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Mechanisms of Action of Differentiation Inducers: Detection of Inducer Binding Protein(s) in Murine Erythroleukemia Cells

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We have shown previously that murine erythroleukemia (MEL) and human neuroectodermal RD/TE-671 cells are induced to differentiate by ureido derivatives of pyridine (UDPs) and may contain inducer binding protein(s). In the present study, we prepared radiolabeled [^{3}H]UDP {2-(3-ethylureido)-6-[^{3}H]-acetylamino-pyridine} as ligand and investigated whether it interacts selectively with novel binding proteins. MEL and RD/TE-671 cells, incubated with the inducer [^{3}H]UDP and subsequently fractionated, yielded a radiolabeled postmitochondrial soluble fraction containing the [^{3}H]UDP–protein complex. We purified the UDP binding protein by using UDP-sepharose affinity chromatography, gel filtration, and SDS-PAGE electrophoresis and analyzed its structure. The data presented here indicate for the first time that the inducer UDP interacts with a 38,333 ± 30 Da binding protein(s) (p38), of unknown function, in both cell lines. Microsequencing and sequence alignment search revealed that the p38 protein(s) contains at least two homologous domains, one being part of ABC-type transporters and another found in the Wingless-type (Wnt) proteins. Kinetic analysis revealed that the p38 forms a relatively stable protein complex with [^{3}H]UDP that accumulates within the cytosol and nucleus of MEL cells during the precommitment period. This complex, however, decays later on after commitment to erythroid maturation has been initiated. De novo protein and mRNA synthesis is needed for the UDP–p38 complex to form, as shown by the use of metabolic inhibitors. Purified p38 was used to develop an anti-p38 polyclonal serum, and Western blot analysis revealed that the level of p38 was quite similar in both UDP-inducible and -resistant MEL subclones that we developed. Although only a portion of the primary structure of the p38 is known from microsequencing, the mechanism by which the UDP–p38 complex contributes to induction of differentiation in both UDP-responsive mouse MEL and human RD/TE-671 cells is discussed.

Key words: Inducers; Differentiation; Inducer binding protein; Murine erythroleukemia (MEL) cells; Human RD/TE-671 cells

Murine erythroleukemia (MEL) and other neoplastic cells that are induced to differentiate into mature nondividing cells serve as model systems for studying various cellular and molecular aspects of the differentiation therapy of cancer (1,2). The precise mechanism(s) by which small molecular weight chemical inducers promote differentiation of leukemic or other neoplastic cells into nondividing cells still remains elusive, although a few concepts have been proposed (3-7). The mechanisms involved in leukemic cell differentiation are of major importance in understanding the molecular basis of this phenomenon. Unfortunately, real progress in this field has been hampered by the structural and physicochemical diversity of many chemical, natural, and pharmacological inducing agents. Nevertheless, quantitative structure–activity relationships (QSARs) obtained for inducers in MEL cells indicated that bisacetamides (4,5) and UDPs (8) share common structural features that may be recognized by cellular components. These developments, taken together with our earlier observations indicating that pentamethylene-bisacetamide (PMBA), a potent inducer, forms complexes with soluble proteins of the postmitochondrial

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fraction of MEL cells (9), prompted us to search for novel inducer binding proteins. Evidence already exists to indicate that ribosomal S3 protein (rpS3) (10) and histone deacetylase (HDAC) (11) serve as binding sites for bisacetamides and HDAC inhibitors (HDACIs), respectively. Hybrid polar compounds (HPCs) have been shown to induce differentiation of MEL cells after binding to 40S ribosomal protein S3 (10). Moreover, other HPCs are potent inhibitors of HDAC activity (12). In addition, experimental data support the notion that HDACIs regulate gene-specific transcription by increasing the accessibility of nucleosomal DNA to *trans*-acting factors and especially in MEL cells by changing the histone acetylation pattern in the β-globin locus (13).

In light of these developments, we have investigated whether there are proteins in MEL and other responsive cells that bind to UDPs acting either as putative receptors, signal transducers, active transporters for the inducers, or exert other functions. MEL and human neuroectodermal RD/TE-671 cells are two cell lines that are differentiated upon treatment with UDP-4 into erythroid and neuron-like cells, respectively (14). In these cells, UDP-4 induces expression of memory for differentiation, commitment to maturation, postmitotic growth arrest, and suppression of c-*myc* and p53 tumor suppressor proto-oncogene expression. Moreover, UDP-4 induced biosynthesis of hemoglobin in MEL cells and of three neurofilament proteins in RD/TE-671 cells (14). In this study, we prepared 2-(3-ethylureido)-6-[^{3}H]-acetylaminopyridine ([^{3}H]UDP) and investigated whether this inducer accumulates intracellularly in both cell types and binds to specific protein(s). We report the detection, isolation, and partial characterization of the primary structure of a ~38 kDa (p38) soluble postmitochondrial protein(s) that binds with [^{3}H]UDP in both MEL and RD/TE-671 cells. Evidence obtained from specific binding and competition binding studies has indicated that this binding protein(s) may also interact with other known inducers. Interestingly, these data impinge on the mechanism(s) by which the UDP–p38 complexes may contribute to the execution of the MEL cell differentiation program.

MATERIALS AND METHODS

Cell Cultures and Development of UDP-Inducible and UDP-Resistant Subclones of MEL-745 Cells

Murine erythroleukemia MEL-745PC-4A cells (3) and human neuroectodermal RD/TE-671 cells (15) used throughout this study were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal calf serum (FCS) (GIBCO, Long Island, NY, USA), penicillin (100 μg/ml), and streptomycin (100 μg/ml) (Sigma) in a 5% CO_2 humidified atmosphere at 37°C.

Subclones of MEL cells expressing resistance to UDP-4 were derived from the MEL-745PC-4A clone after continuous exposure to 0.05 mM UDP-4 for 21 days. By the end of this period, the proportion of inducible cells producing hemoglobin and stained with benzidine-peroxide solution (16) was less than 1% due to the outgrowth of noninducible cells. UDP-resistant subclones were derived by cloning individual cells into 96-well plates in the presence of UDP-4. Each subclone derived was then assessed for its capacity to differentiate and synthesize hemoglobin with 0.25 mM UDP-4 as well as with other inducers at optimum inducing concentration (1.8 mM sodium butyrate, 5 mM HMBA, and 210 mM DMSO). Two highly inducible subclones, designated MEL-UDP-4/4S and MEL-UDP-4/14S, were also isolated by selection in this study and used along with the two UDP-resistant subclones (MEL-UDP-4/8R and MEL-UDP-4/25R) for comparative studies.

Synthesis of [^{3}H]UDP

Radiolabeled [^{3}H]UDP-4 used throughout this study was prepared in two steps. 2-(3-Ethylureido)-6-aminopyridine was synthesized at first. This agent was then acetylated with [^{3}H]acetic anhydride to yield [^{3}H]UDP. The preparation procedures were carried out as follows:

Step A. 2-(3-Ethylureido)-6-aminopyridine was synthesized from ethyl isocyanate (20 mmol) that was added dropwise with vigorous stirring to 2,6-diaminopyridine (100 mmol) dissolved in dried chloroform (50 ml) for 6 h at 22°C. The reaction mixture was evaporated until dry and the crude precipitate obtained was chromatographed in a silica gel column (Silica gel from Merck, Germany, 230–400 mesh ASTM, 2 × 20 cm) using acetic ethylester/methanol (9:1) solution as eluent. 2-(3-Ethylureido)-6-aminopyridine was purified and analyzed by IR and ^{1}NMR. IR spectroscopy gave: 3400 cm^{-1} (NH_2), 3300 cm^{-1} (NH_2), 1680 cm^{-1} (amide I), 1640 cm^{-1} (amide II). The ^{1}NMR ($CDCl_3$) gave: 1.20 (3H, triplet, $NHCONHCH_2C\underline{\mathbf{H}}_3$), 3.36 (2H, fivefold, after addition of D_2O converted to quartet, $NHCONHCH_2C\underline{\mathbf{H}}_3$), 4.69 (2H, wide single, changed with D_2O, NH_2), 6.04 and 6.21 (2H, two doublets, $J = 9$ Hz, H-3 and H-5 of pyridine ring), 7.17–7.41 (1H, multiple, H-4 of pyridine ring), 8.46 (1H, wide single, changed with D_2O, $NHCON\underline{\mathbf{H}}CH_2CH_3$), 8.99 (1H, wide single, changed with D_2O, $N\underline{\mathbf{H}}CONHCH_2CH_3$).

Step B. [^{3}H]UDP was prepared by acetylation of 2-(3-ethylureido)-6-aminopyridine using [^{3}H]acetic anhydride: 2.3 mg of 2-(3-ethylureido)-6-aminopyridine were dissolved in 10 μl glacial acetic acid, mixed with 4.7 μl [^{3}H]acetic anhydride (sp. act. 50 mCi/mmol, Amersham, UK) at 22°C for 4 h and then dried under N_2 stream.

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Samples of radiolabeled compound (0.42 μg, 30,000 cpm) analyzed by TLC (Merck, Darmstadt, Germany) [methanol/acetic ethylester (9:1), ($R_f = 0.6$)] revealed a homogeneous single spot of [^{3}H]UDP that was then extracted from TLC stripes and assessed for radioactivity. The radiochemical purity and specific activity of the radiolabeled compound was found to be 14.2 mCi/mmol.

Construction of an Affinity Chromatography Column With Agarose Bearing UDP Ligand

A constant amount (20 mg) of 2-(3-ethylureido)-6-aminopyridine (used as ligand) was dissolved in 10 ml of 0.1 M $NaHCO_3$ (pH 8.3) and mixed with 0.5 g 6-aminohexanoic acid-activated Sepharose 4B (Sigma). The reaction was carried out for 3 h at 22°C and the product was filtered in a sintered glass filter. Later it was resuspended in 0.1 M ethanolamine (pH 8.0) to inactivate the free binding sites at Sepharose 4B. Sepharose 4B beads bearing the UDP ligand covalently attached were washed first six times with 0.05 M Tris-HCl, 0.05 M NaCl (pH 8.0), and then with 0.05 M HCOONa, 0.05 M NaCl (pH 4.0) to remove any excess unbound ligand. Sepharose 4B-UDP was swollen up in 10 mM phosphate buffer (pH 7.4) containing 10 mM NaCl/0.02% NaN_3 and then stored at 4°C until use.

Uptake, Binding Studies, and Subcellular Distribution of [^{3}H]UDP in MEL and Human Neuroectodermal RD/TE-671 Cells

Uptake of [^{3}H]UDP in MEL Cells. Exponentially growing MEL cells were harvested from cultures, washed with PBS, resuspended in DMEM at a density of 5×10^6 cells/ml, and incubated with 6×10^5 cpm/ml [^{3}H]UDP (sp. act. 14.2 mCi/mmol) at 37°C. At various time intervals, aliquots of cell culture (1 ml) were removed, centrifuged at $500 \times g$ for 5 min, and washed three times with ice-cold PBS, pH 7.4. The cellular pellet was then collected, resuspended in 0.2 ml of 1 N NaOH for 15 min, neutralized with 0.2 ml of 1 N HCl, and counted for radioactivity. All determinations were done in triplicate.

Specific Binding of [^{3}H]UDP in MEL and RD/TE-671 Cells. Because MEL cells were suspension culture and RD/TE-671 were attached cells, we employed a slightly different protocol to determine the specific binding of [^{3}H]UDP. In particular, MEL cells were harvested from culture by centrifugation ($500 \times g$, 5 min) and washed twice with Ca^{2+}- and Mg^{2+}-free PBS. Cells were then resuspended in DMEM without FCS (6.25×10^6 cells/ml) and incubated with varying concentrations of nonradioactive UDP in the presence of 18,400 cpm/ml of [^{3}H]UDP at 37°C for 1 h. By the end of this incubation period, cells were centrifuged ($600 \times g$, 5 min), washed three times with PBS, lysed with 0.2 ml 1 N NaOH, vortexed, and neutralized with 1 N HCl. The amount of [^{3}H]UDP bound to cells was determined according to previously described methods (17–19). The K_d and B_{max} values of [^{3}H]UDP specific binding in MEL cells were then calculated according to Van Zoelen (18) due to the relatively moderate specific activity of [^{3}H]UDP by using EnzFitter computer software (Biosoft, Cambridge, UK). RD/TE-671 cells (1.8×10^6 cells/ml) were exposed to various concentrations of [^{3}H]UDP in the absence or the presence of 20 mM nonradioactive UDP as indicated in Figure 2 legend, and the K_d and B_{max} values of [^{3}H]UDP specific binding were calculated according to Scatchard (19) by using EnzFitter computer software (Biosoft).

Competition Binding Studies. MEL cells collected from cultures and resuspended (10^7 cells/ml) in fresh DMEM without FCS were incubated with [^{3}H]UDP ($5x10^5$ cpm/ml) for 1 hr at 37° C in the presence and/or absence of inducers at the optimal inducing concentrations as follows: 1.25 and 5 mM 2-(3-ethylureido)-6-acetylamino-pyridine (UDP), 0.1 and 1 mM UDP-4, 0.1 and 1 mM UDP-7, 5 mM HMBA, 2 mM sodium butyrate, 10 μM diazepam and 40 μM hemin. Dexamethasone, an inhibitor of MEL cell differentiation (17,20), was also used at 100 μM. After incubation, the amount of [^{3}H]UDP that remained bound to MEL cells was measured as mentioned above.

Subcellular Fractionation and Isolation of [^{3}H]UDP Binding Proteins

Cells (MEL or RD/TE-671) loaded with [^{3}H]UDP (see details in the text for the number of cells and the amount of radioactivity), after 60-min incubation at 37°C, were harvested, washed, and, lysed with a buffer of 10 mM Tris-HCl (pH 7.2), containing 10 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, pepstatin, aprotinin, and 0.5% NP-40. The cell homogenate was centrifuged at $600 \times g$ for 10 min to remove the nuclei. Subsequently, the supernatant was collected and centrifuged at $12{,}000 \times g$ for 20 min at 4°C, which yielded mitochondria and lysosomes in the pellet and the so-called "postmitochondrial fraction" (PMF) in the supernatant. The protein content in each fraction was measured according to Lowry et al. (21).

To detect and then purify soluble protein(s) bound with [^{3}H]UDP in both MEL and RD/TE-671 cells, we applied two different but complementary experimental approaches. Briefly, exponentially growing MEL cells were incubated with [^{3}H]UDP (see details for the number of cells and the amount of radioactivity in the legend to each figure), for 1 h at 37°C, and then collected by

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centrifugation ($600 \times g$, 10 min), washed with PBS, and lysed according to Tsai et al. (22). Nuclei were removed by centrifugation of the cell homogenate at $600 \times g$ for 5 min. The supernatant was collected and adjusted to 10% w/v sucrose prior to centrifugation at $12{,}000 \times g$ for 30 min to remove mitochondria. The resulting supernatant was layered onto a discontinuous sucrose gradient (10/30/45% w/v) and spun at $30{,}000 \times g$ for 30 min. The 30% w/v sucrose fraction, which contained the majority of [^{3}H]UDP–protein complexes, was dialyzed (cutoff 6–14 kDa) for 4 h at 4°C, concentrated with an Amicon apparatus (PM-10 filter), and subsequently fractionated in a Sephadex G-100 column (1×50 cm) eluted with 50 mM Tris-HCl (pH 7.4) solution containing 50 mM NaCl. The conditions applied to purify the UDP binding proteins from postmitochondrial protein extract are presented in detail in the Results section. Fractions of 1.0 ml were obtained and assessed for both protein content (at 280 nm) and radioactivity.

When Nonidet P-40 was used for the purification of postmitochondrial fraction, MEL and/or RD/TE-671 cells were grown as mentioned above, and incubated in the presence or absence of [^{3}H]UDP in serum-free DMEM for 1 h at 37°C. Cells from either culture were lysed in 10 mM Tris-HCl (pH 7.4) containing 1 mM $MgCl_2$, 7% w/v sucrose, 0.1% v/v Nonidet P-40, and protease inhibitors (1 mM PMSF and 1 μg/ml pepstatin A, aprotinin, and leupeptin). The cell homogenates were centrifuged at $600 \times g$ (5 min) to remove the nuclei and collect the supernatants after centrifugation at $12{,}000 \times g$ for 30 min. PMF was analyzed either by gel filtration and/or affinity chromatography (see below).

Similarly, experiments were performed to purify protein complex-enriched [^{3}H]UDP from nuclei. Intact nuclei, isolated through the procedure described above and found to contain portion of [^{3}H]UDP attached, were resuspended in an ice-cold solution of 0.15 M NaCl that was gradually adjusted to 0.4 M NaCl dropwise. Nuclei were then broken with a potter-type homogenizer and centrifuged at $110{,}000 \times g$ for 35 min at 4°C to yield a salt nuclear extract and a chromatin pellet using a Beckman SW 41Ti rotor. The supernatant obtained was dialyzed with membranes (molecular weight cutoff 10–14 kDa) to remove NaCl and chromatographed through a Sephadex G-100 column. Radioactivity and protein content were determined in each fraction. The chromatin pellet was then resuspended in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and centrifuged once again at $110{,}000 \times g$ for 35 min to remove the undissolved material (chromatin pellet) and collect the supernatant fraction. The latter was then analyzed by Sephadex G-100 column chromatography. The analysis of the postmitochondrial fraction with UDP affinity column chromatography is described in the Results section.

Development of Rabbit Antiserum Against the UDP Binding Protein (p38) and Western Blot Analysis

Soluble cytoplasmic protein complexes enriched in [^{3}H]UDP binding proteins were analyzed by SDS-PAGE (5–10%) according to Laemmli (23). Gel was stained with Coomassie Brilliant Blue and the p38 band was visualized as a single band. Next, it was excised from the gel and passed through a 25-gauge needle using 1.0 ml PBS (pH 7.4). After mixing with complete Freund's adjuvant (1:1), the p38 was injected SC into a female white rabbit. Booster immunizations were given SC every 10 days. Rabbit serum was collected after the third booster and assessed for interaction with the p38 in Western blot analysis at a dilution of 1:1000. Serum was also isolated before animal immunization and served as negative control.

Cytoplasmic lysates obtained from MEL and/or RD/TE-671 cells were separated by SDS-PAGE electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes. Blots were then blocked with 5% nonfat milk, 0.1% Tween-20, and 3% normal goat serum in PBS for 1 h at room temperature (RT). Antiserum against the UDP binding protein (1:1000) was added and incubated for 2 h at RT. After washing three times with PBS/Tween-20 (0.1%), blots were incubated with goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (1:1000) for 1 h at RT, washed twice with PBS/Tween-20, once with alkaline phosphatase buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 9.5), and developed with 0.3 mg/ml nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (0.15 mg/ml) in alkaline phosphatase buffer in the dark.

Microsequencing of UDP Binding Protein(s) Purified From MEL-745PC-4A Cells

Highly purified UDP binding protein(s) (p38) derived from MEL-745PC-4A as described (see Fig. 4B) was subsequently analyzed in a SDS-PAGE (5–10%). The p38 was then transferred onto Immobilon-CD (Sigma), stained with Coomassie Brilliant Blue solution, and the band excised. Immobilized protein was then extracted from membranes and digested with endopeptidase (KC). The resulting fragments were resolved by microbore RP-HPLC and subjected to MALDI-MS analysis.

Electrophoretic Analysis of UDP Binding Protein(s) and In-Gel Digestion for MALDI-TOF Analysis

The UDP binding protein(s), isolated from the postmitochondrial fraction of MEL cells after Sephadex G-50 gel filtration as described above, was further analyzed by either SDS-PAGE electrophoresis or 2D gel

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electrophoresis (SDS-PAGE at one dimension and isoelectric focusing at the other) by using the NuPAGER Novex Pre-cast Gel System (Invitrogen Life Technologies) according to the manufacturer's instructions. The SDS-PAGE gel was then stained using Coomassie Brilliant Blue R-250 (BioRad). The protein band migrating to the 38–40-kDa region was cut from the gel. For a control, an additional piece of gel was excised that was protein free and treated the same as the protein band. The bands were then diced into small pieces and placed into siliconized tubes. Fifty microliters of 25 mM ammonium bicarbonate/50% acetonitrile was added to the gel pieces, followed by vortexing for 5–10 min. By using gel loading pipette tips, the supernatant was removed and discarded. This step was repeated three times. The gel pieces were then dried in a speed vacuum (~20 min). Fifty microliters of 10 mM DTT (BioRad) in 25 mM ammonium bicarbonate was added to dried gel pieces. The samples were vortexed and centrifuged briefly. Following incubation for 1 h at 56°C, the supernatant was removed and 25 µl of a 55 mM solution of iodoacetamide (BioRad) in 25 mM ammonium bicarbonate was added to the gel pieces. The samples were again vortexed and centrifuged briefly. The reaction was allowed to proceed in the dark for 45 min at RT. The supernatant was then removed and discarded. The gel pieces were washed with 100 µl of 25 mM ammonium bicarbonate, vortexed for 10 min, and centrifuged. The supernatant was removed and discarded. Fifty microliters of 25 mM ammonium bicarbonate/50% acetonitrile was added to the gel pieces, followed by vortexing for 5–10 min and centrifugation. The gel pieces were then dried in a speed vacuum (~20 min). Fifty microliters of a 15 ng/µl trypsin solution in 25 mM ammonium bicarbonate was added to the gel pieces. Following a 30-min incubation on ice, the solution that remained in the tube with the gel pieces was removed. The samples were incubated at 37°C for 16 h.

Extraction of Peptides. Following the tryptic digestion, 100 µl of 25 mM ammonium bicarbonate was added to the gel pieces. The samples were vortexed for 5–10 min and sonicated for 5 min. The supernatant was removed and saved. This was repeated one time, pooling the supernatant with the supernatant from the previous step. Next, 50 µl of 50% acetonitrile/5% trifluoroacetic acid was added to the gel pieces followed by vortexing for 5–10 min. The gel pieces were centrifuged and the supernatant was removed and pooled with the supernatants from the previous extractions. This step was repeated two times. The pooled solutions were then concentrated to a volume of 20 µl. One microliter of this 20-µl mixture was mixed with 1 µl of matrix solution [alpha-cyano-4-hydroxycinammic acid (Sigma) in 50% acetonitrile/0.1% trifluoroacatic acid (20 mg/ml)] and spotted on the MALDI-TOF plate for mass spectrometric analysis.

MALDI-TOF Mass Spectrometric Analysis. The mass spectrometric analysis was performed on a Micromass Tof-Spec 2e. This instrument is equipped with a nitrogen laser operating at 337 nm. Each mass spectrum was an average of approximately 250–300 shots from the laser. MALDI-TOF spectra were calibrated externally using angiotensin I (Sigma) and adrenocorticotropic hormone (ACTH, Sigma). The *m/z* values generated in the unknown protein tryptic digestion spectrum were searched against the NCBI nonredundant protein database using the programs Profound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) and Protein Prospector (http://prospector.ucsf.edu/). Potential protein IDs were determined by matching the mass of the each peptide in the MALDI-TOF spectra with its corresponding sequence within the protein. The tolerance for the monoisotopic precursor ion for each search was set at 0.3 Da. The mass range cutoff was set to take proteins with masses between 38–39 kDa. This was in accordance to the molecular weight of a MALDI-TOF analysis of the undigested protein, which gave a *m/z* value of 38,333 ± 30 Da.

RESULTS

[^{3}H]UDP Selectively Binds to Components in Intact MEL Cells

By employing [^{3}H]UDP as ligand, we were able to show that this agent accumulates rapidly in MEL cells and reaches a maximum intracellular concentration after 120 min of incubation (Fig. 1). Similar data were obtained with human RD/TE-671 cells (data not shown). Subcellular fractionation of MEL and RD/TE-671 cells loaded with [^{3}H]UDP for 60 min revealed that the majority of radioactivity (Table 1) accumulates in the soluble postmitochondrial fraction (PMF) derived from each cell line (92.5% and 79.4%, respectively). A much smaller proportion of radioactivity (from 5.9% to 18.1%, respectively) was found to be associated with intact nuclei in either cell type. Negligible amounts of radioactivity were found in mitochondria. These data indicate that [^{3}H]UDP enters into both MEL and RD/TE-671 cells and accumulates mainly into the postmitochondrial fraction, suggesting that UDP may form a complex with soluble intracellular proteins. The smaller portion of [^{3}H]UDP found in the nucleus suggests that UDP may also reach the nucleus where it interacts with the nuclear components.

To demonstrate whether [^{3}H]UDP interacts selectively with cellular proteins, we carried out specific binding studies using intact cells and incubating varying concen-

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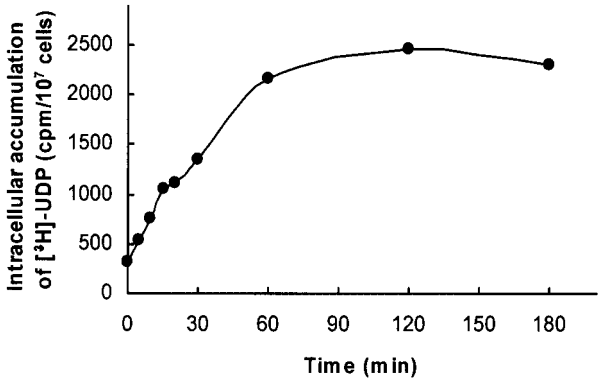

Figure 1. Uptake of [^{3}H]UDP in MEL cells. Exponentially growing MEL-745 PC-4 cells were harvested from cultures, resuspended at a concentration of 5×10^6 cells/ml, and incubated with 6×10^5 cpm [^{3}H]UDP at 37°C. At various times thereafter, samples of 1.0 ml cell suspension were removed, collected by centrifugation ($350 \times g$, 10 min), and washed three times with PBS. The cellular pellet was then resuspended in 0.2 ml 1 N NaOH, neutralized with 0.2 ml 1 N HCl, and counted for radioactivity. Values represent the mean of triplicate samples.

trations of nonradioactive UDP in the presence of a constant amount of [^{3}H]UDP, as shown elsewhere for other ligands (18). These studies revealed that [^{3}H]UDP specifically binds to MEL cells, whereas subsequent analysis led to the estimation of K_d and B_{max} values for MEL cells as follows: $K_d = 167 \pm 31.3$ μM and $B_{max} = 598 \pm 42$ nmol/10^6 cells (Fig. 2). The K_d and B_{max} values for the RD/TE-671 cells were estimated to be 7.9 ± 3.3 μM and 125.8 ± 32.7 nmol/10^6 cells by using a slightly different approach [(18,19), see Materials and Methods]. One must bear in mind that MEL cells are murine erythroleukemia cell suspension culture, in contrast to RD/TE-671 cells that are human neuroectodermal cells attached to the substratum. Therefore, we had to appropriately modify the protocols to estimate K_d and B_{max} values and overcome the relatively moderate specific activity of [^{3}H]UDP.

Detection, Isolation, and Purification of the UDP Binding Protein

The accumulation of the major portion (70–90%) of intracellular radioactive UDP into the soluble PMF in both RD/TE-671 and MEL cells, as illustrated in Table 1, prompted us to fractionate the cells and purify the UDP-enriched protein complexes to isolate the potential UDP binding proteins. This objective was met by carrying out two sets of complementary experiments. First we applied gel filtration column chromatography and secondly affinity column chromatography to purify the binding protein(s), as shown in Figure 3. In either case the purified UDP binding protein(s) derived by either method (gel filtration and/or affinity chromatography) were evaluated by SDS-PAGE electrophoresis and microsequencing.

Isolation of the [^{3}H]UDP–Protein Complexes and Purification of p38 by Gel Filtration. Postmitochondrial proteins enriched in [^{3}H]UDP were derived from MEL and RD/TE-671 cells and analyzed by gel filtration column chromatography on Sephadex G-100 calibrated by using BioRad molecular weight standards (thyroglobin 670 kDa, IgG 150 kDa, ovalbumin 44 kDa, myoglobin 17 kDa, and vitamin B_{12} 1.35 kDa). In the case of MEL cells, as shown in Figure 4, proteins complexed with [^{3}H]UDP were eluted in two peaks, one seen at the void volume and another moving towards the end of the column (Fig. 4A). Comparatively, much more radioactive material was observed in the second peak (fractions 70–90). These fractions, corresponding to the second peak, were further analyzed through Sephadex G-50 column chromatography as shown in Figure 4B. Such data suggested at first that intracellularly accumulated [^{3}H]UDP forms complexes with proteins of relatively large and small molecular weights. Because we reasoned that [^{3}H]UDP may interact with oligomers of the UDP binding protein, we solubilized the high molecular weight [^{3}H]UDP–protein complexes eluted at the void volume of Sephadex G-100 (first peak, Fig. 4A) with Triton X-100 and chromatographed them once again via a Sephacryl S-300 column (Fig. 4C). Fractions corresponding to the small molecular weight peak (Sephadex G-50; fractions 41–48) were pooled and analyzed by SDS-PAGE electrophoresis (see Fig. 5A). To proceed further with the development of a polyclonal antibody against p38,

Table 1. Subcellular Distribution of [^{3}H]UDP in MEL-745PC-4A and RD/TE-671 Cells

	Radioactivity (%)	
Cell Fraction	MEL Cells	RD/TE-671 Cells
Cell homogenate	100.0	100.0
Nuclear pellet	5.9	18.1
Mitochondria and lysosomes	0.5	2.3
Postmitochondrial (PMF)	92.5	79.4

MEL-745PC-4A and RD/TE-671 cells grown in culture were collected and resuspended in 20 and 10 ml of DMEM medium to final concentrations of 1.7×10^7 and 2.5×10^6 cells/ml, respectively. Then they were incubated with 4×10^5 cpm/ml [^{3}H]UDP for 1 h at 37°C. By the end of this period, cells were washed with phosphate-buffered saline (pH 7.4), lysed, and fractionated to various subcellular compartments. Radioactivity was determined in each fraction. Each value represents the average of duplicate measurements.

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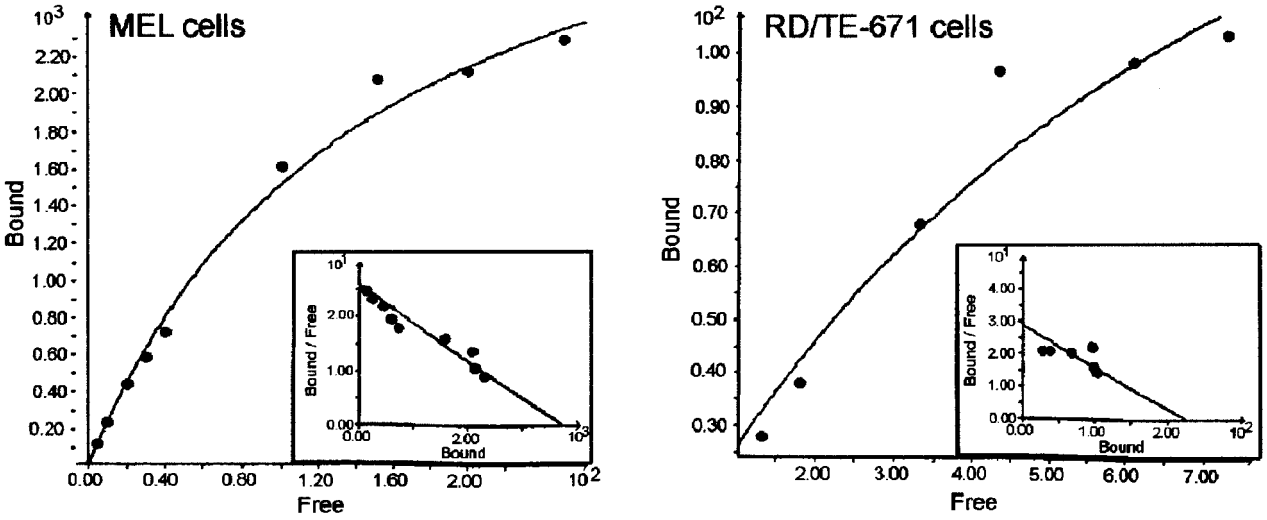

Figure 2. Specific binding of [^{3}H]UDP in intact mouse MEL and human RD/TE-671 cells. Left: MEL-745 PC-4 cells (6.25×10^6 cells/ml) were exposed to 18,400 cpm (0.29 μM) [^{3}H]UDP and various concentrations of nonradioactive UDP for 1 h at 37°C. By the end of this incubation period, cells were washed three times with Ca^{2+}/Mg^{2+}-free PBS and the amount of [^{3}H]UDP bound to cells was determined according to Van Zoelen (18). The K_d value was estimated to be 167 ± 31.3 μM and B_{max} as 598 ± 42 nmol/10^6 cells. Right: RD/TE-671 cells (1.8×10^6 cells/ml) were exposed to various concentrations of [^{3}H]UDP in the absence or presence of 20 mM nonradioactive UDP for 1 h at 37°C. By the end of incubation period, cells were washed three times with Ca^{2+}/Mg^{2+}-free PBS and the amount of [^{3}H]UDP bound to cells was determined according to Scatchard (19). The K_d value was calculated to be 7.9 ± 3.3 μM and B_{max} as 125.8 ± 32.7 nmol/10^6 cells.

the p38 band shown in Figure 5A (lane 2) was then excised from SDS-PAGE electrophoresis and injected into a rabbit for antiserum development following standard procedures. After the appropriate period, rabbit serum was collected and assayed for immunoreactivity, whereas preimmune serum was used as negative control in Western blots to verify polyclonal antibody specificity (data not shown). The development of p38 antiserum allowed us to analyze the fractions corresponding to the small as well as the high molecular weight peaks of UDP–protein complexes by Western blot. In particular, as shown in Figure 5, Western blot analysis of [^{3}H]UDP-enriched protein fractions isolated by Sephadex G-50 column chromatography (see Fig. 4) confirmed the presence of p38 (Fig. 5B). Also, fractions isolated by Sephacryl S-300 column chromatography (see Fig. 4C) contained p38, as shown in Western blots by again using the polyclonal antibody raised against the p38 derived from MEL cells (Fig. 5C).

Following the same methodology, the postmitochondrial fraction from RD/TE-671 cells incubated with [^{3}H]UDP was prepared under similar conditions and analyzed first on Sephadex G-100 (Fig. 6A) and then via Sephadex G-50 column chromatography (Fig. 6B). Western blot analysis of radioactive protein fractions confirmed the presence of p38 in RD/TE-671 cells as well (Fig. 6C).

Isolation of the [^{3}H]UDP Binding Protein by UDP-Sepharose 4B Affinity Column Chromatography. Affinity column chromatography (Sepharose 4B-UDP) of PMF derived from MEL cells was also applied to purify the [^{3}H]UDP binding proteins. A constant amount of protein expressed in $OD_{280\,nm}$ units was loaded and eluted from the affinity column as described below. Each fraction was analyzed by SDS-PAGE electrophoresis thereafter, as shown in Figure 7A. Overall, different elution conditions were applied separately to remove proteins attached to the UDP-Sepharose 4B affinity column: a) after loading and flushing out the nonbound proteins that passed throughout, the affinity column was eluted with urea alone, which removed all the proteins bound (Fig. 7A, lane 1); b) alternatively, elution was done first under acidic conditions followed by urea to initially remove positively charged proteins and then detach the rest of the proteins bound to the column (Fig. 7A, lanes 2 and 3); and c) elution of the affinity column was also carried out separately by using solution containing each one of the two inducers, HMBA or UDP-4, thus expecting the selective removal of p38 among a few other proteins (Fig. 7A, lanes 4, 5).

To investigate whether the radioactive material bound to nuclear fraction contained [^{3}H]UDP–p38 binding protein complexes loosely associated with nuclei, nucleoplasm was extracted from MEL cell nuclei and analyzed

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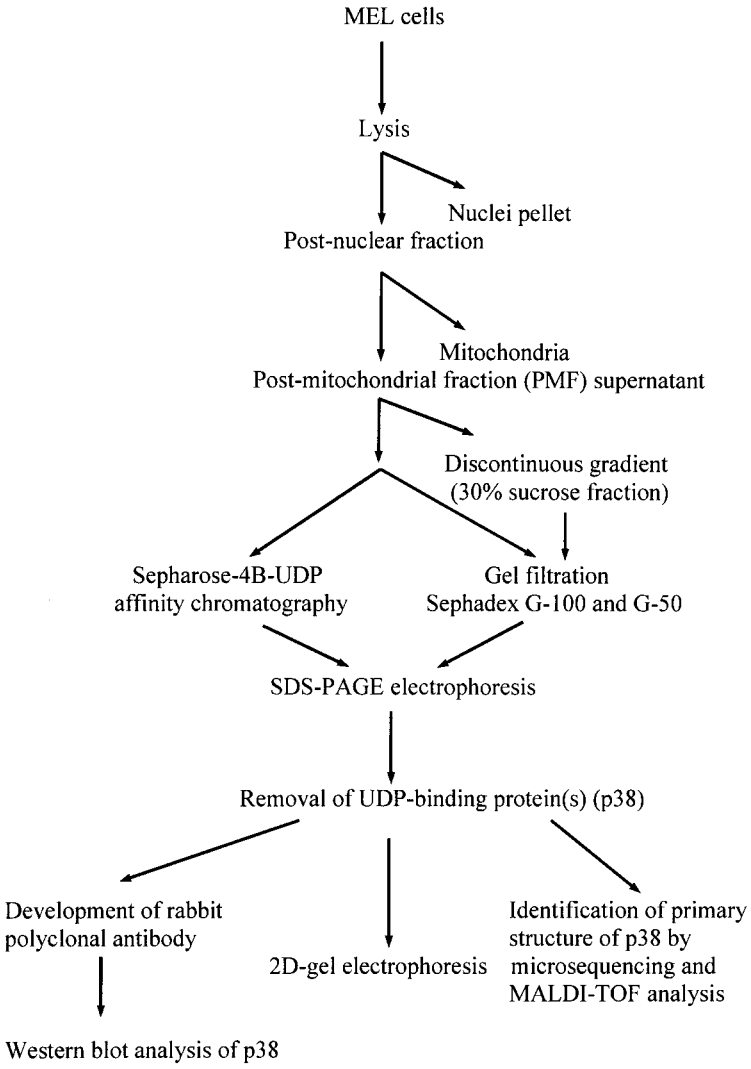

Figure 3. Diagram illustrating the methodology applied for the isolation and purification of UDP binding p38 (see details under Materials and Methods in the text).

by molecular gel filtration as previously described. These experiments revealed the presence of p38 in the nuclear compartment as shown by SDS-PAGE analysis and Western blot (Fig. 7B, lanes 2 and 3). These findings are consistent with the data illustrated in Table 1, which show detectable quantities of [^{3}H]UDP found in the nucleus. To further confirm the presence of p38 in fractions isolated from MEL cells by various methods, samples from all fractions were analyzed by Western blot using the p38 rabbit antiserum. As illustrated in Figure 7B, p38 was found in the postmitochondrial fraction (Fig. 7B, lane 1), in nuclear extracts fractionated by gel filtration (Sephadex G-100 and G-50) (lane 2), as well as in chromatin (lane 3). The presence of p38 was also confirmed in the 30% w/v sucrose fraction of postmitochondrial supernatant (lane 4). Finally, p38 was detected in the postmitochondrial fraction from MEL cells that was loaded on affinity chromatography column (Sepharose 4B-UDP) and subsequently eluted with UDP-4 (0.1 M) (lane 5).

These data indicate that different elution with either HMBA or UDP-4 led to the purification of p38 along with a small number of proteins. This protein was also among the proteins eluted by urea under neutral or acidic conditions, as expected. Overall, both methodologies (molecular sieve analysis and affinity chromatography) confirmed the existence of p38 as the UDP binding protein that forms stable complexes with [^{3}H]UDP in both MEL-745PC-4A and RD/TE 671 cells. It was interesting that murine MEL and human RD/TE-671 cells

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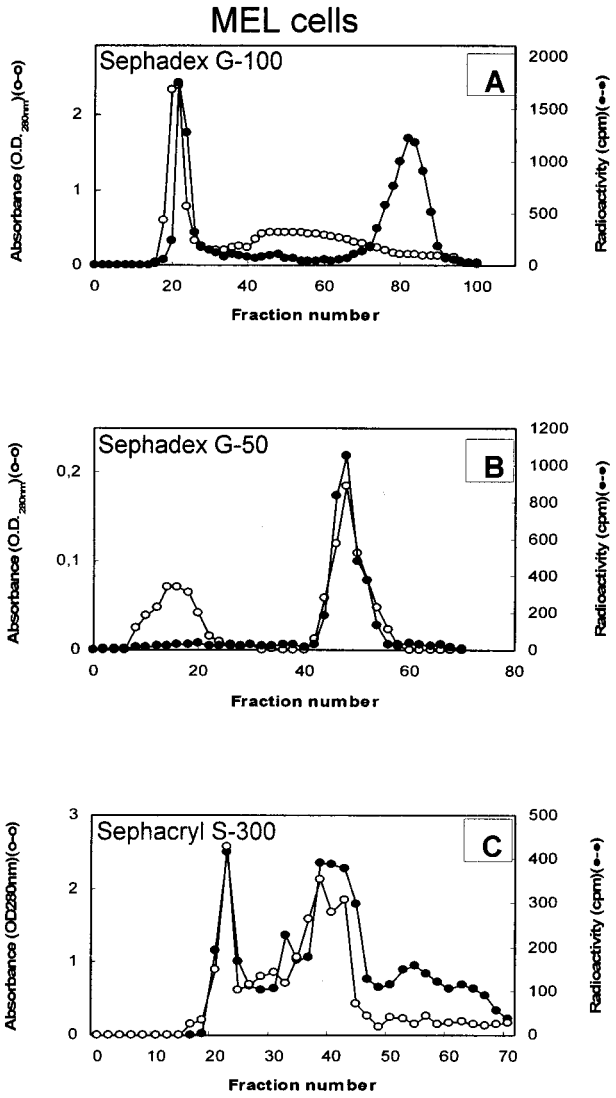

Figure 4. Gel filtration of postmitochondrial fraction (PMF) from MEL cells enriched in [^{3}H]UDP–protein complexes. MEL cells (2×10^7 cells/ml; 40 ml in total) were incubated with [^{3}H]UDP (3×10^5 cpm/ml) and then the postmitochondrial fraction was isolated as described under Material and Methods. Proteins of the postmitochondrial fractions were analyzed by Sephadex G-100 calibrated by using BioRad molecular weight standards (bovine thyroglobulin 670 kDa, bovine γ-globulin 158 kDa, ovalbumin 44 kDa, horse myoglobin 17 kDa, and vitamin B_{12} 1.35 kDa). Fractions containing the [^{3}H]UDP–protein complexes were eluted in two peaks (A). The low molecular weight peak was further analyzed by Sephadex G-50 column (B) and radioactive fractions were collected. The high molecular weight peak fractions (eluted at the void volume of the column) were pooled together, treated with Triton X-100 to dissolve possible aggregates, and then analyzed by a Sephacryl S-300 column to further analyze the high-molecular weight UDP–protein complexes (C). Fractions containing radioactivity were collected and assessed for both protein content ($OD_{280\ nm}$) and radioactivity.

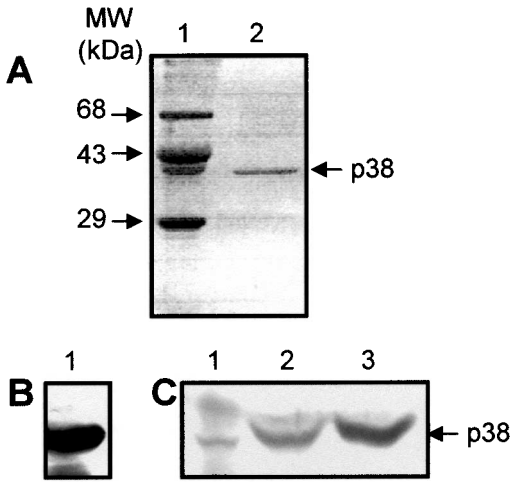

Figure 5. SDS-PAGE electrophoresis of [^{3}H]UDP–protein complexes derived from purified postmitochondrial fraction of MEL cells and Western blot analysis of cellular protein extracts purified by Sephacryl S-300 column chromatography. (A) Postmitochondrial fraction enriched in [^{3}H]UDP–protein complexes was purified by gel filtration as shown in Figure 4A and B (fractions 70–90 from Sephadex G-100 and 41–48 from Sephadex G-50) and then analyzed by SDS-PAGE electrophoresis. The SDS-PAGE gel was then stained using Coomassie Brilliant Blue R-250 (BioRad) and the predominant protein band of p38 shown in lane 2 was excised, removed, and used for the development of a rabbit antiserum. Lane 1: molecular weight markers whose size is indicated by the arrows. (B) Protein fractions (41–48 shown in Fig. 4B) containing radioactive [^{3}H]UDP were collected by Sephadex G-50, electrophoresed, and then transferred onto PVDF membranes. Next membranes were blotted with a rabbit anti-p38 antiserum and p38 was visualized. (C) Protein fractions (lane 1: fractions 26–31; lane 2: fractions 34–39, and lane 3: fractions 41–42) isolated from the Sephacryl S-300 column described under Figure 4C were electrophoresed and then transferred onto PVDF membranes. p38 was detected in fractions containing radioactivity.

contain a UDP binding protein of similar molecular weight.

The Specific Binding of [^{3}H]UDP Is Affected by Other Inducers and Inhibitors of MEL Cell Differentiation

To demonstrate whether the MEL and RD/TE-671 cells contain [^{3}H]UDP binding proteins that may serve as potential molecular targets for other known inducers of differentiation, we performed binding studies using other known inducing agents as competitors. Our reasoning was as follows. If p38 serves as a potential molecular binding agent common for inducers other than UDPs, then these inducers will compete with each other for the same site. We loaded MEL cells with [^{3}H]UDP for 60 min, harvested them, washed them to remove the

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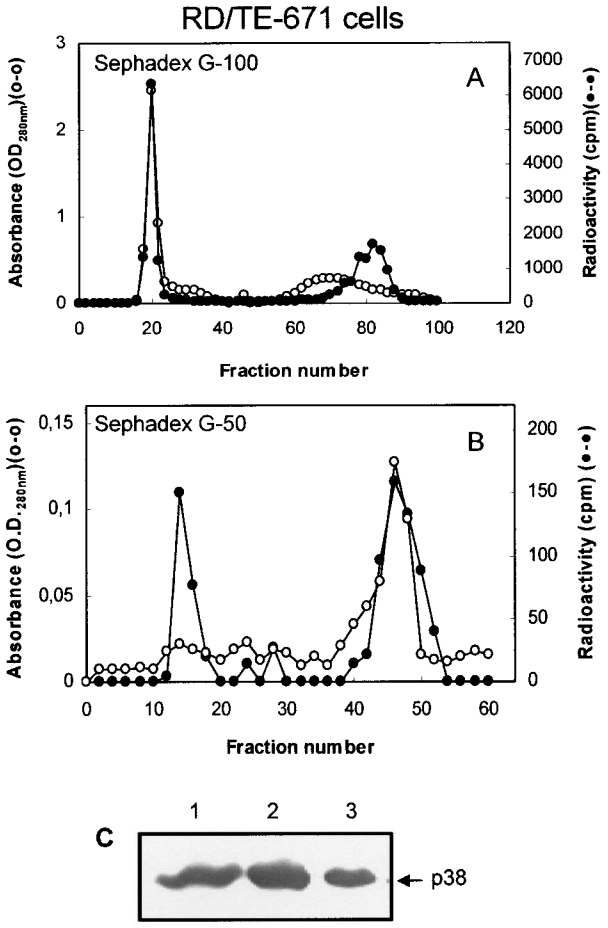

Figure 6. Gel filtration of subcellular fractions enriched in [^{3}H]UDP–protein complexes and Western blot analysis of p38 isolated from RD/TE-671 extracts. RD/TE-671 cells (7×10^6 cells/ml; 10 ml in total) were incubated with 3×10^5 cpm/ml [^{3}H]UDP for 1 h at 37°C and then the postmitochondrial fraction was isolated and analyzed by Sephadex G-100. Protein fractions enriched in [^{3}H]UDP were eluted in two peaks (A). The low molecular weight peak fraction containing radioactivity was further analyzed by Sephadex G-50 (B). Each fraction was assessed for both protein content ($OD_{280\ nm}$) and radioactivity. (C) Proteins purified by Sephadex G-100 and G-50 column chromatography (A and B) were further analyzed by SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membranes, and blotted with rabbit anti-p38 serum. Lane 1 represents purified extracts with the low molecular weight protein fraction containing radioactivity (fractions 72–88 from Sephadex G-100 column shown in A) pooled together. Similarly, lane 2 represents purified extracts containing the pooled fractions 13–17 and lane 3 the fractions 44–50 purified by Sephadex G-50 (B).

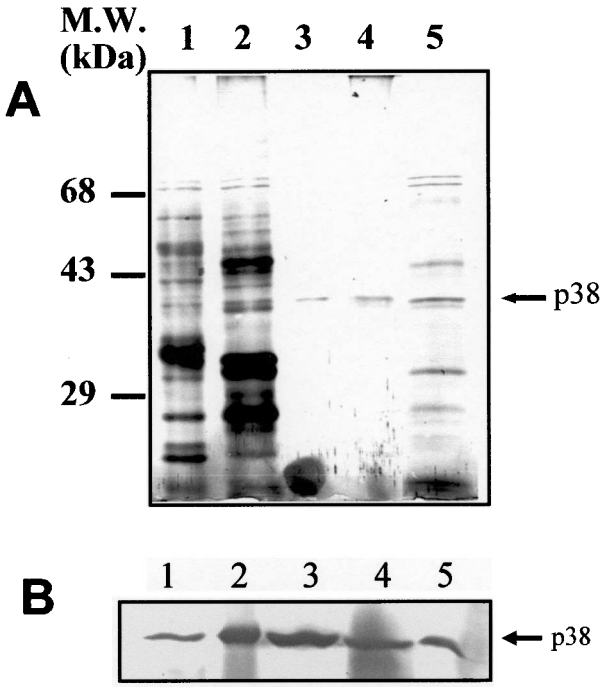

Figure 7. SDS-electrophoresis and Western blot detection of the p38 in subcellular fractions prepared from MEL cells and purified by affinity chromatography and gel filtration. (A) SDS-PAGE gel electrophoreris of samples derived from affinity column chromatography loaded with proteins of postmitochondrial fraction of MEL cells (~45 OD_{280} units) and eluted by using four different elution conditions as follows: lane 1, the proteins eluted with 8 M urea; lane 2, proteins eluted at first with 10 mM HCOONa/0.5 M NaCl (pH 4.5) solution; lane 3, proteins remained attached and eluted with 8 M urea under acidic elution; lane 4, proteins eluted with 0.1 M HMBA; lane 5, the proteins eluted with 0.1 M UDP-4 under acidic conditions (pH 1.5). The gel was stained with Coomassie Brilliant Blue solution and the results obtained are shown above. (B) Detection of p38 by Western blot analysis, as described under Figure 5B, in proteins derived from postmitochondrial fraction (lane 1), nucleoplasm (lane 2), and dissolved chromatin pellet (lane 3) of MEL cells as described under Figure 4A and B. Proteins being present in the 30% w/v sucrose subcellular fraction of MEL cells and purified by Sephadex G-100 were also analyzed (lane 4) along with proteins of the postmitochondrial fraction eluted by UDP-4 in affinity chromatography (lane 5).

unbound radiolabeled UDP, and resuspended them in the presence of another inducer or an inhibitor, like dexamethasone. The extent to which each inducing agent displaced the [^{3}H]UDP from its binding protein in MEL cells was estimated. According to the data shown in Table 2, unlabeled UDP decreased by 50.0% and 82.7% [^{3}H]UDP binding when added at 1.25 and 5.0 mM concentration, respectively. Slightly lower reduction of ligand binding (~30%) was achieved with UDP-4 and UDP-7, two potent inducers of the UDP class (8) added at 1.0 mM concentration (Table 2). Significant reduction of [^{3}H]UDP binding (35–40%) was also observed when four other known inducers of MEL cells (HMBA, sodium butyrate, diazepam, and hemin) were employed. However, a pronounced reduction in binding of [^{3}H]UDP

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Table 2. Effect of Different Differentiation Inducers and Dexamethasone on [^{3}H]UDP Binding to Intact MEL Cells

Inducer or Inhibitor of Differentiation	Concentration (mM)	Reduction of [^{3}H]UDP Binding (% of control)	[^{3}H]UDP Remained Bound (% of control)
None	—	100.0	0.0
UDP	1.25	50.0	50.0
	5.00	82.7	17.3
UDP-4	0.10	2.1	97.9
	1.00	32.3	67.7
UDP-7	0.10	5.4	94.6
	1.00	29.7	70.3
HMBA	5.00	37.8	62.2
Sodium butyrate	2.00	35.7	64.3
Diazepam	0.01	36.9	60.1
Hemin	0.04	41.1	58.9
Dexamethasone*	0.10	64.4	33.6

Highly inducible MEL-745PC-4A cells were suspended in a final concentration of 5×10^6 cells/ml and incubated with 4×10^5 cpm/ml [^{3}H]UDP for 1 h at 37°C in the absence or presence of inducers of differentiation and/or dexamethasone. The cells were then centrifuged, washed three times with PBS, and the cellular pellet was treated with 0.2 ml 1 N NaOH. After neutralization with 0.2 ml 1 N HCl, the amount of radioactivity entrapped in cells was determined. UDP: 2-(3-ethylureido)-6-acetylamino-pyridine; UDP-4: 2-(3-ethylureido)-6-methyl-pyridine; UDP-7: 2,6-bis-(3-ethylureido)-pyridine; HMBA: hexamethylene-bisacetamide. Each value represents the average of duplicate measurements.
*Dexamethasone is an inhibitor of MEL cell differentiation.

was observed with dexamethasone (64.4%), a potent inhibitor of MEL cell differentiation (17,20). These data indicate that: (a) all inducers tested decreased the binding of [^{3}H]UDP, and (b) remarkably, dexamethasone reduced [^{3}H]UDP binding by ~65%. It was interesting to observe, however, that none of the agents employed increased the total binding of [^{3}H]UDP in MEL cells. Although it is quite difficult to explain how each one of the agents tested affected the binding of [^{3}H]UDP to its binding site, directly or indirectly, it is proposed that: a) the UDP binding protein may serve as potential binding protein for other inducers with different affinities, and b) inhibition of differentiation by dexamethasone may impinge on the possible interaction of UDP with p38 protein.

Characterization of UDP Binding Protein(s) by 2D Gel Isoelectric Focusing, MALTI-TOF Analysis, and Microsequencing

UDP binding protein(s) purified to a single band level, as shown by SDS-PAGE electrophoresis, was also subjected into 2D gel isoelectric focusing analysis, as shown in Figure 8. The 2D gel analysis revealed the existence of three proteins of the same molecular weight, differing slightly in charge (Fig. 8B). All had relatively acidic isoelectric points (pIs), suggesting that they represented three different forms of the same protein. Their purification one by one was not possible. However, MALDI-TOF analysis complemented with LC-MS/MS revealed that UDP binding protein(s) were 38,333 ± 30 Da molecular weight (p38) (Fig. 9), whereas two peptides were identified at the same time, as shown in Table 3 (peptides 7 and 8). In addition, microsequencing of these highly purified proteins by different methods (gel filtration and affinity column chromatography) resulted in the identification of six additional peptide fragments of different sizes, shown in Table 3 (peptides 1–6). Interestingly, the estimated pI values are acidic for most of these peptides (Table 3). Comparing these amino acid sequences with known protein sequences submitted in protein databanks revealed that p38 is an unknown protein bearing domains with structural homology to the ABC-type transporters (peptide 5) and the Wingless-type (wnt) proteins (peptide 8) (Fig. 10). In addition, degenerate primers were synthesized and are currently being used to clone and identify the cDNA sequence corresponding to p38. In the meantime, based on the analysis data presented, p38 appears to be a protein associated with the membrane protein fraction, being part of the postmitochondrial proteins.

p38 Exists in Both UDP-Inducible and -Resistant MEL Cells

To investigate whether p38 interacts with [^{3}H]UDP equally well in MEL cells both UDP inducible and resistant to differentiation, we estimated the intracellular dis-

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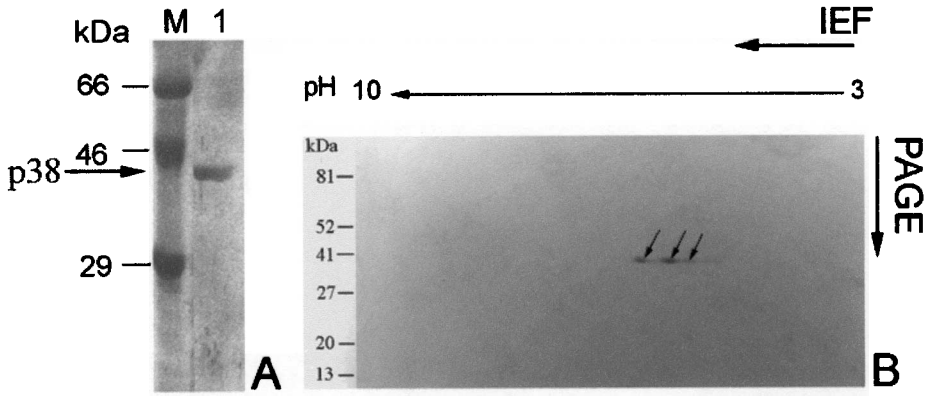

Figure 8. Two-dimensional gel electrophoresis of p38 isolated from MEL cells by gel filtration. UDP–protein complexes isolated from MEL postmitochondrial cell extracts by Sephadex G-50 gel filtration column chromatography as described under Figure 4 were analyzed either in SDS-PAGE (A; 30 μl of sample loaded) or 2D gel (B; 70 μl of sample loaded) electrophoresis, as described in Materials and Methods. (A) The gel stained with Coomassie Brilliant Blue solution shows the proteins complexed with [^{3}H]UDP and eluted through Sephadex G-50 (see Fig. 4B) (lane 1). Lane M indicates the protein molecular weight markers used as standards (size is shown at the left in kDa). Note that p38 is the predominant protein detected. (B) The 2D gel stained with silver solution revealed three proteins of similar molecular weight being present in the same sample having different pIs (shown by the arrows). IEF: isoelectric focusing.

tribution of [^{3}H]UDP in either cell type (Table 4). The data shown in Table 4 indicate no substantial differences in the level of [^{3}H]UDP fractions accumulated in each subcellular compartment in inducible and resistant MEL cells. Western blot analysis using anti-p38 serum revealed that the level of p38 was similar in both the UDP-inducible and -resistant subclones of MEL cells (Fig. 11B). These data indicate that although the interaction of [^{3}H]UDP with the binding protein occurs within both cell types, it is not sufficient to initiate commitment of MEL cells to erythroid maturation. More evidence supporting this conclusion is illustrated in Figure 11A, where the level of p38 was found to be similar in [^{3}H]UDP-treated MEL cells.

Exploring the Role of p38 in MEL Cells: [^{3}H]UDP Forms Stable Protein Complexes During the Precommitment Period

The experimental data described thus far encouraged us to further explore the role of p38 in UDP-induced MEL cell differentiation. We asked the following questions: (a) Does the inducer UDP form a complex with p38 both in the postmitochondrial fraction and in the nucleus and how soon does this occur following induction? (b) Is this complex maintained throughout the entire course of the differentiation process or decayed after commitment to differentiation has been initiated? (c) Is the UDP–p38 complex affected by inhibitors of MEL cell differentiation that act at the level of new protein and mRNA synthesis? (d) Do UDP-resistant subclones of MEL cells (which are also cross-resistant to HMBA, DMSO, and sodium butyrate) express p38 like the inducible cells? (e) Is the level of p38 altered in differentiating MEL cells?

As shown in Figure 12, [^{3}H]UDP formed a soluble complex with p38 in both the cytotolic and nuclear fractions. Kinetically, these complexes were observed and isolated by gel filtration after 12 h in the nuclei and 12–36 h in the cytosolic fraction, when they reached their highest steady-state level. The [^{3}H]UDP–p38 protein complexes were maintained stable for several hours but decayed later on when the majority of the cells (>80%) were committed to differentiation (Fig. 12C). Exposure of MEL cells to cycloheximide and cordycepin, two inhibitors of new protein and RNA synthesis that block differentiation [for review see (5,6)], decreased and delayed, respectively, the accumulation of UDP–protein complexes substantially, suggesting that new mRNA and protein synthesis is involved for UDP–protein complex formation to occur (Fig. 12A, B). These data indicate that the inducer UDP forms a complex with p38 during the period prior to commitment (latent period) (2,7). As soon as commitment was initiated, the UDP–p38 complexes no longer existed. However, a threshold level of the UDP–p38 complex is needed for the induction of MEL cell differentiation to occur, because a lower level of [^{3}H]UDP–p38 complex seen in

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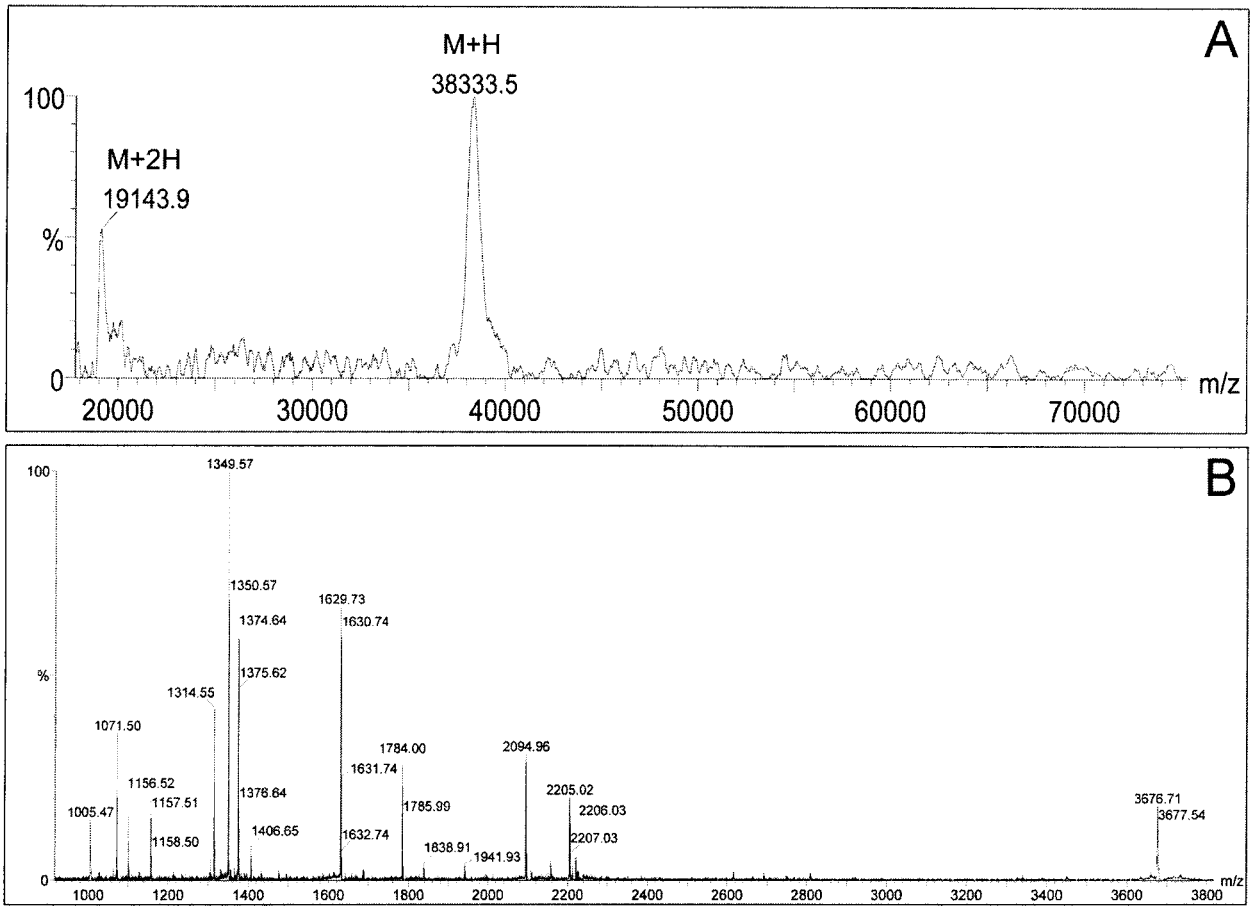

Figure 9. MALDI-TOF analysis of p38 protein(s) associated with UDP. One portion of UDP–protein complexes isolated from MEL postmitochondrial cell extracts by Sephadex G-50 gel filtration column chromatography, as shown in Figure 8, was further analyzed by MALDI-TOF as described in Materials and Methods. The profile obtained from undigested (A) along with that of in-gel-digested p38 protein(s) (B) is shown. Note that in (A) only one peak at a mass of 38,333 ± 30 Da is observed, thus indicating the molecular weight of p38 protein.

Table 3. Amino Acid Sequence of Peptide Fragments Obtained From Microsequencing of p38 Derived From MEL-745PC-4A Cells

No.	Peptide Amino Acid Sequence	Theoretical pI	Calculated MW
1	DVDGNGATLP	3.56	957.99
2	ATANNDA	3.80	675.65
3	NVHWAG	6.74	682.74
4	LIQVPSVATXVAIPFN	5.52	1687.74
5	AGTAGVVAYPTSGYPILGFT	5.57	1942.20
6	DANVDLSVQEL	3.49	1202.28
7	RACNKTSRL	10.86	1048.23
8	RCHCRFHWCCYVLCDECKV	7.80	2403.87

Peptides 1, 5, and 6 were identified at Molecular Biology Unit, University of Newcastle upon Tyne, UK (Dr. Joe Gray) and the rest of them (peptides 2, 3, 4, 7, and 8) at Protein Chemistry Laboratory, Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA (Dr. John Lambris).

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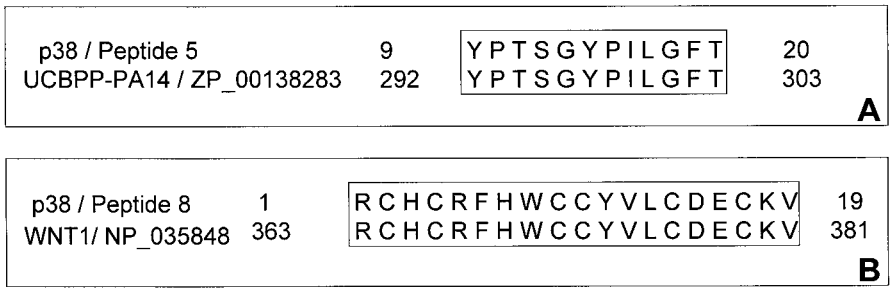

Figure 10. Structural homology of the identified peptide sequences derived from p38 with known proteins submitted to protein databanks. Protein sequence alignment search of the peptide fragments illustrated in Table 3 was performed by using the BLASTp program to identify structural homology with known proteins submitted in databanks, as described previously (31). Note that only peptides #5 and #8 show structural homology with the ABC-type transporter (Accession # ZP_00138283) and the wnt (Accession # NP_035848) proteins, respectively, as shown above.

cycloheximide-treated MEL cells was associated with blockade of erythroid differentiation (5,6).

To demonstrate whether the gradual disappearance of the [^{3}H]UDP–protein complex in committed MEL cells was attributed to a gradual reduction in the level of p38 or other factors, we assessed the level of UDP binding protein(s) at various time intervals during differentiation. Postmitochondrial fractions prepared at various times during differentiation were purified by molecular gel filtration and analyzed by Western blot, indicating that the steady-state level of UDP binding protein(s) in differentiating MEL cells remained practically unchanged (Fig. 11A), despite the decay of [^{3}H]UDP–p38 complexes seen in committed cells (Fig. 12).

DISCUSSION

Development and study of potent chemical inducers other than DMSO and polar solvents with defined chemical structure and physicochemical properties by several groups (8,12,24–27) has been a fruitful approach to explore the mechanisms of their transport and action inside cells. Analysis of quantitative structure–activity relationships (QSAR) demonstrated that inducers of one class may share common structural features responsible for the induction process. This led us to propose that different classes of inducers, despite their structural diversity and similarities [e.g., benzodiazepines, bisacetamides, and ureido-derivatives of pyridine (UDPs)], may have structural features recognized by cellular components in leukemia or other neoplastic cells in common with transporters or putative receptors for many drugs in tissues (9,12,24,25).

The development of UDPs in our laboratory (8) allowed us to further test this hypothesis. In this study, we prepared radiolabeled UDP and used it as a ligand to search for binding proteins in two highly UDP-responsive cell lines (MEL and RD/TE-671 cells). Uptake and specific binding studies demonstrated that [^{3}H]UDP selectively binds to p38 in intact MEL and/or RD/TE-671 cells but with different affinity. Subcellular distribution and fractionation studies revealed that [^{3}H]UDP forms

Table 4. Subcellular Distribution of [^{3}H]UDP in UDP-Inducible and -Resistant MEL Subclone Lines

	UDP-Inducible Subclones		UDP-Resistant Subclones	
Fraction	4S	14S	8R	25R
Cell homogenate	100.0	100.0	100.0	100.0
Nuclear	9.3	6.8	7.5	7.0
Mitochondrial and lysosomal	2.4	3.5	2.3	3.3
Postmitochondrial	88.3	89.6	90.1	89.2

Exponentially growing MEL cells were collected, washed, and resuspended in DMEM without FCS at a final concentration of 2×10^6 cells/ml for all clones tested. The cells were incubated with [^{3}H]UDP (7×10^4 cpm/ml) for 3 h at 37°C. Cells were then collected, washed, and fractionated as described under Materials and Methods. MEL-UDP-4/4S and MEL-UDP-4/14S are highly inducible while MEL-UDP-4/8R and MEL-UDP-4/26R are subclones of cells resistant to UDP-4. Each value represents the average of duplicate measurements.

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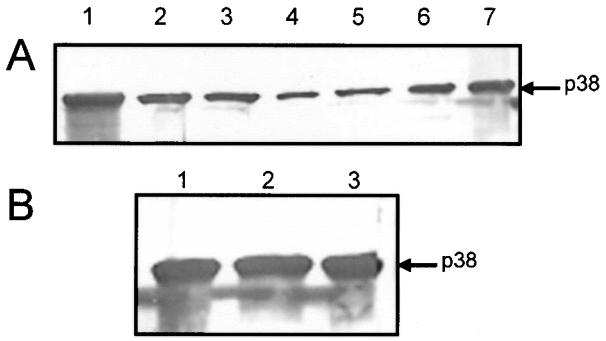

Figure 11. Time-dependent accumulation of p38 in [^{3}H]UDP-treated MEL cells as well as in UDP-inducible (sensitive) and UDP-resistant MEL cells by Western blot analysis. (A) Exponentially growing MEL cells (4×10^4 cells/ml, total volume 200 ml) were incubated with [^{3}H]UDP (8.7×10^4 cpm/ml). At various times, aliquots of cells were removed from the culture, washed the DMEM medium, and lysed. Postmitochondrial fractions containing a constant amount of protein enriched in [^{3}H]UDP–p38 complexes were isolated and analyzed by Sephadex G-100 and G-50. Fractions enriched in UDP–p38 complexes eluted at the last part of the column were lyophilized, analyzed by SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membranes, and assessed by Western blot analysis using rabbit anti-p38 serum. Lanes 1 to 7 indicate the level of p38 in postmitochondrial fractions isolated from MEL incubated with [^{3}H]UDP for 3, 6, 12, 24, 48, 72, and 96 h, respectively. (B) Highly UDP-inducible or resistant MEL cells were incubated with [^{3}H]UDP for 3 h. The cells were then harvested, lysed, and the postmitochondrial fractions containing a constant amount of protein were loaded and analyzed by Sephadex G-100 column chromatography as indicated in Figure 4A. Radioactive protein fractions eluted at the last part of the column were further analyzed by SDS-PAGE electrophoresis, transferred onto PVDF membranes, and incubated with anti-p38 rabbit antiserum. The p38 was found to be at similar levels in both UDP-inducible (MEL-UDP-4/4S) (lane 1) and UDP-resistant (MEL-UDP-4/8R, lane 2; MEL-UDP-4/25R lane 3) subclones of MEL cells.

complexes with protein(s) located not only in the soluble cytosolic fraction but in the nucleus as well. In fact, the accumulation of [^{3}H]UDP-enriched protein complexes reached maximum values within the first 24–36 h of incubation with the inducer (DMSO or UDP-4). However, these complexes decayed at later stages in both the cytosol and nucleus. Treatment of cells with known inhibitors of protein and mRNA synthesis, which block initiation of commitment (2–5), reduced and delayed, but did not completely abolish, the formation of [^{3}H]UDP–protein complexes in MEL cells. This finding suggests that UDP–p38 complexes are required during the early phase of the induction process but not at the later stages of differentiation, indicating that they may be related to initiation of commitment.

Moreover, we highly purified the novel p38 that selectively binds to UDP. Whether this protein serves as a

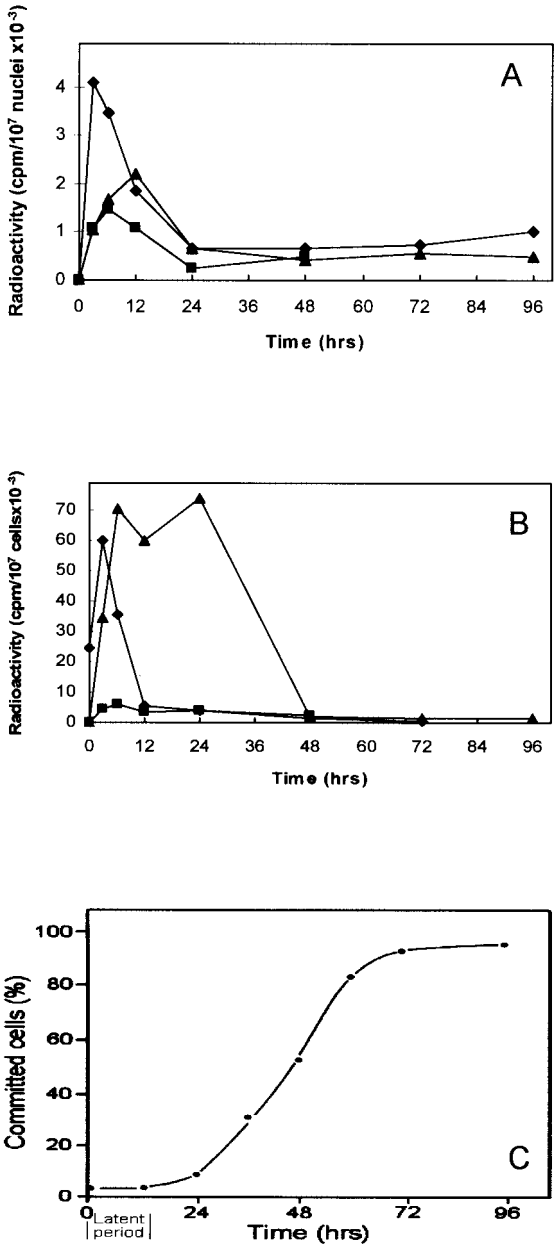

Figure 12. Time-dependent accumulation of the [^{3}H]UDP–p38 complex in the nucleus and cytosol of MEL cells exposed separately to inhibitors cordycepin and cycloheximide. MEL cells (5×10^4 cell/ml) were incubated with [^{3}H]UDP (9×10^5 cpm/ml) in the absence (◆) and/or the presence of either cycloheximide (2 μM) (■) or cordycepin (20 μM) (▲). At various times, as indicated, cells were collected, washed with DMEM, and lysed as described under Material and Methods. Cell homogenates were fractionated and [^{3}H]UDP-enriched protein complexes in the nuclear (A) and postmitochondrial fractions (B) were purified by gel filtration as described in the text. Radioactivity was measured in control cells (untreated) as well as in cycloheximide- and cordycepin-treated cultures. The number of committed cells determined, as previously published (32), is shown (C).

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potential transporter (carrier) protein or a receptor-like binding protein for inducers other than UDPs is not known. Kinetic analysis of the appearance of [^{3}H]UDP–p38 complexes in MEL cells by using other inducers revealed that p38 exists in both the cytosolic and nuclear fraction. If p38 acts like a putative drug receptor, it may translocate from the cytosol into the nucleus, like many other cytosolic receptors upon interaction with the inducer. Alternatively, p38 could translocate from the membrane into the nucleus if it acts as a potential transporter of the inducer. This possibility requires further investigation.

The fact that inducing agents such as HMBA, benzodiazepines, and sodium butyrate affected the binding of [^{3}H]UDP indicates that such agents somehow alter [^{3}H]UDP affinity to p38. Whether this results from direct competition at the binding site or by an indirect effect (of a noncompetitive mode) is not clear from the presented study, because this work was carried out with whole cells and not with isolated p38. Unfortunately, the amount of purified p38 actually isolated was insufficient to perform extensive binding studies. It may well be that the agents tested modulate the interaction of UDP with p38 indirectly. Alternatively, structure–activity relationship studies indicate that UDP and HMBA share similar structural groups (e.g., -NH-CO-CH_3) being recognized by the p38 binding protein. The fact that p38 was efficiently purified by elution with HMBA as well as UDP suggests that UDP class agents and bisacetamides may recognize the same binding protein but to a different extent. Moreover, the observation that UDP-resistant MEL cells also exhibited resistance to HMBA for induction directly suggests that HMBA and UDP at least may share similar mechanism of action.

Unfortunately, the precise biological role of p38 in MEL cell differentiation is still unknown. The observation that this protein exists in both UDP-responsive and UDP-resistant MEL cells to the same extent (see Western blot analysis data) makes its role in the initiation of commitment quite complicated. Apparently, this novel protein functions at an early phase of the differentiation process (precommitment period) when the inducer enters the cell and interacts specifically with p38 binding protein and it is not needed after the cells have passed the commitment event and begin to mature. Therefore, the interaction of p38 with the inducer UDP is necessary but not sufficient to promote terminal differentiation. The presence of p38 in UDP-resistant MEL cells supports this conclusion. It is apparent from earlier studies (4,5,28) that at least more than two events are involved in the differentiation process for commitment to occur. One is related to the synthesis of an intracellular protein factor (29), while the other refers to DNA damage. Currently we do not know to what extent such an intracellular factor is related to p38. Our observation that new protein and mRNA synthesis is needed for the assembly of the UDP–p38 complex is consistent with the role of an intracellular component. Indirect evidence suggesting that p38 may be the potential binding protein for HMBA and sodium butyrate is derived from the observation that MEL cells resistant to UDP are also resistant to HMBA, sodium butyrate, and other inducers. Unfortunately, the limited quantities of p38 that we isolated from MEL cells, mouse brain, and RD/TE-671 cells of human origin did not allow us to identify the entire primary structure of p38. We did succeed in isolating small quantities of p38 from MEL cells and determined its partial amino acid sequence, as shown in Table 3. Such information will allow us to clone the gene that encodes p38. Cloning experiments aimed to isolate the p38 gene are now in progress in our laboratory. However, the fact that S3 ribosomal protein and histone deacetylase serve as receptors for inducers of differentiation of the hydroxamic acid class (10,11) suggests that there is possibly more than one binding protein for chemical inducers of erythroid differentiation. Finally, our observation that potent inducers of MEL cell differentiation work via potential soluble binding protein receptors is consistent with other leukemia cells, such as HL-60, where commitment to granulocytic maturation is carried out through retinoic receptors (30).

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