A role for Poly (ADP-ribose) polymerase (PARP) in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression.

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Running Title: Role of PARP in the transcriptional activation of CXCL1.
Abstract

The melanoma growth stimulatory activity/growth regulated protein, CXCL1, is constitutively expressed at high levels during inflammation and progression of melanocytes into malignant melanoma. It has been previously shown that CXCL1 over-expression in melanoma cells is due to increased transcription as well as stability of the CXCL1 message. The transcription of CXCL1 is regulated through several cis acting elements including Sp1, NF-κB, HMGI(Y) and the IUR element (−94 to −78 nt) which lies immediately upstream to the NF-κB element. Earlier it has been shown that the IUR is necessary for basal and cytokine-induced transcription of the CXCL1 gene. UV-crosslinking and South-western blot analyses indicate that the IUR oligonucleotide probe selectively binds a 115 kDa protein. In this study, the IUR element has been further characterized. We show here that proximity of the IUR element to the adjacent NF-κB element is critical to its function as a positive regulatory element. Using binding site oligonucleotide affinity chromatography, we have selectively purified the 115 kDa IUR-F. MS/MS/MALDI spectroscopy and amino acid analysis as well as microcapillary reverse phase chromatography electrospray ionization tandem mass spectrometry identified this protein as the 114 kDa, Poly (ADP-ribose) polymerase (PARP 1). Furthermore, 3-aminobenzamide, an inhibitor of PARP-specific ADP-ribosylation, inhibits CXCL1 promoter activity and reduces levels of CXCL1 mRNA. The data point to the possibility that PARP may be a co-activator of CXCL1 transcription.
The abbreviations used are

CXCL1. Melanoma growth stimulatory activity/growth related protein; IUR, immediate upstream region; IL-1, interleukin-1; TNF, tumor necrosis factor, LPS, lipopolysaccharide; RPE, retinal pigment epithelial; NF-κB, nuclear factor κB; I-κB, inhibitor of NF-κB; IKK, I-κB kinase; PARP, poly ADP-ribose polymerase; 3-AB, 3-amino benzamide; EMSA, electrophoretic mobility shift assay.
Introduction

The melanoma growth stimulatory activity/growth regulated protein (CXCL1) chemokine plays an important role in wound healing, inflammation and tumorigenesis (1). The CXCL1 gene is not constitutively expressed in normal retinal pigment epithelial cells (RPE) but can be induced by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF α) and bacterial products such as lipopolysaccharide (LPS). In contrast, Hs294T malignant melanoma cells exhibit high, constitutive levels of CXCL1 mRNA. IL-1 treatment of Hs294T cells does not significantly increase the transcription of the gene, although it does appear to stabilize CXCL1 mRNA (2).

Transcription of the CXCL1 gene is regulated through a 306 bp minimal promoter comprising four cis-acting elements which include the TATA box (-25 to –30 nt), a NF-κB binding site (-67 to – 77 nt), an AT-rich HMGI(Y) binding element nested within the NF-κB site, an immediate upstream region, IUR, (-78 to – -93 nt) and a GC-rich SP1 binding site (-117 to –128 nt) (3). In RPE cells, IL-1 increases nuclear levels of NF-κB p65 (Rel A) and NF-κB p50 subunits (4). This is due to the IL-1 induced activation of the IκB kinases (IKK1/IKK2) and subsequent phosphorylation, ubiquitination and degradation of the IκB protein, I-κB. In Hs294T cells, constitutively high nuclear levels of the p50 and p65/RelA proteins can be correlated with constitutive activity of IKK1/ IKK2 and enhanced degradation of the I-κB protein. (5,6). The NF-κB element therefore represents a crucial, inducible component of the putative CXCL1 enhanceosome.

The IUR is an approximately 20 bp sequence which is located immediately upstream of the NF-κB site in the CXCL1 promoter. Previously we demonstrated that
this element is essential for basal as well as cytokine expression of the CXCL1 gene. In particular, point mutations within a putative TCGAT motif abolished basal and IL-1 induced transcription in reporter gene assays with RPE and Hs294T cells. Furthermore, in electrophoretic mobility shift assays (EMSA), these mutations blocked the ability of this element to compete with a constitutive, IUR-specific complex in RPE and Hs294T nuclear extracts (7). UV-crosslinking and Southwestern blot analyses revealed that at least one protein having a relative molecular size of 115 kDa bound the IUR element in a sequence-specific (8). In this study the 115 kDa IUR-specific protein has been purified by binding-site oligonucleotide affinity chromatography. Peptide sequence analysis identifies this protein as Poly (ADP-ribose) polymerase (PARP).

PARP is a 114-115 kDa nuclear DNA binding protein, which catalyses the transfer of long, branched ADP-ribose chains to either itself or different classes of target proteins involved in chromatin decondensation, DNA replication, DNA repair and gene expression (9,10) ADP-ribosylation by PARP affects such cellular processes as apoptosis, necrosis, cellular differentiation and malignant transformation (11). PARP appears to have dual functions in the regulation of transcription. PARP-mediated ADP-ribosylation of the transcription factors TATA-binding protein (TBP), Yin-Yang (YY1), Sp1 (12), NF-κB (13), and p53 (14) and alters the sequence-specific DNA binding of these protein and is thought to cause a reversible silencing of transcription. On the other hand, PARP has been shown to enhance activator-dependent transcription in vitro (15). More recently, PARP has been shown to activate muscle-specific gene expression by interacting with sequences in the MCAT element of the cardiac troponin T promoter and by ADP-ribosylating the MCAT-specific Transcription Enhancer Factor 1 (TEF-1) (16).
In addition, PARP increases the on-rate binding of nuclear factors to the PAX-6 gene enhancer (17). Furthermore, PARP has been shown to activate transcription of genes involved in cell proliferation in cooperation with the transcription factor, B-MYB (18) and potentiates the transcriptional activation by the human T-cell Leukemia virus type 1 Tax protein (19).

In this study, the IUR element of the CXCL1 promoter has been further characterized. Evidence presented here indicates that the IUR is a positive cis-acting element and its activity is dependent on its contiguity with the adjacent NF-κB element A 115 kDa IUR-specific activity has been purified by binding site oligonucleotide affinity chromatography and identified as PARP. A specific PARP inhibitor, 3-aminobenzamide, inhibits CXCL1 promoter activity and decreases the endogenous levels of CXCL1 mRNA in a dose-dependent manner. We propose that PARP is a potential co-activator of CXCL1 gene transcription.
Materials and Methods

Cell Lines and treatments

Hs294T melanoma cells are a continuous cell line established from a human melanoma metastatic to the lymph node. These cells were obtained from American Type Culture Collection (Rockville, MD). RPE cells are normal retinal pigment epithelial cells that were cultured by Dr. Glenn Jaffe from the North Carolina Organ Donor and Eye Bank within 24 h of death. RPE and Hs294T cells were cultured as previously described (7). In one series of experiments, Hs294T cells were incubated at 37 °C in the absence of serum for 48 h, during which period, cells received the indicated doses of 3-aminobenzamide (Sigma-Aldrich, St. Louis, MO) at 24 h intervals.

Sequence of oligonucleotides

The wildtype IUR probe, 2xIUR, used in electrophoretic mobility shift assays and Southwestern blot analyses had the upper strand sequence

5’ccatcgatcgactccgtatcgatatcgactccgtatcgatactccgtatcgatactccgtatcgatccgtatcgatccgatgc 3’

and contained two copies of the IUR sequence which are underscored. Sequences between the two IUR repeats and those flanking the probe were included to optimize binding. A mutant IUR oligonucleotide, 2xmIUR, which contained mutations in the TCGAT motif of the IUR element had the upper strand sequence, 5’ccaAGTaCctggaactccgatatcCgtggaactccggatgc 3’. Upper case characters indicate nucleotide replacements in the TCGAT motif while the underscored sequences define the two copies of IUR element.

The oligonucleotide used to multimerize the IUR sequence for DNA affinity chromatography had the upper strand sequence 5’-
The IUR sequence used in this study was 5'-ggatcgatctggaactccggatcgatctggaactcc-3' and the lower strand sequence 5'-cccgaggattccagatcgatccggaggattccagatcgat-3'. The underscored characters represent the two copies of the IUR sequence. The two strands when annealed form cohesive ends and were ligated with T4 Polynucleotide Kinase (Promega, Madison, WI) to generate the multimerized IUR DNA comprising up to 24 tandem repeats of the 2x IUR sequence. A similar strategy was employed to generate the multimerized mutant IUR (mIUR) DNA using the upper strand sequence 5'-gggaAGTaCctggaactccgggaAGTaCctggaactcc-3'. The upper case characters represent nucleotide replacements in the TCGAT motif, while the underscored characters outline the IUR element repeats.

**Reporter and Expression vectors**

The CXCL1 minimal promoter region (-306 to +45 nt) was inserted in the pGL2 Basic vector (Promega) either in the correct orientation (pWT.Luc) or in the opposite orientation (pREV.Luc) relative to the transcription start site. To separate the IUR element from the NF-κB site, pWT.Luc was modified to include an Nco I site between the two elements using the GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI). The mutagenic oligonucleotide employed for this purpose had the sequence: 5' tcgggatcgatctggaactccATgggaatttccctggcc 3'. (The characters in upper case represent the two nucleotide insertion that was necessary to create the Nco I site which is underscored). The modified construct was termed pIN:2 to represent a two nucleotide insertion between the the IUR and NF-κB sites. Similarly, mutant promoters containing a 6 bp (pIN:6) and 12 bp insertion (pIN:12) were generated using the mutagenic oligonucleotides 5’ gggagatcctggaactccGGATCCgggaatttccctggcc 3’ and 5’
tcggatcgcagtgagcgcaacgcaattac 3’ (Characters in upper case represent the BamHI site and the BamHI/XbaI sites introduced to create the 6 and 12 bp separations). To generate the promoter with a 25 bp insertion (pIN: 25), a 25 bp oligonucleotide having the sequence 5’ catggcagtgagcgcaacgcaattac 3’ and flanked by Nco I sites, was inserted in the Nco I site of pIN:2. A clone pIN:50, in which the 25 bp insert had dimerized, was also selected. To generate the pGL2.mIUR construct, the TCGAT motif in the wild type promoter was mutated to AGTAC by using a mutagenic oligonucleotide 5’ ttccttccggatcgcagtgagcgcaacgcaattac 3’. All constructs were confirmed by restriction analysis and automated plasmid sequencing.

**Reagents:**

Anti-PARP antibodies were from Santacruz Biotechnology, Santacruz, CA. The PARP enzyme was purchased from R & D Systems Minneapolis, MN.

**Electrophoretic Mobility Shift Assays.**

Nuclear extracts were prepared as described previously (7). All extracts contained a 1 x concentration of Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The 2xIUR or the 2xmIUR probes were radio-labeled by extension of an annealed primer, 5’ gcactccgagag 3’ with the Klenow fragment of *E. coli* DNA polymerase, dNTPs and α<sup>32</sup>P dCTP. A typical binding reaction involved a 15 min pre-incubation with 10 μg of nuclear extract, 2 μg of non-specific competitor poly dIdC, 200 ng single-stranded oligonucleotide, 20 mM Hapes-NaOH (pH 7.6), 100 mM NaCl, 1 mM DTT, 2% glycerol, followed by a 20 min incubation with 50,000 cpm (40 femtomoles) of radio-
labeled probe. In oligonucleotide competitions, 1000-fold molar excess of cold, double-stranded oligonucleotide was added to the pre-incubation mix. Complexes were resolved by electrophoresis for 2 h at 170 volts on a 6% native, polyacrylamide gel, which was later dried and processed for autoradiography. EMSA with affinity-purified fractions contained 50 ng of poly dIdC and 100 ng of cold, double-stranded 2mR oligonucleotide to ensure specificity of binding in the fractions. In cases where purified PARP was used, the reaction mixture contained 50 ng of poly dIdC and about 5-10 femtomoles of pure enzyme (cat # 4667-50-01) obtained from R&D Systems, Minneapolis, MN.

**Southwestern and Western Blot Analysis**

Nuclear extracts (25 µg) were heated at 90 °C for 3 minutes in 50 mM Tris.Cl (pH:6.8), 100 mM DTT, 2% SDS and 10% glycerol, resolved on 4% stacking/8% resolving SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Biorad Laboratories). The membranes were rocked for 30 minutes in PBS, blocked for 4 h at RT with buffer A (20 mM Hepes-NaOH, 50 mM NaCl, 12.5 mg/ml skim milk powder, 2.5 mg/ml bovine serum albumin, 100 µg/ml native salmon sperm DNA) and incubated overnight at RT in up to 2 ml of Buffer A + 10^7 cpm (8-10 pmoles) of radio-labeled 2xIUR or 2xmIUR probes. Membranes were subjected to three washes of 15 minutes each at RT in a buffer containing 20 mM Hepes-NaOH, 50 mM NaCl, 1g/L skim milk powder 0.025% Nonidet P40, prior to drying and autoradiography.

For western analysis, membranes were probed with 800 µg/10 ml of anti-PARP rabbit polyclonal antibody (cat #: sc-7150) from SantaCruz Antibodies, Santa Cruz, CA using procedures recommended by the antibody manufacturer and the signal was
visualized by enhanced chemiluminescence (ECL) assay (Amersham Pharmacia Biotech) according to the manufacturer’s recommendations.

Northern Analysis:
Total RNA from Hs294T cells treated over a 48 h period with various doses of 3-aminobenzamide was isolated using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX) according to the manufacturer’s protocol. 20 µg/lane of purified RNA was used in northern analysis which was performed exactly as described previously (2). After probing for levels of CXCL1 mRNA, blots were stripped by boiling for 10 minutes in water and re-probed with radiolabeled, cyclophilin cDNA. Bands corresponding to the CXCL1 and cyclophilin were analysed by phosphorimager using ImageQuant software (Molecular Dynamics). Values for CXCL1 mRNA were normalized against cyclophilin mRNA. The experiment was repeated three times in triplicate. Mean normalized values obtained from samples receiving the highest dose of 3-AB were compared with values from samples that received DMSO alone and were found to be statistically different as determined by Student’s T-test (paired).

Transient Transfection and Reporter Activity assay:
Hs294T were plated in 60-mm dishes at a density of 2 X 10^5. The following day, cells at nearly 70% confluency were transfected using the Lipofectamine/Plus method (Life Technologies, Rockville, MD) with 1 µg of the appropriate reporter construct containing either the wild type CXCL1 promoter (WT) or promoters containing insertions between the IUR and NF-κB elements or nucleotide replacements within the TCGAT motif of
the IUR element (mIUR.Luc). In addition, all samples received 1µg of pRSV-β-gal. Cells were harvested 48 h after transfection and luciferase activity was measured using the luciferase assay system (Promega) and Monolight 2010 Luminometer (Analytical Luminiscence Laboratory, San Diego, CA)

In transfection experiments where 3-Aminobenzamide was used, cells were first transfected with 1 µg of pRSV-β-gal reporter as well as reporter constructs driven by either the wild type (CXCL1.Luc) or the mutant CXCL1 (mIUR.Luc) promoters. Six hours after transfection, the medium was replaced by medium containing either DMSO or 3 -Aminobenzamide within a dose range of 1-5 M. A second change of DMSO or 3-AB containing medium was given at 24 h after transfection. Cells were harvested 48 h after transfection and processed as described above.

All values were normalized to β-galactosidase expression to correct for transfection efficiency. Each experiment was repeated three separate times in triplicate. In promoter mutagenesis experiments, mean normalized values from samples showing the highest inhibition of promoter activity were compared to those obtained from samples transfected with the wild type promoter and were found to be significantly different according to the Student’s paired T-test. Similarly, mean normalized values of the samples treated with the highest dose of 3-AB were compared with values obtained of the sample containing the wild type promoter treated with DMSO alone and were found to be significantly different according to Student’s paired T-test (p < 0.01). Statistical measurements were performed using Microsoft Excel software.
**Purification of the 115 kDa IUR-specific protein:**

Binding site oligonucleotide affinity chromatography was performed using a modified protocol of Kadonaga *et al.* (20). Briefly, multimers of the IUR were cross-linked to cyanogen bromide-activated agarose to generate the IUR-agarose column. Similarly, a control column containing mIUR multimers (mIUR–agarose) was made. Cells equivalent to 60 L HeLa suspension culture was obtained from National Cell Culture Center, Minneapolis, MN. Approximately 2.5 g of nuclear extract was prepared from these cells. First, non-specific DNA binding proteins were competed with poly dI:dC at a protein : DNA ratio of 30 : 1, incubated at room temperature for 6 h and eliminated by high speed centrifugation. Unbound proteins in the supernatant were subjected to binding site oligonucleotide chromatography which involved two passages through a 3ml settled bed-volume of the mutant IUR-agarose column, an overnight incubation with a 3 ml bed-volume of IUR-agarose resin, followed by two passes through the same column. After 5 washes of 3ml each in a 50 mM NaCl containing buffer, fractions were eluted from the IUR-agarose column by a step-gradient of buffers containing 0.2 to 1.0 M NaCl. In EMSA and Southwestern assays, the 400 mM NaCl fraction consistently tested positive for an activity with a relative molecular size of 115 kDa that specifically bound the 2xIUR probe. SDS-PAGE and silver staining revealed selective enrichment of the 115 kDa protein.

In order to further purify the 115 kDa protein, the 400 mM NaCl fraction was pooled, dialyzed against water, lyophilized and reconstituted in the 1x SDS-PAGE loading dye. The sample was then subjected to continuous-elution SDS-PAGE on a 7% polyacrylamide gel using a Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA).
Electro-elution was performed at a flow-rate of 0.1 ml/min. Aliquots of electro-eluted fractions were screened by electrophoresis on 4% stacking/10% resolving SDS-PAGE gels and polypeptides were detected by silver-staining. Fractions that contained the 115 kDa protein were pooled and lyophilized. Reconstituted sample was re-electrophoresed through a 4% stacking/8% resolving SDS-PAGE gel and the band containing the 115 kDa protein was excised and sent for sequencing. Two different, nearly homogenous, preparations of the 115 kDa protein were sequenced separately in two different laboratories; by microcapillary reverse phase chromatography electrospray ionization tandem mass spectrometry using an ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) in the laboratory of Dr. R. Aebersold at the University of Washington, Seattle, WA and by MS/MS MALDI at the sequencing facility at the W.M. Keck Foundation, Yale University, New Haven, CT.
Results

The activity of the CXCL1 promoter depends on the position and orientation of the IUR element:

The IUR element in the CXCL1 promoter plays an important role in the basal and cytokine induced expression of the CXCL1 gene. The IUR contains a TCGAT motif designated here as T1C2G3A4T5. Previously, we showed that a T5-C5 point mutation inhibits basal and IL-1-induced CXCL1 promoter activity. In order to further characterize the IUR element the effect of multiple replacements in the TCGAT motif on CXCL1 promoter activity was examined. In addition we investigated whether dislocating the IUR relative to the NF-κB site has any effect on transcriptional activity of the CXCL1 promoter. We created reporter gene constructs which either retained the 306 bp CXCL1 promoter in the wild type configuration (WT) or had a 2, 6, 12, or 25 bp insertion between the IUR and NF-κB elements (IN:2, IN:6, IN:12 and IN:25, respectively). Two additional promoter mutants were also constructed, one which reversed the entire 350 bp CXCL1 promoter relative to the luciferase reporter (Rev) and a second, which had a T1C2G3A4T5 to A1G2T3A4C5 conversion in the IUR element (mIUR). These constructs were then transfected into Hs294T malignant melanoma cells and luciferase activity measured 48 hours later. Results in Figure 1A indicate that transcription from the CXCL1 promoter is inhibited by nearly 90% when the entire promoter is oriented in a direction opposite to the transcription start site (Rev). Furthermore, a 75% inhibition in promoter activity is observed when the T1C2G3A4T5 motif in the IUR element is replaced by A1G2T3A4C5 (mIUR). A similar inhibition of promoter activity is seen when the IUR and NF-κB elements are separated by insertions (Figure 1B). Although promoter activity
appears to be unaffected when the IUR and NF-κB elements are separated by a 2 bp insert. However, inserts that dislocate the IUR element from the NF-κB site, by 6, 12, and 25 bp strongly inhibit promoter activity in a distance dependent manner. This inhibition, however, is independent of helical phase of the two elements, because introduction of a half (IN:6) or two and a half helical turn (IN:25) has the same effect as a full helical turn (IN:12), ruling out the possibility that the insert-mediated inhibition of CXCL1 promoter activity is due to a change in the helical phase between the two elements. Together, the data suggest that the IUR is a positive cis acting element and that the transcriptional activity of the CXCL1 promoter is strictly dependent on the distance of the IUR element relative to the NF-κB site.

Purification and identification of 115 kDa IUR binding protein:

We have previously demonstrated that the IUR element binds a 115 kDa protein in Southwestern blot assays (8). In order to identify the 115 kDa protein, which binds the IUR, we purified the IUR specific factor by binding site oligonucleotide affinity chromatography. HeLa nuclear extract proved to be a convenient and abundant source for both proteins. We obtained frozen pellets of HeLa cells from 63 L suspension cultures from the National Tissue Culture Center. The yield of nuclear extract from a 63 L suspension culture was about 1.5 – 2.0 g. The IUR-specific activities were reproducibly purified to near homogeneity in three separate experiments. Making use of the following protocol, nuclear extract from HeLa cells was first challenged with non-specific competitor DNA and chromatographed through the mutant-IUR-agarose column which contained multimers of the double-stranded mIUR oligonucleotide. The unbound fraction
was then chromatographed through an IUR-agarose column, which contained multimers of double-stranded IUR oligonucleotide. Bound fractions from both columns were washed and eluted by a step gradient of NaCl ranging from 50 mM to 1000 mM. Proteins specific to the IUR element emerged in the unbound (flow-through) fraction of the mutant-IUR column and eluates from the bound fraction of this column consistently tested negative for IUR-specific activity in EMSA (data not shown). Eluates from the IUR-agarose column were assayed for IUR-specific activity by EMSA and Southwestern blot analysis. Figure 2 is a representative of three separate experiments. The results from an EMSA (Figure 2A) indicate that an IUR-specific activity reproducibly eluted in the 400 mM fraction of the IUR-agarose column. This correlated well with results from Southwestern blot analysis (Figure 2B) where a 115 kDa protein in the 400 mM fraction bound the 2xIUR element in a sequence specific manner. Proteins from bound and unbound fractions from the IUR-agarose column were resolved by SDS-PAGE (Figure 2C) and detected by silver-staining. SDS-PAGE analysis consistently showed that the 400 mM fraction of the IUR-agarose column essentially contains 5 bands with relative molecular sizes of 180 kDa, 115 kDa, 85 kDa, 42 and 29 kDa. Of these, the 115 kDa band was considerably enriched in this fraction.

In order to further purify the 115 kDa protein, the 400 mM IUR-agarose fractions were pooled, dialyzed and lyophilized to dryness. The reconstituted 400 mM fraction was then electrophoresed through a preparative SDS-PAGE miniprep cell and 265 fractions of 1.0 ml each were collected. Electro-eluted fractions were analyzed by SDS-PAGE/silver-staining. Figure 2D shows results of such an experiment. The 115 kDa protein electro-elutes reproducibly in fractions 135-160 of the minprep cell.
Two separate preparations of the 115 kDa protein, purified to near homogeneity, were independently sequenced at two different institutions: by MS/MS/MALDI mass spectroscopy at W.M. Keck Foundation, Yale University, New Haven, CT and by microcapillary reverse phase chromatography electrospray ionization tandem mass spectrometry using an ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) at University of Washington, Seattle, WA by S. Gygi and R. Aebersold). Each preparation had about 10 - 20 picomoles of the 115 kDa protein. Both sources identified the 115 kDa as the human poly ADP ribose polymerase (PARP) (EC Number: 2.4.2.30).

**The DNA-affinity purified 115 kDa protein is PARP:**

We tested the 400 mM fraction from the IUR agarose column by Western blot analysis (Figure 3A) to determine whether or not the 115 kDa is the poly ADP ribose polymerase (PARP). Anti-PARP antibodies successfully detected a 115 kDa protein in the fraction thus confirming that the affinity purified 115 kDa protein is PARP.

**Recombinant PARP binds the CXCL1 IUR element in a sequence-specific manner.**

Results in Figure 2 demonstrate that the 115 kDa protein (PARP) emerged in the unbound fraction of the mutant-IUR agarose column and eluted in the 400 mM fraction bound to IUR agarose column. This strongly suggested that the PARP protein could discriminate between the mutant and wild-type IUR sequences. To verify this, we used purified, recombinant PARP in EMSA and compared PARP binding to 2xIUR and 2xmIUR probes (Figure 3B). The use of poly dIdC in the reaction mixture was found to be necessary since the recombinant protein was not a homogenous preparation.
Specificity to IUR was observed at relatively high concentrations of the non-specific competitor DNA. Three shifted complexes could be detected with the 2xIUR probe. Two complexes, which were specific to 2xIUR, did not bind the 2xmIUR probe. This result indicates that PARP can form a sequence-specific complex with the IUR element of CXCL1. We would like to point out that the complex generated by the purified recombinant protein appears to differ in mobility and intensity to the one obtained from crude nuclear extracts. Moreover, there are two IUR specific complexes generated with the recombinant protein as opposed to a single complex observed with crude nuclear extracts. The reasons for these discrepancies are unclear at this time. However, it is likely that the complex observed in crude nuclear extract could be a composite of more than one protein in addition to PARP.

3-aminobenzamide (3-AB) decreases levels of CXCL1 mRNA in Hs294T cells.

PARP catalyses the transfer of multiple ADP-ribose units to target nuclear proteins. This ADP-ribose transferase activity is believed to inhibit DNA binding as well as trans-activation functions of the target proteins. 3-Aminobenzamide (3AB), a specific PARP inhibitor, blocks ADP-ribose transferase functions of PARP. We asked the question whether inhibition of PARP catalytic activity by 3AB might have any effect on CXCL1 expression in Hs294T cells.

Hs294T cells were either left untreated or treated with 3AB in a dose range of 1.0 mM to 5.0 mM. Forty-eight hours later, cells were harvested and RNA from these samples was isolated. CXCL1 mRNA levels were measured by Northern analysis (Figure 4A). Values, normalized against levels of an internal control cyclophilin B, are graphically represented in Figure 4B. Results indicate that 3-AB treatment inhibited
CXCL1 mRNA levels by nearly 60% over untreated or DMSO-treated controls. The data suggest that PARP-mediated ADP-ribosylation is essential for CXCL1 gene expression. The ADP-ribose transferase activity of PARP, has been shown to be involved in negatively regulating gene expression by abrogating the DNA binding of such factors as NF-κB, YY1, C/EBPβ and Sp1. In addition ADP-ribosylation of these factors does not appear to require DNA binding of PARP. It is therefore possible that the effect of 3-AB on CXCL1 mRNA levels may be indirect and could involve other trans activators of the CXCL1 promoter such as NF-κB and Sp1.

To test the possibility that the effect of 3-AB on CXCL1 mRNA levels is at the level of the CXCL1 promoter and involved the IUR element, luciferase reporter constructs driven by either the wild type CXCL1 promoter or a promoter containing replacements in the IUR element of this promoter were transiently transfected in Hs294T cells and cells were treated with 3-AB in a dose range of 1-5 mM. Cells were harvested 48 h after transfection and luciferase activity was measured. Figure 5 shows a representative of four independent experiments performed in duplicate. The data indicate that 3-AB inhibited CXCL1 promoter activity in a dose dependent manner. Maximum inhibition (50%) was observed at 5 mM 3-AB. There was no effect on the mutant IUR promoter (mIUR.Luc) although the mIUR promoter contained intact binding sites for NF-κB, HMG(Y) and Sp1. The results in Figure 2 show that the 115 kDa protein identified to be PARP bound the wild type IUR but not the mutant mIUR sequence and that recombinant PARP specifically bound the IUR but not the mIUR sequence (Figure 4). Results shown in Figure 5 further demonstrate that the PARP specific inhibitor, 3-AB specifically inhibits the wild type CXCL1, but not the mIUR promoter, indicating that
PARP mediated ADP ribosylation is involved in the trans-activation of the CXCL1 promoter. This rules out the possibility that 3-AB mediated inhibition of CXCL1 mRNA levels is due to abrogation of other trans-acting factors of the CXCL1 promoter.

**Binding of PARP to the IUR element does not require ADP-ribosylation:**

Since 3-AB treatment had a dramatic effect on endogenous CXCL1 mRNA levels as well as on the CXCL1 promoter activity in reporter assays, we considered the possibility that binding of PARP to the IUR element might involve the ADP-ribosylation activity of the factor. Hs294T cells were treated with 3-AB within a dose range of 1-5 mM. Cells were harvested after 48 h of 3-AB treatment and nuclear extracts were prepared. Figure 6 shows a representative EMSA experiment in which nuclear extracts from DMSO treated or 3-AB treated cells were probed with radiolabeled 2xIUR oligonucleotide. The results indicate that there was no difference in the binding of the IUR-specific protein, PARP, with 3-AB treatment. The data suggest that ADP-ribosylation may not be involved in binding of PARP to the IUR element.

The data presented in this study provide evidence that the IUR element is a positive cis-acting element in the CXCL1 promoter whose function depends on its contiguity with the adjacent NF-kB element. The IUR binds a 115 kDa factor which has been purified and identified as PARP. Although binding of PARP does not require the ADP ribose transferase function of PARP, this activity appears to be important for its ability to transactivate the CXCL1 promoter and induce CXCL1 mRNA levels. A model for the regulation of CXCL1 transcription is proposed.
Discussion

The CXCL1 gene is expressed at a high constitutive level in several disorders including acute and chronic inflammation and malignant melanoma. Our earlier findings indicate that the CXCL1 gene is under the control of a 306 bp minimal promoter containing four cis acting elements, including a 20 bp region called the IUR, located immediately upstream to the NF-κB binding sequence (7). In an effort to understand the mechanism by which the CXCL1 gene is regulated, we have further characterized the IUR element in the CXCL1 promoter and examined its role in CXCL1 gene regulation.

To examine whether the IUR has a positive or negative role in CXCL1 regulation, we first made mutations in the CXCL1 promoter which either altered the TCGAT motif or separated it from the adjacent NF-κB element. When tested in luciferase reporter assays, these mutations effected an almost 50% inhibition in CXCL1 promoter activity. The data indicate that the IUR is a positive regulatory sequence in the CXCL1 promoter and that the contiguity of the IUR and NF-κB element is critical for optimal promoter activity.

In electrophoretic mobility shift assays, we previously identified constitutive binding to the IUR element and detected a 115 kDa protein which bound to the IUR element in southwestern blot analyses. Mutations which altered the TCGAT motif eliminated binding in both assays suggesting that these interactions were specific to the TCGAT motif of the IUR element (8). Taken together with our current observations from reporter assays, the evidence points to the possibility that p115 is a positive transcriptional regulator of the CXCL1 gene.
In this study we have purified the 115 kDa protein binding to the IUR element using a two-step procedure involving binding site oligonucleotide affinity chromatography and electrophoretic separation through a Biorad mini-prep cell. Pre-incubation of the nuclear extract through a mutant-IUR-sepharose matrix eliminated non-specific proteins and ensured that bound fractions eluting from the wild-type IUR-sepharose matrix represented proteins that selectively bound the wild-type but not the mutant IUR oligonucleotide.

Peptide sequence analysis has established the identity of the 115 kDa IUR-specific protein as the Poly (ADP-ribose) polymerase (PARP). We show here by western blot analysis that the 115 kDa PARP is enriched in the IUR affinity purified fraction, that peptide sequence of the purified 115 kDa matches that of PARP, that a commercial preparation of the PARP enzyme can discriminate between the wild-type and mutant IUR sequences and treatment of cells with 3-Amino benzamide (3-AB), a specific PARP inhibitor, significantly reduces CXCL1 mRNA levels in Hs294T cells and inhibits CXCL1 promoter activity in transient transfection assays. These data indicate that PARP transactivates the CXCL1 promoter and its ADP ribose transferase activity is essential for this process.

The ability of PARP to activate or suppress specific gene expression appears to be dependent on at least two factors: (1) the ADP-ribose transferase activity of PARP (12). (2) DNA binding of PARP (13). Reversible repression of gene expression by PARP can be correlated with its catalytic activity and may not involve DNA binding of the enzyme. PARP-catalyzed ADP-ribosylation of the TATA binding protein (TBP), Yin-Yang-1 (YY1), p53, NF-kB, Sp1, CREB (12), Oct-1 (21) and retinoid X receptors (22) has been
shown to occur before DNA binding and prevented the formation of transcriptionally active complexes. In contrast, DNA-bound PARP appears to be associated with transactivation. PARP has been identified as the active component of the transcription factor TFIIC (19). In addition, PARP has been demonstrated to enhance activator-dependent transcription during which, DNA binding, but not the ADP-ribose transferase activity, of the unmodified PARP seemed to be necessary for transcriptional activation (15). More recently, PARP has been shown to trans-activate the cardiac Troponin T promoter by binding to a 5′ TGTTG 3′ sequence in the MCAT cis element and cooperating with the muscle-specific TEF-1 transcription factor (16). In the latter case, both DNA binding and ADP-ribose transferase functions appear to be essential for coactivation of the troponin gene promoter.

We examined the possibility that PARP-mediated ADP-ribosylation plays a role in the regulation of CXCL1 gene expression. We addressed this question by examining the effects of 3-Aminobenzamide, a specific inhibitor of PARP-mediated ADP-ribosylation, on (1) CXCL1 mRNA levels, (2) CXCL1 promoter activity and (3) specific binding to the IUR oligonucleotide probe. We show here that inhibition by 3AB reduces CXCL1 mRNA levels and blocks CXCL1 promoter activity up to 50% over untreated controls in a dose dependent manner. The level of inhibition seen with 3AB appears to be similar to that observed when the TCGAT motif in the CXCL1 promoter is altered. In addition, the activity of the mutant-IUR promoter appears to be insensitive to 3-AB treatment indicating that 3AB effects target events associated with the TCGAT motif. Inhibition of PARP-mediated ADP-ribosylation, however had no effect on the complex associated with the IUR probe in EMSA indicating that although ADP-ribosylation is
required for trans-activation of the CXCL1 promoter, it may not be necessary for binding to the IUR element in the promoter.

Our data indicate the following: (1) contiguity with NF-κB element appears to be necessary for trans-activation through the IUR element; (2) the 115 kDa PARP binds to the IUR element in the CXCL1 promoter; (3) PARP is associated with trans-activation of the CXCL-1 promoter; (4) the ADP ribose transferase activity is necessary for trans activation; (5) this activity is not necessary for binding of PARP to the IUR element.

Based on these findings we have proposed a model for the regulation of the CXCL1 promoter.

We have uncovered a novel role for the PARP in the regulation of the CXCL1 chemokine gene. The CXCL1 minimal promoter, under the control of five cis-acting elements including the Sp1, IUR, NF-κB, HMG1(Y) and TATA box binding sites, is a template for a multi-protein complex, the CXCL1 enhanceosome. The TCGAT motif of the IUR element and the 115 kDa PARP, which specifically binds the IUR, appear to possess distinct positive roles in CXCL1 regulation. PARP appears to be associated with the trans-activation of the gene. One mechanism of PARP-mediated trans-activation of the CXCL1 promoter may involve the NF-κB site because dislocation of the IUR element from this site inhibits trans-activation of the promoter, possibly by disrupting interactions between PARP and Rel factors. We have no evidence of protein-protein interactions between PARP and the NF-κB factors at this time. However, a recent report has demonstrated that NF-κB dependent transcription activation is severely compromised in mice that are deficient in PARP (13). PARP deficient mice appear to have extensive skin disorder and extreme sensitivity to X-ray irradiation, a phenotype closely resembling the
one observed in mice deficient for IKK2, the kinase involved in the NF-κB activation pathway. Based on the findings by Hassa et al., PARP deficiency may contribute to a failure in NF-κB signaling (13).

In a separate study we have identified a second protein binding to the IUR element which is also specific to the TCGAT motif. Preliminary evidence indicates that the 170 kDa IUR-specific factor could be a repressor of CXCL1 transcription. A second mechanism of PARP mediated trans-activation could involve displacement or inactivation by ADP-ribosylation of the putative 170 kDa trans-repressor of the CXCL1 promoter binding to a site at or near the IUR element.

Tumor models involving PARP are yet to be developed. In this context, the role of PARP as an anti-apoptotic factor may be important. During the onset of apoptosis, PARP is cleaved into two fragments, 85 and 29 kDa, by caspase-3, a crucial component of the apoptotic cascade and this is thought to contribute to cell death (24). However, direct evidence for PARP as an agent of cell proliferation is forthcoming. In this regard, the DNA binding and transcriptional regulatory properties of PARP may be more revealing. PARP has been previously shown to modulate DNA binding and specificity of the anti-tumor factor, p53 (14). More of such evidence will be crucial towards our understanding of its role in cell proliferation and tumorigenesis. Our findings that PARP binds the CXCL1 promoter and is likely involved in its trans-activation, therefore, present an important step in that direction.

Future investigations in this area could examine interactions between PARP and other constituents of the CXCL1 enhanceosome, compare levels or post-translational modification status of these factors in normal and melanoma cells and trace the signaling
pathways that modulate their activity. These investigations could elucidate the role for PARP in disorders such as chronic inflammation and melanoma.

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List of References


Figure Legends.

Figure 1. The IUR element is a positive cis element which requires the TCGAT motif as well as contiguity with the adjacent NF-κB element: (A) $5 \times 10^5$ Hs294T cells were transfected with RSV-β-Gal vector and one of the following luciferase reporter constructs (1) a reporter construct lacking the CXCL1 promoter (pGL2); (2) a reporter construct driven by the wild-type CXCL1 promoter in the correct orientation relative to the transcription start site (WT); (3) a reporter construct with the CXCL1 promoter in the opposite direction (REV); (4) a mutant CXCL1 promoter in which the TCGAT motif was altered to AGTAC (mIUR). (B) $5 \times 10^5$ Hs294T cells were transfected with RSV-β-Gal vector as well as luciferase reporter constructs driven by the wild type CXCL1 promoter or mutant promoter which had an insertion of 2 bp (IN:2), 6 bp (IN:6), 12 bp (IN:12) or 25 bp (IN:25) between the IUR and the NF-κB elements. 48 h after transfection, cells were harvested and luciferase activity was measured and normalized to β-galactosidase activity. Shown here is a representative of three independent experiments performed each time in triplicate. Mean normalized values obtained from the sample showing the highest inhibition in promoter activity were compared to those from samples transfected with the wild type promoter and were found to be significantly different according to Student’s T-test (paired). The asterisk (*) indicates $p < 0.01$.

Figure 2. Binding site oligonucleotide affinity purification of the 115 kDa IUR-specific protein. In Electrophoretic mobility shift assays (A), crude HeLa nuclear extract (2 μg) (lane 1) or 200 ng bound fractions eluting at 0.2 M NaCl (lanes 2-5), 0.4 M NaCl
(lanes 7-10), 0.6 M NaCl (lanes 11-14) or 1 M NaCl (lanes 16-18) from the IUR-agarose column, were tested for binding to the 2R probe in EMSA. All samples contained a 250-fold molar excess of cold oligonucleotide corresponding to the 2mR probe. (B) Southwestern blot analysis: Polypeptides in crude HeLa nuclear extract (25 µg) (lanes 1 and 3) or the 0.4 M NaCl fraction (100 ng) eluted from the IUR-agarose column were separated on 8% SDS-PAGE gels, trans-blotted on nitrocellulose membranes and probed with radio-labeled oligonucleotides corresponding to either the 2R (lanes 1 and 2) or the 2mR (lanes 3 and 4) probes. The relative molecular size is indicated on the left. (C) Silver-stained SDS-PAGE profile of the 0.4 M NaCl fraction. analysis: Polypeptides in crude HeLa nuclear extract (500 ng) or the 0.4 M NaCl fraction (100 ng) eluted from the IUR-agarose column was separated on 8% SDS-PAGE gels and the protein was visualized by silver-staining. (D) SDS-PAGE electro-elution: The 0.4 M IUR-agarose eluate was then fractionated by electro-elution through a 7% SDS-PAGE using the Model 491 Prep Cell (Biorad). Fractions were collected and every 8th fraction was electrophoresed on an 8% SDS-PAGE gel and proteins were stained by silver-staining.

**Figure 3.** PARP can bind the IUR element: (A) Western Blot analysis: crude HeLa nuclear extract (25 µg), unbound/flow-through fraction from the IUR-agarose column (25 µg) and 100 ng of 0.2 M (lanes 3 and 4) or 0.4 M (lanes 5 and 6) IUR-agarose fractions were separated on 8% SDS-PAGE gels and probed with anti-PARP antibody. Arrow (→) indicates the relative mobility of the PARP protein. (B) EMSA: Partially purified, commercially available PARP was tested for binding to the 2xIUR (lanes 1-3) or the 2xmIUR (lanes 4-6) probes. Non-specific complexes were
competed with poly dI:dC in the range of 0.2-1.0 µg. The arrow (→) indicates the specific complex formed by PARP with the 2xIUR probe, while NS represents non-specific complexes.

**Figure 4.** 3AB inhibits MGSA/GRO expression (A) Northern analysis: Hs294T cells were treated for 48 h with indicated concentrations of 3-aminobenzamide (3-AB). RNA from samples was resolved on a 1.4% formaldehyde-agarose gel, trans-blotted to nitrocellulose membrane and probed with an MGSA/GRO cDNA probe (A, top panel). Blots were stripped and re-probed with a cDNA probe for cyclophilin B transcripts (A, bottom panel). Blots were densitometrically scanned using a phosphorimager (Molecular Dynamics). Values for MGSA/GRO mRNA were normalized against those for cyclophilin mRNA. (B) A graphical representative of 6 independent experiments, each performed in triplicate is shown. The results in all 6 experiments was qualitatively identical. Error bars represent SD values. The mean normalized value obtained from samples receiving the highest dose of 3AB was compared to those from samples receiving DMSO and were found to be significantly different according to Student’s paired T-test. The asterisk indicates p<0.01.

**Figure 5.** 3-AB inhibits CXCL1 promoter activity: 5 x 10^5 cells were first transfected with 1 µg of pRSV-β-gal reporter as well as reporter constructs driven by either the wild type (MGSA.Luc) or the mutant CXCL1 promoter (mIUR.Luc). Six hours after transfection, the medium was replaced by medium containing either DMSO or 3-AB within a concentration range of 1-5 mM. Cells were harvested 48 h after transfection and luciferase activity was measured. Values obtained were normalized to β-
galactosidase activity. The experiment was performed three times in triplicate. Error bars represent standard deviations. The mean normalized values obtained from samples receiving the highest dose of 3AB as compared to those from samples receiving DMSO were significantly different according to Student’s paired T-test The asterisk indicates p<0.01.

**Figure 6.** 3-AB has no effect on binding to the IUR element. Hs 294T cells were treated with either DMSO alone or with 1-5 mM 3-AB for 48 h. Cells were harvested and nuclear extracts were made. 10 µg of nuclear extract was incubated with $^{32}$P labeled 2xIUR oligonucleotide probe in the presence of 1 – 2 µg of poly dI.dC. The reaction mixtures electrophoresed on 6% native polyacrylamide gels which were then dried and processed for autoradiography. The arrow indicates the specific complex associated with the IUR probe. Shown here is a representative of three independent experiments. Results were qualitatively similar in all three experiments.
(A)

![Bar Chart](chart_A)

Relative Light Units

WT | mU1R | REV | pGL2

Constructs

(B)

![Bar Chart](chart_B)

Relative Light Units

WT | IN:2 | IN:6 | IN:12 | IN:25

Constructs
(A)

3 Amino Benzamide (mM):

0 1 3 5

CXCL1

Cyclophilin B

(B)

CXCL1 mRNA Levels

0 10000 20000 30000 40000 50000 60000

0 1 3 5

3-Amino benzamide (mM)

Hs294T

*
A role for poly ADP-ribose polymerase (PARP) in the transcriptional regulation of melanoma growth stimulatory activity (CXCL1) gene expression
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