer is unknown. We find that the human CDC34 UBC protein is expressed at a 3–4 fold higher level (P < 0.001) in pediatric T cell than in pre-B-cell acute lymphoblastic leukemia (ALL) before treatment in two independent patient sets. The level of CDC34 mRNA was similar in both types of leukemia. CDC34 expression levels in normal resting T cells, B cells, and activated T lymphocytes was comparable with pre-B-cell ALL. CDC34 protein (but not mRNA) was also increased in T-cell ALL compared with pre-B-cell ALL cell lines. The difference in expression was not attributable to mutation or associated with altered CDC34 stability. Immunohistochemistry and cellular fractionation reveals a heterogeneous CDC34 expression pattern including cells containing primarily cytoplasmic or nuclear protein. Thus, a feature of pediatric T-cell ALL is posttranscriptional up-regulation and heterogeneous localization of the human CDC34 UBC.

Introduction
Ubiquitin-mediated proteolysis has been implicated in control of diverse processes including cell cycle regulation, signal transduction, and transcription. Destruction by ubiquitination of cellular regulators including cyclins A, B, and E, cyclin-dependent kinase inhibitor p27

KIP1, tumor suppressor protein p53, and inhibitor of NF

κB has been documented (1). The human CDC34 protein is a ubiquitin-conjugating enzyme (2, 3) Enzymes of this family are involved in the transfer of ubiquitin to target proteins that consequently become degraded by the 26S proteasome. A large multisubunit complex termed the E3 or ubiquitin protein ligase often mediates recognition of a specific ubiquitination target. The CDC34 enzyme uses the SCF E3 complex for ubiquitination of multiple targets (reviewed in Ref. 1).

CDC34 was originally identified in Saccharomyces cerevisiae as an essential gene required in the late G1 phase of the cell cycle because of the requirement for CDC34-SCF-mediated degradation of p40

SIC, a potent cyclin-dependent kinase inhibitor (4). CDC34 was shown to have a similar role in higher eukaryotic organisms including Xenopus laevis (5). The degradation of p27

KIP1 by the human CDC34-SCF complex is analogous to the degradation of Sic1 in yeast (6). In addition to cell cycle regulators it has been demonstrated that transcriptional activators MyoD1 (7), repressors hICE-RII and hATF5 (8), and the IκB (9) proteins can be targeted for degradation by human CDC34.

Although significant work on identifying the components of the human SCF complex has been accomplished, little is known about the regulation of the human CDC34 protein in normal or malignant cells. Alterations in the rate of ubiquitination in tumor cells have been described for p27

KIP1 in leukemia and colon cancer cells (10). In addition, the products of a number of tumor suppressor genes and oncogenes are regulated by ubiquitination. Thus, it is important to know if the human CDC34 ubiquitin-conjugating enzyme is differentially regulated in human cancers. Here we report that human CDC34 protein is differentially expressed and heterogeneously localized in the most common pediatric malignancies, pre-B-cell and T-cell ALL.

Results
Human CDC34 Protein Is Differentially Expressed in Pre-B-Cell versus T-Cell Pediatric Leukemias. We analyzed expression of human CDC34 RNA and protein in excess samples obtained from sequentially diagnosed patients with acute leukemia at TCCC under an Institutional Review Board-approved protocol. These samples were obtained at initial clinical presentation, and none of the patients had received antileukemic therapy before obtaining the sample. The specific type of leukemia was defined by immunophenotype studies performed on the same initial sample. Com-
complete protein degradation (based on integrity of the diatric population). Samples that demonstrated partial or (this reflects the relative ratio of pre-B:T-cell ALL in the pe-

with pre-B-cell ALL and 6 samples from T-cell ALL patients

patients were used for analysis: 30 samples from patients

actin and CDC34 bands in the lysate) were not included in

of myeloid and lymphoid markers on immunophenotype. The

comparison in different types of pediatric leukemias demonstrated

that CDC34 protein (normalized to β-actin) was expressed at

a higher level in T-cell ALL samples than in the more common

pre-B-cell ALL (see Fig. 1, top panels). Northern blot hybrid-

ization of the mRNA extracts obtained from the same pa-

tients samples demonstrated that CDC34 protein levels did

not correlate with mRNA levels (Fig. 1, bottom panel). In

particular, samples with high CDC34 mRNA levels could

have either low or high CDC34 protein levels. Therefore, the

observed differential expression of CDC34 protein in these

two types of leukemia seems to be regulated postranscrip-

tionally.

To obtain statistically significant data in regard to CDC34

protein expression, multiple patient samples were run in

Western blots with the same control pre-B-cell ALL sample,

and CDC34 expression was normalized to this sample and

β-actin. Altogether 36 samples from lymphoblastic leukemia

patients were used for analysis: 30 samples from patients

with pre-B-cell ALL and 6 samples from T-cell ALL patients

(this reflects the relative ratio of pre-B:T-cell ALL in the pe-

diatric population). Samples that demonstrated partial or

complete protein degradation (based on integrity of the β-

actin and CDC34 bands in the lysate) were not included in

the analysis. There was an ~4-fold higher level of CDC34

protein detected in T-cell ALL samples compared with pre-

B-cell ALL samples (Fig. 2A). Using Mann-Whitney statistics,

this difference proved to be highly significant (P < 0.001)

despite the small number of T-cell ALL samples from TCCC.

Patients with T-cell ALL are more likely than pre-B-cell ALL

to present with very high WBC counts at the time of diag-

nosis requiring leukapheresis. However, acute myelogenous

leukemia is rare in children and represents a very heteroge-

neous set of cell precursors. Therefore, for the remainder of

this study we have focused on pre-B-cell and T-cell ALL.

CDC34 protein expression in T-cell ALL was analyzed in a

larger and independent set of T-cell ALL samples obtained

through a POG study. Expression of CDC34 protein in these

samples was analyzed in Western blots with 3 pre-B-cell ALL

samples from the TCCC (as described above). Consistent

with previous results, the level of CDC34 protein was higher

in the POG T-cell ALL samples than in pre-B-cell ALL cells

(Fig. 2B). The mean expression of CDC34 in the two patient

sets was similar at 6642 for the Baylor College of Medicine

patients and 5976 for the POG patients. With the larger

number of POG samples there was also a larger variability in

expression level of the T-cell cases. This variability may be

attributable to differences in handling, because these sam-

ples are collected from multiple medical centers and sent for

central processing. Another potential difference is that the

lysis method used for the POG samples resulted in a cyto-

parison of CDC34 protein levels using Western blot hybrid-

ization in different types of pediatric leukemias demonstrated

that CDC34 protein (normalized to β-actin) was expressed at

a higher level in T-cell ALL samples than in the more common

pre-B-cell ALL (see Fig. 1, top panels). Northern blot hybrid-

ization of the mRNA extracts obtained from the same pa-

tients samples demonstrated that CDC34 protein levels did

not correlate with mRNA levels (Fig. 1, bottom panel). In

particular, samples with high CDC34 mRNA levels could

have either low or high CDC34 protein levels. Therefore, the

observed differential expression of CDC34 protein in these

two types of leukemia seems to be regulated postranscrip-

tionally.

To obtain statistically significant data in regard to CDC34

protein expression, multiple patient samples were run in

Western blots with the same control pre-B-cell ALL sample,

and CDC34 expression was normalized to this sample and

β-actin. Altogether 36 samples from lymphoblastic leukemia

patients were used for analysis: 30 samples from patients

with pre-B-cell ALL and 6 samples from T-cell ALL patients

(this reflects the relative ratio of pre-B:T-cell ALL in the pe-

diatric population). Samples that demonstrated partial or

complete protein degradation (based on integrity of the β-

actin and CDC34 bands in the lysate) were not included in

the analysis. There was an ~4-fold higher level of CDC34

protein detected in T-cell ALL samples compared with pre-

B-cell ALL samples (Fig. 2A). Using Mann-Whitney statistics,

this difference proved to be highly significant (P < 0.001)

despite the small number of T-cell ALL samples from TCCC.

Patients with T-cell ALL are more likely than pre-B-cell ALL

to present with very high WBC counts at the time of diag-

nosis requiring leukapheresis. However, acute myelogenous

leukemia is rare in children and represents a very heteroge-

neous set of cell precursors. Therefore, for the remainder of

this study we have focused on pre-B-cell and T-cell ALL.

CDC34 protein expression in T-cell ALL was analyzed in a

larger and independent set of T-cell ALL samples obtained

through a POG study. Expression of CDC34 protein in these

samples was analyzed in Western blots with 3 pre-B-cell ALL

samples from the TCCC (as described above). Consistent

with previous results, the level of CDC34 protein was higher

in the POG T-cell ALL samples than in pre-B-cell ALL cells

(Fig. 2B). The mean expression of CDC34 in the two patient

sets was similar at 6642 for the Baylor College of Medicine

patients and 5976 for the POG patients. With the larger

number of POG samples there was also a larger variability in

expression level of the T-cell cases. This variability may be

attributable to differences in handling, because these sam-

ples are collected from multiple medical centers and sent for

central processing. Another potential difference is that the

lysis method used for the POG samples resulted in a cyto-

simultaneous expression of lymphoid and myeloid antigens

on leukemic blasts that have staining and karyotypic features

of ALL is a common phenomenon (11). Perhaps consistent

with the high expression in this sample, parallel analysis of

the five acute myelogenous leukemia lysates with intact pro-

tein collected during the same period revealed very high but

variable expression (Fig. 2A). However, acute myelogenous

leukemia is rare in children and represents a very heteroge-

neous set of cell precursors. Therefore, for the remainder of

this study we have focused on pre-B-cell and T-cell ALL.

CDC34 protein expression in T-cell ALL was analyzed in a

larger and independent set of T-cell ALL samples obtained

through a POG study. Expression of CDC34 protein in these

samples was analyzed in Western blots with 3 pre-B-cell ALL

samples from the TCCC (as described above). Consistent

with previous results, the level of CDC34 protein was higher

in the POG T-cell ALL samples than in pre-B-cell ALL cells

(Fig. 2B). The mean expression of CDC34 in the two patient

sets was similar at 6642 for the Baylor College of Medicine

patients and 5976 for the POG patients. With the larger

number of POG samples there was also a larger variability in

expression level of the T-cell cases. This variability may be

attributable to differences in handling, because these sam-

ples are collected from multiple medical centers and sent for

central processing. Another potential difference is that the

lysis method used for the POG samples resulted in a cyto-

Fig. 1. Top panel, Western blot analysis of human CDC34 protein ex-

pression in a representative set of five pre-B-cell ALL(Lanes 1–5) and

T-cell ALL (Lanes 6–10) samples. Middle panel, results of hybridization of

the same membrane with anti β-actin antibody. The sample in Lane 3 is a

pre-B-cell ALL sample that expresses both lymphoid and myeloid markers

on immunophenotype analysis. Lower panel, Northern blot analysis of

total RNA from the same samples as shown in top panel using a 32P-
labeled human CDC34 probe.

Fig. 2. A, quantitative analysis of CDC34 protein expression in a set of

sequentially diagnosed acute leukemia patients from the TCCC. All sam-

dles were run with the same pre-B-cell ALL sample in each gel, and

CDC34 expression was normalized to β-actin expression. ✽, the three

pre-B-cell ALL samples from patients undergoing leukapheresis. B, quan-
titative analysis of CDC34 protein expression in a set of T-cell ALL sam-

ples from the POG. Also shown are three representative pre-B-cell ALL

samples analyzed in A and three samples of FACS-sorted normal resting

t cells.
Increased Expression of CDC34 Protein in Pre-B-Cell Lymphoblastic Cells Is Limited to Leukemia. To determine whether the difference in expression between pre-B-cell and T-cell leukemia reflects their cell of origin we analyzed expression of CDC34 protein in normal resting B and T lymphocytes and activated T lymphocytes. Resting lymphocytes obtained from healthy donors were purified using FACS. FACS-purified T and B lymphocytes were >99% pure and yielded intact protein. The level of CDC34 protein in the FACS-sorted resting T and B cells was comparable with each other (Fig. 3A) and that seen in the pre-B-cell ALL samples (Fig. 2B).

The higher level of CDC34 protein in T-cell leukemia cells compared with normal T lymphocytes could be the result of their different proliferative status, because the leukemia cells represent cycling blast cells whereas normal T lymphocytes isolated from peripheral blood are in the quiescent stage of the cell cycle. To test whether CDC34 protein becomes up-regulated on T-cell activation, we studied its expression in lymphocytes isolated from peripheral blood of healthy donors and then probed with α-cyclin D3 antibodies and α-p27 antibodies without stripping. Therefore, the upper band in the middle panel represents residual staining for CDC34 protein.

dCDC34 was originally identified in yeast because of its requirement for entry into S phase. Therefore, the differences seen in primary leukemia samples from T-cell and pre-B-cell ALL patients may reflect different cell cycle kinetics or some other aspect of the disease process. To determine whether higher level of CDC34 protein in T-cell ALL is a function of growth conditions, we analyzed several human cancer cell lines of lymphoid origin: Jurkat and MOLT-4 were derived from pediatric T-cell ALL patients, and Reh and JM1 were derived from pre-B-cell ALL. The immunophenotype of these four cell lines obtained from the American Type Culture Collection was confirmed in our laboratory and is consistent with the description of the cell lines. Growth rates including doubling times were determined for these four cell lines grown in culture under similar conditions. Protein and RNA lysates were prepared from cells on 4 sequential days during the exponential phase of growth and averaged. The doubling times for the four cell lines demonstrate that the proliferation rates of these cell lines were practically identical (data not shown). Propidium iodide staining of the four cell lines (Table 1) demonstrated that all four cell lines were actively cycling with the two pre-B-cell lines having somewhat more cells in S phase. Western blot analysis of these samples demonstrated that CDC34 protein level was 3–4-fold higher in the two T-cell ALL lines than in the pre-B-cell ALL lines (Fig. 4A). Consistent with our observations of primary leukemia samples, there was no correlation between levels of CDC34 RNA and protein isolated from parallel samples in the four cell lines (Fig. 4B). Taken together, these data

![Image](https://example.com/image.png)
indicate that the difference in CDC34 protein levels between pre-B-cell and T-cell leukemia cells does not simply reflect a difference in growth rates or cell cycle kinetics but may reflect differential regulation of CDC34 protein in these leukemic cells.

There Is No Evidence for Mutations in the CDC34 Gene in Leukemia Cell Lines. Somatic or germ-line mutations in the human CDC34 gene have not been reported in any disorder. However, differences in protein expression might reflect missense mutations as is commonly seen for the p53 tumor suppressor gene (12). On the basis of the CDC34 genomic sequence we analyzed all 7 exons that code for the human CDC34 open reading frame in the four leukemia cell lines (Reh, JM1, MOLT4, and Jurkat). No sequence changes consistent with a missense mutation were detected. We detected several silent polymorphisms and a small intronic deletion in the Reh cell line but no mutations that would result in an altered protein sequence.

CDC34 Protein Is Stable in Both T-Cell and Pre-B-Cell Leukemia Cell Lines. Another possible explanation for the observed difference in protein expression would be higher stability of CDC34 protein in T-leukemia cells. To test this hypothesis, we compared stability of CDC34 in pre-B-cell (Reh) and T-cell (Jurkat) leukemia cell lines by determining the half-life of the CDC34 protein on incubation with cycloheximide. Within 30 min after addition of cycloheximide to the cellular suspension >95% of [35S]methionine incorporation into protein was abolished. Under these conditions CDC34 protein proved to be relatively stable in both Reh and Jurkat cells. The half-life of the protein determined on the basis of three independent experiments was 10.6 h for Reh and 10.3 h for Jurkat. Thus, there is no evidence for significant difference in stability of the protein. Consistent with this finding we did not see any change in the steady-state level of CDC34 when cells were treated with proteasome inhibitors (data not shown).

Localization of the CDC34 Protein in Leukemia Cells. The above assays of CDC34 protein expression with the exception of the POG samples were all performed with whole cell lysates. The POG samples represent a primarily cytoplasmic extract. Recent reports in a number of systems suggest that the cellular localization of proteins is crucial to both regulation of activity and ubiquitin-mediated degradation. Shuttling between the nucleus and cytoplasm is a direct method of regulation of activity for proteins that are ubiquitinated including p27KIP1 (13), IκB (14), and MDM2 (15). To test whether there were differences in localization of CDC34 protein, the four cell lines described above were grown in culture during the exponential growth phase. Cell pellets were analyzed by IHC with the anti-CDC34 monoclonal antibody used in the prior analyses. Fig. 5 shows representative fields for staining of the Jurkat and Reh cell lines. The overall level of CDC34 expression is consistent with the Western blot analyses. The T-cell ALL line shows a considerably higher level of staining with the anti-CDC34 antibody. Surprisingly, the cellular localization in both the Jurkat and Reh cells is extremely heterogeneous. For Jurkat, cells show either a strong signal detected in the nucleus, cytoplasm, or both. For example, note the two large cells in the center with no nuclear signal but moderate staining in the cytoplasm. For the Reh cells, CDC34 staining is weak and present in relatively fewer cells, as compared with Jurkat cells. The heterogeneous localization in Reh cells appears similar to those seen in the Jurkat cell line. Western blot analyses of fractionated cell lysates also reveal that in all four of the cell lines CDC34 protein is present in both nuclear and cytoplasmic fractions (data not shown).

Table 1. Cell cycle profiles of four cell lines used to analyze CDC34 expression

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>56.9</td>
<td>35.9</td>
<td>7.2</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>47.8</td>
<td>37.6</td>
<td>14.7</td>
</tr>
<tr>
<td>Reh</td>
<td>48.3</td>
<td>42.5</td>
<td>9.2</td>
</tr>
<tr>
<td>JM1</td>
<td>42.4</td>
<td>46.2</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Fig. 4. A, Western blot analysis of CDC34 protein expression in four leukemia cell lines. The cells were grown in exponential culture and aliquots removed for protein and RNA analysis on each day. The average expression over 4 consecutive days is shown for each cell line. B, Northern blot analysis of CDC34 mRNA expression of the same samples shown in A.

Fig. 5. Immunohistochemical staining for CDC34 in Jurkat and Reh leukemia cell lines. The signal was developed using 3,3′-diaminobenzidine chromogen (brown) with a methyl green counter stain. Jurkat cells (left) and Reh cells (right). ×400 for both panels.
Discussion

Regulation of multiple cellular processes by ubiquitin-mediated degradation has been well established in all eukaryotes. More recently, the results of a number of investigations demonstrate that ubiquitination of either oncogenes or tumor suppressor genes is a significant feature of the regulation of both normal and malignant human cells (7, 16). Despite these findings there has been little research on the expression of ubiquitin-conjugating enzymes in human tissues. In this paper we report evidence that there is differential regulation of human CDC34 ubiquitin-conjugating enzyme in the most common pediatric malignancy, ALL. Analysis of clinical samples demonstrates that at the time of diagnosis there is a 3–4-fold increase in total CDC34 protein in T-cell ALL versus pre-B-cell ALL. In contrast, we find no increase in expression of CDC34 in either normal resting T lymphocytes or activated T lymphocytes when compared with normal B lymphocytes or pre-B-cell leukemia. Subsequent analysis of leukemia cell lines confirms this difference between T-cell and pre-B-cell leukemia. More recently, the results of a number of investigations demonstrated that ubiquitination of either oncogenes or tumor suppressor genes is a significant feature of the regulation of multiple cellular processes by ubiquitin-mediated activity. The identification of specific targets of each ubiquitin-conjugating enzyme is incomplete, and some targets require additional modification, e.g., phosphorylation, to be recognized by the appropriate ubiquitin protein ligase complex (1). However, we have previously reported that the availability of CDC34 is rate limiting in regard to ubiquitination of some targets in choriocarcinoma cell lines (8). This dependence on ubiquitin-conjugating enzyme expression has also been reported for the UbcH10 enzyme (21). Our study in pediatric ALL demonstrates that for different cell types both the overall amount and localization of CDC34 protein differ. Differences in CDC34 expression may alter ubiquitination efficiency and be responsible for altering more subtle aspects of the biology of the leukemia cells or their response to treatment.

Materials and Methods

Leukemia Samples. Bone marrow, peripheral blood, and leukapheresis samples were obtained from sequentially diagnosed ALL patients of the TCCC under a Baylor College of Medicine Institutional Review Board-approved protocol. Only excess samples obtained for clinical purposes at initial diagnosis and before initiation of treatment were analyzed. Leukemic cells were isolated from the samples by centrifugation in a ficoll density gradient. Whole cell protein extracts were prepared by lysis of 4 × 10⁷ cells in 100 μl of buffer containing 25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 2.5 mM EDTA, 0.05% SDS, 0.5% NP40, 0.5% sodium deoxycholate, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 1 mM Na₂-p-tosyl-L-lysine chloromethyl ketone, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 mM benzamidine, 0.5 mM sodium orthovanadate, 80 μM β-glycerophosphate, and 50 mM NaF. Samples were sonicated 2 × 30 s in a Cophorn Sonicator (Fisher Scientific) and centrifuged at 100,000 × g at 4°C for 15 min. Samples from patients diagnosed with T-cell ALL enrolled in one POG study were lysed in a buffer containing 50 mM Tris (pH 8.0), 1% NP40, 150 mM NaCl, 1 mM EGTA, 10 mM NaPO₄, 10 mM NaF, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 5 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 μg/ml Trans-epoxy succinyl L-Leucyl-Lysylamido, and 0.5 μg/ml NαP (Sigma Chemical Co.). Cellular debris (including intact nuclei) was removed by centrifugation at 13,000 g for 10–30 min and the supernatant used for analysis. Direct comparison of the two methods in the T-cell ALL cell line MOLT4 revealed ~2-fold more CDC34 (normalized to β-actin) protein detected on Western blots using the whole cell lysate (TCCC) versus the cytoplasmic procedure (POG).

Cell Lines and Culture Conditions. MOLT4, Jurkat, JM1, and Reh cells were purchased from American Tissue Culture
Collection. All of the cells were grown in suspension culture at 37°C in 5% CO₂, humidified incubators in RPMI 1640 supplemented with 10% fetal bovine serum plus antibiotics. As indicated cycloheximide (Sigma Chemical Co.) was dissolved in water and added directly to the culture medium to a final concentration of 20 μg/ml.

**Lymphocyte Studies.** Normal human lymphocytes were isolated from Leukocyte Sources obtained from the Gulf Regional Blood Center, Houston, TX. Diluted samples were layered over Histopaque gradients (Sigma Chemical Co.) and centrifuged according to the manufacturer’s instructions. PBMCs were collected, washed twice with HBSS, and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum. Monocytes were depleted by incubating the cell suspension for 1 h at 37°C. The final suspension contained 80–85% T cells and 6–9% B cells. B- and T-cell-enriched fractions were obtained by FACS.

**T-Cell Activation.** PBMCs were incubated in complete RPMI 1640 either in the presence or absence of PHA (2 μg/ml). Stimulated and control cells were collected at daily intervals, and protein lysates were prepared from them as described above. The morphology of the cells was evaluated on Cytospins (Shandon) stained with Wright-Giemsa. Aliquots for FACS analysis were taken at the same time. In some experiments cells were washed 72 h after stimulation with HBSS, and fresh medium with or without interleukin 2 (50 units/ml) was added.

**Western Blotting.** Monoclonal antibodies against human CDC34, cyclin D3, and p27Kip1 were purchased from Transduction Laboratories. Western blots were performed using cell extracts (25–30 μg of total protein) separated on 12% SDS-PAGE and electroblotted onto Immobilon-P membranes (Millipore). After transfer membranes were blocked overnight with 5% nonfat milk in Tris buffer saline, 0.1% Tween 20 incubated with primary (using 1:1000 dilution for anti-CDC34) and secondary antibodies and then detected using an enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions. After scanning of autoradiograms and quantification of protein bands, CDC34 protein levels in the extracts were normalized by comparison with expression of β-actin. The nonparametric Mann-Whitney test was applied for statistical analysis.

**Northern Blotting.** Total RNA was extracted from the cells using RNeasy kit (Qiagen). RNA (10–15 μg) was separated in 1% agarose gels, transferred to polyvinylidene difluoride membrane, and hybridized to a 32P-labeled human CDC34 probe obtained by random-primer hybridization (2). After hybridization, the filters were washed and detected using a Molecular Dynamics PhosphorImager.

**Sequencing.** The genomic sequence for the human CDC34 gene on chromosome 19p13 (2) was obtained from GenBank. Primers were designed to amplify the exons containing the entire coding region of the gene. The sequence of primers and PCR conditions are available on request. DNA sequencing was performed on each PCR product from both strands and the sequence obtained compared with that reported in GenBank.

**IHC.** The IHC studies were performed on the indicated cell lines actively growing in culture. Cells pellets were prepared, fixed, and sectioned. For CDC34 IHC, the sections were deparaffinized in xylene and heat treated for epitope retrieval using 0.1 M Tris-HCl buffer (pH 9.0) for 5 min in a pressure cooker. Endogenous peroxidase was blocked with 3% hydrogen peroxide and additionally blocked with avidin-biotin block solution (Dako). Anti-CDC34 monoclonal antibody was used at 1:200 for 1 h at room temperature followed by secondary biotinylated antibody (Dako) at 1:200. Horseradish peroxidase-labeled streptavidin (Dako) was used followed by 3,3’-diaminobenzidine + as the chromogen. Signal was enhanced by 30 s incubation with 0.2% osmium tetroxide. Sections were then counterstained for 30 s with 0.05% methyl green.

**Acknowledgments**

We thank Shirley Waldon and Yvonne Tsang for aid in obtaining leukemia samples promptly at the time of diagnosis, and Anuradha Ganavarapu for sequencing.

**References**


14. Karin, M., and Ben Neriah, Y. Phosphorylation meets ubiquitina-


17. Hangst, L., and Reed, S. I. Translational control of p27Kip1 accumu-


