Induction of Protein Kinase C mRNA in Cultured Lymphoblastoid T Cells by Iron-Transferrin But Not by Soluble Iron

By O. Alcantara, M. Javors, and D.H. Boldt

Iron-transferrin (FeTF) is an essential growth factor required for proliferation of lymphoid cells. FeTF activates protein kinase C (PKC) in the lymphoblastoid T-cell line, CCRF-CEM. We have treated CEM cells with human FeTF, then examined levels of PKC mRNA by hybridization analysis using cDNA probes specific for α- and γ-PKC subtypes. CEM cell mRNA hybridized with the β-subspecies probe but not with probes for α- or γ-PKC subtypes. PKC-β mRNA was detectable at 10 minutes, peaked at 12 hours, and was sustained for 72 hours. Nuclear transcription assays demonstrated that rates of PKC-β mRNA transcription were increased in FeTF-treated cells. By contrast, steady state levels of PKC-β mRNA did not increase after treatment of cells with apotransferrin or gallium TF. Similarly, treatment with soluble iron as ferric ammonium citrate did not increase steady state levels of PKC-β mRNA, despite producing a marked increase in cellular ferritin content. Ferritin increased from a baseline value of 63 ng/10⁶ cells to 98 and 100 ng/10⁶ cells in CEM cells treated for 1 hour with ferric ammonium citrate or FeTF, respectively. FeTF did not increase cytoplasmic-free calcium in CEM cells loaded with fura-2, indicating that binding of FeTF to transferrin receptors did not open membrane Ca²⁺ channels or release intracellular Ca²⁺. In addition, pretreatment of cells with desferrioxamine, but not ferrioxamine, blocked the FeTF-induced increase in PKC-β transcripts. Therefore, iron as FeTF (not soluble iron or nonferric TF) stimulates transcription of the CEM cell PKC-β gene. Transcriptional rates of the PKC-β gene do not correlate with cellular iron content as judged by ferritin measurements. Furthermore, the requirement for FeTF does not appear to reflect activation of a classic agonist pathway as judged by stable cellular Ca²⁺. These data suggest that delivery of iron by FeTF to one or more specific cellular compartments may stimulate PKC-β gene transcription in CEM cells.

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Iron-Transferrin (FeTF) is the principal iron transport glycoprotein in vertebrates. FeTF is an essential growth factor required by all cells in culture. For lymphoid cells growing in vitro, binding of FeTF to cell surfaces is required for proliferation. T-lymphocyte proliferation also is associated with protein kinase C (PKC)-dependent pathways. For example, autocrine growth in T lymphocytes is associated with activation via the CD3-antigen receptor complex, which correlates with enhancement of PKC activity. Activation of PKC during ligand-receptor interactions may play a key role in the induction of certain T-cell functions in addition to proliferation. Previously, we have shown that FeTF induced a mean 10-fold increase in the activity of PKC in CCRF-CEM cells. The kinetics of appearance of this increased activity, as well as its inhibition by cycloheximide or actinomycin D, suggested an effect of FeTF on transcription of PKC genes. We now report results of studies to examine transcriptional rates and steady state mRNA levels of PKC subtypes in CCRF-CEM cells treated with FeTF. Induction of PKC-β gene transcription, increased levels of PKC-β mRNA, and increased PKC enzyme activity all occur in T-lymphoblastoid cells treated in vitro with FeTF. Additional studies were performed to examine mechanisms by which this increased rate of PKC-β gene transcription may be mediated.

MATERIALS AND METHODS

Cell cultures. Experiments were performed with CCRF-CEM cells, a T-lymphoblastoid cell line established from a patient with acute lymphoblastic leukemia. Cells in logarithmic growth phase were seeded in culture at 5 x 10⁶ to 1 x 10⁹/mL in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 200 mmol/L L-glutamine, and antibiotics.

For FeTF treatment, plateau-phase cells were washed twice in 1 vol of ice-cold RPMI-1640 medium. The cells were resuspended in the same medium supplemented with 200 mmol/L L-glutamine and 0.5% bovine serum albumin (BSA) at a concentration of 1.0 x 10⁶ cells/mL. The cells were equilibrated at 37°C for 30 minutes, then were treated with 100 µg/mL of human FeTF for the times indicated. Experiments with TF derivatives, desferrioxamine, and ferrioxamine were performed in the same manner. For all cell culture experiments, control cells were treated and incubated under identical conditions without addition of FeTF or other compounds to the culture medium.

TF derivatives. Apotransferrin (apo-TF) was obtained from Calbiochem-Behring, La Jolla, CA. Before use this preparation was dialyzed against 50 mmol/L Na citrate, pH 5.0, then incubated with prewashed Chelex 100 (BioRad, Richmond, CA) for 1 hour at room temperature with mixing to ensure removal of trace iron. FeTF was prepared by adding ferric citrate to apo-TF to a final stoichiometry of 2 mol Fe³⁺ per mol protein. Gallium-TF was prepared as described by Chitambar and Seligman. Briefly, gallium nitrate (Alfa Products, Danvers, MA) was mixed with apo-TF in a 3:1 molar ratio in 20 mmol/L acetic acid, 150 mmol/L NaCl, pH 3.5. The pH of the solution was raised in increments to 7.4 by adding 1 mol/L NaHCO₃ to achieve a final NaHCO₃ concentration of 30 mmol/L. Saturation was confirmed by monitoring change in absorbance at 242 nm.

RNA hybridization analyses. Total cellular RNA was isolated by lysis in a guanidinium isothiocyanate solution and pelleting through cesium chloride. Integrity of RNA was assessed by visualization of ethidium bromide-stained gels. For blot analysis, 5 µg of total RNA was immobilized on nitrocellulose filters using a dot-blot apparatus. The filters were hybridized with 32P-labeled cDNA probes.

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Submitted May 14, 1990; accepted November 14, 1990.

Supported by Veterans Administration Research Funds.

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Nitrocellulose filters presoaked in 6X SSC (0.15 mol/L NaCl, 15 mmol/L sodium citrate dihydrate) were baked (80°C for 2 hours under vacuum), then prehybridized for 1 hour at 45°C. Hybridization was performed overnight at moderate stringency in hybridization solution containing 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 100 µg/mL sheared carrier DNA, 6X SSC, 2X Denhardt’s, and 10% dextran sulfate. After hybridization the filters were washed for 10 minutes each in three changes of 2X SSC, 0.1% SDS and in two changes of 0.2% SSC, 0.1% SDS (45°C, 1 hour). After washing, filters were exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY) at −70°C with Dupont Cronex intensifying screens. Relative intensities of blots on autoradiographs were quantitated by scanning densitometry.

For Northern analyses, total cellular RNA was electrophoresed through a 1% agarose gel containing formaldehyde. The gel was rinsed in water and 500 mL of 10X SSC to remove formaldehyde, then RNA was transferred to nitrocellulose filters presoaked in 20X SSC. Hybridization and autoradiography were performed as described above.

Nuclear transcription assays. Measurements of relative rates of transcription of PKC-α, β, and γ were performed by nuclear run-on transcription assays performed as we previously have described.8 Nuclei isolated by lysis of cells in 0.1% Triton X-100 (Sigma, St Louis, MO) were used for in vitro transcription assays with [α-32P] uridine triphosphate. After 45 minutes, incubation mixtures were treated sequentially with RNASE-free DNASE I and Proteinase K. RNA was extracted with phenol:chloroform, ethanol precipitated, and retreated with DNASE I. Unincorporated label was removed by ethanol precipitation and two subsequent washings in 70% ethanol. Volumes of transcription reaction products containing equal amounts of radioactivity (10 to 20 × 10⁶ cpm per filter) were incubated for 3 days with prehybridized nitrocellulose filters onto which linearized plasmid cDNAs had been immobilized using a dot-blot apparatus. After washing, filters were dried and autoradiographed. Relative intensities of blots on autoradiographs were quantitated by scanning densitometry. In preliminary experiments (not shown), a-amanitin, 2 µg/mL, inhibited specific mRNA transcription but not transcription of 28S rRNA.

**Table 1.** [H]-PDBU Binding in FeTF-Treated CCRF-CEM Cells

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<th>[H]-PDBU Bound (cpm/10⁶ cells)</th>
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<tr>
<td>Control</td>
<td>9,579 ± 519</td>
</tr>
<tr>
<td>FeTF</td>
<td>20,814 ± 1,585</td>
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CCRF-CEM cells were treated with or without 100 µg/mL of FeTF for 60 minutes. [H]-PDBU binding was performed as described in Materials and Methods. Numbers are mean ± SEM for five experiments.

**Fig 1.** PKC activity in CCRF-CEM cells treated with FeTF. CEM cells, 10⁶/mL, were treated with FeTF, 100 µg/mL, for the times indicated. PKC activity in cell sonicates was measured as described in Materials and Methods. PKC activity, plotted on the ordinate, is reported in units (U), where 1 U is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of 32P into H1 histone during 5 minutes per 10⁶ cells. (●) control. (■) FeTF.

**Fig 2.** Northern analyses of whole cell mRNA hybridized with α, β-, and γ-PKC subtypes [32P] probes. Twenty micrograms of total mRNA were fractionated in each lane of a formaldehyde denaturing agarose gel. The total RNA was immobilized onto nitrocellulose and hybridized to [32P]-labeled α, β, and γ-PKC plasmids. Autoradiography was conducted for 1 to 4 days using two Dupont-Cronex intensifying screens at −70°C. Ribosomal RNA bands were visualized by ethidium bromide staining and corresponding mobilities are indicated on the left of the autoradiogram. Filters probed with the β-PKC subtypes cDNA showed one major 3.4-kb band and minor 9.2- and 1.2-kb hybridization bands.
cells were incubated at 37°C for 30 minutes with 20 nmol/L 3H-PDBU (10 to 20 Ci/mmol) in 500 μL medium. Reactions were terminated by addition of 4 mL of ice-cold RPMI-1640 medium. Cells were collected by filtration through GF/A glass fiber filters. The filters were air dried overnight and radioactivity was counted using Du Pont (Boston, MA) Aquasol universal cocktail. Specific binding was calculated as the difference between total binding and that measured in the presence of 10 μmol/L unlabeled PDBU.

PKC activity. The activity of PKC in cell sonicates was assessed by measuring the incorporation of 32P from [32P] adenosine triphosphate (ATP) into Histone in the presence of Ca2+ and phosphatidyserine, essentially as described by Ogawa et al.29

Ferritin assay. Cellular ferritin was measured in cell lysates using a commercial radioimmunoassay purchased from Becton Dickinson, Sunnyvale, CA.

Determination of cytosolic [Ca2+] using fura-2. Cytosolic-free Ca2+ was measured by a modification of the method of Tsien et al.28 CEM cells were incubated at 37°C with 1 μmol/L fura-2 AM for 30 minutes. Loading of the cells with fura-2 was verified by lysing the cells with 0.1% Triton X-100, then sequentially adding 1 mmol/L Ca2+ and 5 mmol/L EGTA and observing the appropriate shifts in excitation spectra. Cytosolic [Ca2+] was determined using a Delta scan fluorometer (PTI, Inc, Princeton, NJ) specifically designed for use with fura-2. During the assay, the cells were stirred at 800 rpm with a teflon stirring bar, and additions to the reaction cuvette were made through an injection port directly into the cell suspension using a Hamilton syringe. Cytosolic [Ca2+] was calculated in nanomoles of Ca2+ using the computer software provided by PTI, Inc.

RESULTS

PKC activity and 3H-PDBU binding. PKC activity in CEM cells after exposure to human FeTF, 100 μg/mL, is shown in Fig 1. 3H-PDBU binding also increased in cells treated with FeTF for 60 minutes (Table 1). Several studies have demonstrated that PKC is a phorbol ester receptor and that PDBU binding can be used to detect and quantify presence of PKC.28 The marked increase in PKC enzyme activity is similar to data we have reported previously.3 The fact that enzyme activity increased more than 10-fold compared with the twofold increase in PDBU
INDUCTION OF PKC mRNA BY IRON-TRANSFERRIN

Fig 3. Increase in PKC-β transcript requires Fe^{3+} delivery by FeTF. CCRF-CEM cells were treated with the agents indicated or without additions under conditions described in Materials and Methods. After 1 hour total cellular RNA was extracted and PKC-β mRNA was detected by dot-blot hybridization as described in Materials and Methods. Dots illustrated for each experimental condition represent triplicate determinations.

relative rate of PKC-β gene transcription in these cells was approximately seven times that in parallel untreated cells. By contrast, the transcription rate of α-actin did not change under identical conditions. These data are consistent with earlier observations that increased PKC activity in FeTF-treated CEM cells could be abolished by actinomycin D or cycloheximide. They provide direct confirmation that FeTF may induce transcription of the PKC-β subspecies in lymphoblastoid T cells.

Increased mRNA levels were maintained up to 72 hours in the continued presence of FeTF. α-actin levels did not change similarly during exposure to these agents (Fig 3). Although apo-TF would be predicted to have a lower affinity for binding to the TF receptor (TFR), the affinity of the gallium-TF-TFR interaction has been reported to be equivalent to that of the FeTF-TFR interaction. Therefore, the failure of these two compounds to

Table 2. Cellular Ferritin Levels in CCRF-CEM Cells Treated With FeTF or Ferric Ammonium Citrate

<table>
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<tr>
<th>Cells</th>
<th>Ferritin (mg/10^6 cells)</th>
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<tr>
<td>Basal</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>FeTF-treated</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Ferric ammonium citrate-treated</td>
<td>98 ± 16</td>
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CCRF-CEM cells were incubated for 1 hour in serum-free medium with no addition; FeTF, 100 μg/mL; or ferric ammonium citrate, 75 μmol/L. Lysates were prepared in distilled water and cellular ferritin was measured using a commercial radioimmunoassay. Numbers are mean ± SEM for six experiments.

binding may reflect high specific activity of newly synthesized PKC enzyme.

Selective expression of PKC-β by CCRF-CEM cells. PKC comprises a family of serine/threonine-specific protein kinases. To date, a number of isoforms, α, β, γ, δ, ε, and ζ, have been identified by molecular cloning. We used cDNA probes specific for α-, β-, and γ-subspecies to probe CEM cells for expression of these three isoforms. Results are shown in Fig 2. PKC-β was the only PKC subspecies detected in our CEM cell lines. Identical results were obtained when hybridization was performed under a wide range of stringencies.

Although we have not tested for expression of PKC-δ, -ε, or ζ, clearly PKC-β is a major subspecies expressed by the CEM cells used in these studies. Therefore, additional studies were performed to examine regulation of PKC-β in CEM cells during treatment with FeTF.

Expression of PKC-β mRNA during treatment with FeTF. Figure 3 illustrates results of a representative experiment in which blot hybridization analyses were used to determine steady state levels of PKC-β and α-actin mRNA in CEM cells exposed to 100 μg/mL of human FeTF for the times indicated. These data demonstrate a rapid increase in PKC-β mRNA detectable at 10 minutes and reaching a maximum approximately three times baseline levels after 12 hours. This threefold increase in PKC-β mRNA is very similar to the twofold increase in PDBU binding capacity seen in FeTF-treated cells (Table 1). The observed increase in PKC-β mRNA levels was maintained up to 72 hours in the continued presence of FeTF. α-actin levels did not change similarly during exposure to FeTF (Fig 3).

Nuclear run-on transcription assays. To determine directly the effect of FeTF on PKC-β gene transcription, we performed nuclear run-on transcription assays. Figure 4 illustrates results of a representative transcription assay in which cells were exposed to FeTF, 100 μg/mL, for 3 hours.
stimulate PKC-β gene transcription is not explained by failure to bind to cell surface TFR. These data suggested that the effect may depend upon delivery of Fe\textsuperscript{3+}.

To test this possibility, cells were treated with 75 μmol/L ferric ammonium citrate under otherwise identical conditions. As shown in Table 2, iron accumulation, judged by cellular ferritin concentration, was similar in cells treated with either FeTF or ferric ammonium citrate. The data in Table 2 refer to ferritin accumulation during a 60-minute incubation. This time frame is relevant to the studies at hand because increased PKC-β transcripts were detected as early as 10 minutes after addition of FeTF to the cultures (Fig 3). In other experiments not shown, both FeTF and ferric ammonium citrate induced equivalent accumulations of ferritin in cells monitored during more prolonged incubation periods up to 180 minutes. However, ferric ammonium citrate failed to stimulate PKC-β gene transcription (Fig 5).

These data suggest that iron delivered in the form of FeTF, but not soluble ferric ion, is required for enhancement of PKC-β gene transcription in CEM cells.

Additional experiments were performed with the iron chelator, desferrioxamine (Fig 6). CEM cells preincubated with 50 μmol/L desferrioxamine failed to demonstrate an increase in PKC-β transcripts when treated with FeTF. By contrast, 50 μmol/L ferrioxamine did not block the expected increase. Because desferrioxamine can enter the cells and chelate iron released from FeTF, these data provide strong evidence that this effect of FeTF is mediated by iron.

**Effect of FeTF on cytosolic [Ca\textsuperscript{2+}].** To test whether binding of FeTF to cell surface TFR might induce a second messenger, CEM cells loaded with fura-2 were exposed to FeTF, 100 μg/mL, and cytosolic [Ca\textsuperscript{2+}] was measured in a fluorometer.\textsuperscript{18} As indicated in Fig 7, fura-2–loaded CEM cells loaded with fura-2 respond appropriately to alterations in [Ca\textsuperscript{2+}]. CEM cells were loaded with fura-2 as described in Materials and Methods. Cytosolic [Ca\textsuperscript{2+}] was determined using a fluorometer while the indicated agents were added to the cell suspension.
cells responded predictably to manipulations of \([\text{Ca}^{2+}]\). Results shown in Fig 8 indicate that FeTF added to incubations with fura-2-loaded CEM cells had no effect on cytosolic \([\text{Ca}^{2+}]\). These cells remained appropriately responsive to extracellular calcium concentrations. These data indicate that FeTF does not activate a classic lymphocyte activation pathway.²⁴

**DISCUSSION**

Binding of TF to TFR during lymphocyte activation has been shown to be essential for proliferation.²³ Lymphocytes may produce TF and it has been suggested that lymphoid cell TF and TFR may constitute an autocrine system controlling lymphocyte growth.²³,²⁶ Therefore, it is of interest that binding of FeTF to lymphocyte TFR produces enhancement of PKC activity.

Our results indicate that among the three PKC subtypes that we studied, CCRF-CEM cells in our laboratory expressed only the PKC-\(\beta\) isoform. PKC-\(\beta\) mRNA has been shown to be the sole species in rat spleen tissue and murine T lymphocytes.²²,²⁹ Beyers et al, using immunologic probes, have identified both \(\beta\) and \(\gamma\) isoforms of PKC in human T lymphocytes.³⁰ Sawamura et al demonstrated both PKC-\(\beta\) and \(-\alpha\) in a series of human T- and B-lymphoid and nonlymphoid leukemia cell lines using immunochemical analyses.³¹ More recently, Koretzky et al examined six leukemia cell lines, including CCRF-CEM, for PKC-\(\alpha\), -\(\beta\), and -\(\gamma\) mRNA transcripts.³² Although none expressed PKC-\(\gamma\) and all expressed PKC-\(\alpha\), there was considerable variability in expression of PKC-\(\beta\) among the cell lines tested. CEM cells were noted to express predominantly PKC-\(\beta\), but detectable amounts of PKC-\(\alpha\) were also identified. Immunophenotype of the CEM cells used by Koretzky et al (CD4⁻CD8⁻)³³ differed from that of the CEM cells in our laboratory (CD4⁺CD8⁻), indicating that different CEM sublines reside in the two different laboratories. This
PKC is increasingly recognized as an important second messenger mediating effects of cell agonists in a variety of different systems. In general, in these systems interaction of agonists with their cell surface receptors activate phospholipase C, causing hydrolysis of phosphatidyl inositol to diacyl glycerol and inositol triphosphate. The latter mobilizes intracellular calcium whereas diacyl glycerol directly activates PKC, resulting in phosphorylation of various protein substrates. However, the effect of FeTF on lymphoid-cell PKC appears to occur through a different mechanism.

First, the time course is different from that of transient diacyl glycerol-mediated enzyme activation. In classic agonist-induced PKC activation, membrane translocation of PKC and enhancement of activity occur within minutes as opposed to 1 hour as observed in our system (Fig 1). Second, there is no mobilization of intracellular [Ca\(^{2+}\)] as might be expected from an agonist-receptor interaction causing activation of phospholipase C (Fig 8). Third, we had noted previously that the increased PKC activity induced by FeTF was inhibited by treatment of cells with cycloheximide or actinomycin D. This observation suggested that FeTF caused increased transcription and translation of PKC protein.

Therefore, we have examined steady state levels of PKC mRNA in CEM cells after exposure to FeTF. These data confirm that levels of PKC-\(\beta\) transcripts increase rapidly after binding of FeTF, and that these levels are sustained over a period of many days in the continued presence of FeTF (Fig 3).

Nuclear run-on transcription assays confirmed that increased PKC-\(\beta\) transcripts resulted at least in part from an increased rate of transcription (Fig 4). Our studies with various TF derivatives and ferric ammonium citrate have demonstrated that the increase in PKC-\(\beta\) transcripts requires ferric iron delivered in the form of FeTF (Fig 5). The data in Fig 6, showing that desferrioxamine also can abolish this increase, provide additional strong evidence to implicate iron in the observed effect.

Other proteins dependent upon iron regulation, such as ferritin and TFR, are regulated mainly through mRNA stability. We have not investigated PKC-\(\beta\) mRNA t\(_{1/2}\) in our system and cannot exclude such an effect. However, the similar increases in PKC-\(\beta\) gene transcription, mRNA steady state levels, and PDBU binding seem to imply that transcription is largely responsible for the changes we have observed.

It is of interest that Obeid et al recently have demonstrated transcriptional regulation of PKC-\(\beta\) in HL-60 cells treated with dihydroxy vitamin D\(_3\),. Taken together with the results in this report, this observation indicates that transcriptional regulation may represent a mechanism used by cells of different lineages to increase PKC enzyme activity under certain conditions. Such a mechanism would provide added flexibility to the mechanism of transient PKC activation by diacyl glycerol and might ensure again against enzyme depletion during conditions requiring sustained PKC activity such as cell differentiation and/or proliferation.

The mechanism by which the FeTF stimulus may function to increase PKC gene transcription is of interest. The possibility that FeTF may work as a classical agonist in a ligand-receptor interaction seems excluded by the fact that cytosolic [Ca\(^{2+}\)] is not increased (Fig 8). The data in Figs 5 and 6 define a requirement for iron delivered in the form of FeTF but not soluble ferric ammonium citrate. Iron stores assessed by cellular ferritin content do not differ whether iron is provided as FeTF or ferric ion (Table 2). However, only FeTF is capable of supporting appearance of increased PKC-\(\beta\) transcripts (Fig 5). These data suggest that a compartmentalization of iron must exist in CEM cells. Delivery of iron to certain compartments appears to be highly dependent on the form in which it is initially presented to the cell, with iron from FeTF being rapidly channeled to regulatory functions and soluble Fe\(^{3+}\) being channeled preferentially to storage sites. Both the mechanism by which iron may be selectively distributed within the cell milieu and that by which iron regulation of gene transcription may function are unknown. Whether iron requiring regulatory proteins, alterations in intracellular redox potential, or other mechanisms are involved will require additional studies.

ACKNOWLEDGMENT

The authors thank Elizabeth Castillo-Bailey for technical assistance and Cheryl Adams for typing the manuscript.

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Induction of protein kinase C mRNA in cultured lymphoblastoid T cells by iron-transferrin but not by soluble iron

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