Suppression Subtractive Hybridization (SSH) identifies prolactin stimulation of p38 MAP kinase gene expression in Nb2 T lymphoma cells: Molecular cloning of rat p38 MAP kinase.

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ABSTRACT. Suppression Subtractive Hybridization (SSH) has been used to compare rat Nb2 cells treated with prolactin for 1 hour with untreated cells. This new method for identifying differentially expressed genes showed that the mRNAs for at least three genes were elevated by such treatment, including a p38 mitogen activated protein (MAP) kinase. The p38 MAP kinase was cloned and the full length cDNA sequence was determined.

INTRODUCTION

The Nb2 cell line has been used as a model system to test a new PCR-based technique called Suppression Subtractive Hybridization (SSH) (1) by comparing untreated, quiescent cells with those treated with prolactin for 1 h. SSH would selectively amplify cDNAs of differentially expressed genes while suppressing amplification of sequences common to the two populations.

Prolactin stimulates proliferation and differentiation of a variety of cell types and regulates diverse physiological processes such as immune function, reproduction and osmoregulation (2). The rat Nb2 T lymphoma cell line has been extensively used to study the action of prolactin. Their growth can be arrested in the early G₁ phase of the cell cycle by lactogen deprivation and addition of prolactin reinitiates the cell cycle, causing rapid induction of gene transcription. Prolactin has been shown to induce transcription of IRF-1, c-fos, c-myc, hsp 70, ornithine decarboxylase, cyclins D2 and D3 and a serine/threonine kinase pim-1 (3-7). These effects of prolactin are mediated through its cell-surface receptor and the ensuing receptor activation is transduced through a number of intracellular pathways. Jak2 tyrosine kinase enables rapid signal transduction to the nucleus via phosphorylation of signal transducers and activators of transcription (STATs) (8). An alternative route has been proposed which involves a complex cascade via SHC, Grb2, Sos, Ras and Raf-1 resulting in the activation of MAPK (Erk 1) (2,8,9). In addition, protein kinase C and p59^{lyn} have also been implicated in prolactin signal transduction (9,10).

We show that the SSH technique can be successfully applied to this problem and that prolactin stimulates the

expression of three genes: a p38 MAP kinase and two as yet unidentified genes. The full-length nucleotide and deduced amino acid sequence of the rat p38 MAP kinase was determined and has been deposited with Gen Bank under Accession Number U73142.

MATERIALS AND METHODS

Cell Culture. The Nb2 rat T lymphoma cell line (clone 11C) was maintained as described by (11). Cells were rendered quiescent for 20 h and then treated for 0.5, 1, 2 and 4h with 10 ng/ml prolactin (NIDDK-hPRL-SIAFP-B-2) (3).

Northern and Southern analysis. Total cellular RNA was extracted using TRIZOL (Life Technologies, Grand Island, NY) and poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography. Procedures for Northern and Southern blots and hybridizations were as described (12). The following probes were used in Northern/Southern analyses: 1.6 kb rat c25 IRF-1 cDNA, 3.1 kb p38 rat MAP kinase cDNA, 600 bp SSH fragment and 330 bp SSH fragment. These were labeled by the RadPrime labeling system (Life Technologies). The ribosomal RNA probe (CLONTECH, Palo Alto, CA), labeled by 3'end labeling with terminal deoxynucleotidyl transferase (Promega, Madison, WI) was used as a loading control for total RNA blots. Densitometric analysis of the Northern blots was performed using Scanalytics/Ambis, Image Acquisition and Analysis System (Scanalytics, Billerica, MA). In the time-course experiment, the mRNA normalization was performed by quantifying the ethidium bromide-stained mRNA. Prolactin affects common housekeeping genes like actin (5) and cyclophilin (data not shown) and thus could not be used for normalization of mRNA.

Suppression Subtractive Hybridization (SSH). Subtraction was carried out using the PCR-Select

cDNA Subtraction Kit (CLONTECH) following the manufacturer's protocol. Briefly, two populations of cDNAs were made: "tester" cDNA was made from 2 µg of mRNA of prolactin-treated Nb2 cells and "driver" cDNA from mRNA of untreated Nb2 cells. Both cDNA populations were digested with *RsaI*. Tester fragments were then divided into two samples and ligated with two different adaptors. Two hybridizations followed: in the first hybridization, an excess of driver is added to each sample of the tester leading to equalization and enrichment of differentially expressed sequences; in the second hybridization, the two samples from the first hybridization are mixed together, generating templates for PCR amplification. Hybridization is followed by two rounds of PCR resulting in suppression of sequences common to the two cDNA populations and exponential amplification of the differentially expressed sequences. PCR products were analyzed on agarose gels, bands excised and individually reamplified with nested primers. The bands were purified by QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA) and cloned with the TA cloning system (Invitrogen, San Diego, CA). Plasmid DNAs were isolated using QIAGEN Plasmid Mini Kit.

Cloning of the Full-length cDNA. The Marathon cDNA Amplification Kit (CLONTECH) was used to generate the 5' and 3' fragments of the p38 cDNA, which then enabled the cloning of the full-length cDNA. The gene specific primers (GSPs) for the 5' and 3' ends and the multiple internal primers for sequencing the full-length p38 cDNA were chosen using the Gene Runner program (Hastings Software, Inc., Hastings, NY). All PCR products were purified (Qiagen) and cloned with the TA cloning system.

DNA Sequencing and Sequence Analysis. The SSH products, the 5'- and 3'-RACE products and the full-length p38 cDNA clone were sequenced using the PRISM dyeDeoxy Terminator Cycle Sequencing kit of Applied Biosystems (Foster City, CA). Sequence comparisons (FASTA), alignments (PILEUP) and translation (TRANSLATE) programs from the Wisconsin Genetics Computer Group (Madison, WI) were used throughout this work.

RESULTS

SSH identifies three transcripts as differentially expressed in prolactin-treated Nb2 cells. Before proceeding to the SSH method, mRNA from control and prolactin stimulated cells were hybridized with the IRF-

1 probe to confirm prolactin's upregulation of the IRF-1 mRNA in the tester population of Nb2 cells to be used for SSH (data not shown). In addition, the Northern blots used to confirm the differential expression of the three genes found in this study were also probed with IRF-1. In all cases, the prolactin treated cells showed approximately 20-fold upregulation of the IRF-1 gene expression.

The prolactin-stimulated Nb2 cells were used as the tester population and control unstimulated Nb2 cells as the driver population. At the end of the SSH procedure, an agarose gel showed multibands with a background smear of differentially expressed cDNAs. Three distinct bands (600, 450 and 330bp) were chosen for further analysis without proceeding onto a high throughput differential screening (13). After sequencing and GCG analysis, the 600 bp and 330 bp products showed no significant homologies to any current entries in the GenBank database. However, the 450 bp product had over 90% similarity with the 3' untranslated region (UTR) of the p38 mouse MAP kinase.

Cloning the full-length p38 rat MAP kinase cDNA. The rapid amplification of cDNA ends (RACE) strategy (Marathon cDNA Amplification Kit, CLONTECH) was used to obtain the full-length cDNA of p38 MAP kinase. Prolactin-treated Nb2 cell mRNA was used for the synthesis of ds cDNAs thus representing an uncloned cDNA library from which different genes could be amplified using the appropriate sets of primers. Gene specific primers for 5' and 3'-RACE amplification of the p38 MAP kinase were designed based on the sequence of the 450 bp SSH fragment. 5' and 3'-RACE generated single bands which were 1.5 and 1.8 kb in size, respectively. Both products were sequenced and these also showed over 90% homology to the p38 mouse MAP kinase. Based on the sequence of the extreme 5'-end of the 5'-RACE product and 3'-end of the 3'-RACE product, two gene-specific primers were then designed for direct amplification of the full-length cDNA.

The full-length p38 MAP kinase cDNA clone is 3132 bp long and contains the complete coding region spanning 1079 bp (Figure 1). This would encode a protein product of 360 amino acids (Figure 1) with a predicted molecular mass of 41.3 kDa and an isoelectric point of 5.77. The protein has a TGY sequence in the putative phosphorylation site which characterizes the p38 group of MAP kinases (14). Comparison of the rat p38 amino acid sequence with the human (15), mouse (16), frog (17) and yeast (18) homologues shows a high degree of identity: rat p38 is 99.2% identical to mouse p38, 98.6% to human p38,

1	GCCGCTGGAATAATGTCGACGAAAGGCCACGTTCTACCGGCAGGAGCTGAACAAGACCGCTCTGGGAGGTGCCGAGCGA	80
81	TACCAGAACCTGTCCCGGCTGGGCTCGGAGCCTACGGCTCGGTGTGTGCTGCTTTTGATACAAAGACGGGACATCGTGT	160
161	YQNLSPVGS GAYGSVCAAFD TKTGHRV	240
241	GGCAGTGAAGAAGCTGTCGAGACCGTTTCAGTCCATCATTCACGCCAAAAGGACCTACAGGGAGCTGCGGCTGCTGAAGC	320
321	ACATGAAGCACGAGAATGTGATGGTCTGTTGGATGTGTTTACACCTGCAAGGTCCTGGAAGAATTCACCGATGCTAC	400
401	CTGGTGACCCATCTCATGGGGCAGAGCTGAACAACATCGTGAAGTGTGCAAGCTTACCGATGACCCAGTTTCAGTTCT	480
481	LVTHLMGADLNNIVKCKLTDDHVF	560
561	TATCTACCAGATCCTGCGAGGGCTGAAGTATATACACTCGGCTGACATAATCCACAGGGACCTAAAGCCAGCAACCTCG	640
641	IYQILRLKYLHRSADIIHRDLKPSNL	720
721	CTGTGAATGAAGATGTGAGCTGAAGATTTTGGGCTGGCTCGGCACACTGATGACGAAATGACCGCTACGTG	800
801	VNEDECELEKILDFGLARHTDDEMTGYV	880
881	GCTACCCGGTGGTACAGAGCCCCGAGATTATGCTGAATGGATGCACTACAACAGACAGTGGATATTTGGTCCGTGGG	960
961	ATRWYRAP EIMLRNPMHYNQTVDIWSVG	1040
1041	CTGCATCATGGCTGAGCTGTTGACCGAAGAACGTTGTTTCTGGTACAGACCATATTGATCAGTTGAAGCTCATTTAA	1120
1121	CI MAELLTGR TLFPGTDHIDQLKILR	1200
1201	GACTCGTTGGAACCCAGGGGCTGAGCTTCTGAAGAAATCTCCTCAGAGTCTGCAAGAACTACATTCAGTCTCTGGCC	1280
1281	LVGLTPGAELELKKKISSEESARNYIQSLA	1360
1361	CAGATGCCAAGATGAACCTCGCAAAATGATTTATGGTGCCAAATCCCGTGTGCAAGCTGCTGGAAGAGATGCTGGT	1440
1441	QMPKMFANVF IGANPLAVDLLEKMLV	1520
1521	TTTGGACTCGGATAAGAGGATCACAGCAGCCCAAGCTCTTGCGCATGCGCTACTTTGCTCAGTACCACGACCTGATG	1600
1601	LDSDKRI TAAQA LAHA YFAQYHDPDDE	1680
1681	AGCCATGGCTGAACCTTATGACCACTCTTTGAAAGCAGGGACTTCCTTATAGACGAATGGAAGAGCTGACCTACGAT	1760
1761	PVAEPYDQSFE SRDFLIDEWKSLTYD	1840
1841	GAAGTCATTAGCTTTGTGCCACCGCCCTTGACCAAGAAATGGAGTCTGAGCACCTTGCTTCTGTTCTGTCATCC	1920
1921	EVISFVPPPLDQEEEMES	2000
2001	CACCTTCACTGTGAGGGGAAGGCTGTTTATGGGAACCTCCAAATACCATTCAGTGCCTCTGTTGAAAGATTCCTTCA	2080
2081	TGGTGGAAGGGGTGCATGTATGTGCGTAGTGTTTGTGTGTCTGTCTGTCTGTCGCTTTGTCATGTATCTTTGTGGA	2160
2161	AGTCATTGTGATGGCAGTGACTTTCATGAGTGGTAGATGCTCCTTGCGAGTCTGCCTGCTCTCAGAGTCCGGGACGGCC	2240
2241	GATGGAACTGCCGCTCCTTAGGGATGTGTGTGTATGTTAAGTGCAAAGTAAGAATATTAATAATATCCCTGTTCCTA	2320
2321	GTTCACCTTGCCACTTCGGCTTCTCCTGTGGCCCTGCCTTTACCATATCACAGTGACAGAGAGGGCTGCTTCAGGTCTGA	2400
2401	GGCTATTCCTCAGCCATGCATAAAGCCCAAGAGAACCAACTGGCTCCTGGGCTCTAGCCTGTGATCGGCTTGCTCATGTC	2480
2481	CTCAGAACCTGTCAGTCTGTTTGTGCCTTAAAGGAGAGAGGGGCGCTGTGGTGTAGTACAGAATCTCAGTTGCTGGCG	2560
2561	TTCTGAGCCAGGCAAGGCACAGGGCTGTGGATGGCCAGTGGGAGCTGGACAAAACAAGGCAGCCTTCAAGGAGGCCAT	2640
2641	GGGTGCATGTTTGCATGAGTGTATGTGCAACCGCCCTCCCTCACCTCCAGGAGCAAGCTGTTTCTATGCTTACCTAAGT	2720
2721	TCACCTCAGTGCAGAGGTCTCCAGTGCCAGGCACAGGCTCCTGCCATCAGTAGCTTCTCATGTGTCATCTTCACTCATGCG	2800
2801	GGTGTTCGATGCTGTGCTCGGAGCTTGCTCCTGTCTTCTGGAAGCCCTGGGCGGGCGTGTGAAGACTTCCAGCAGCTC	2880
2881	CTATCCACGCACCTCAGCTGAGGGCACGGGCACACTGCTGCTTCTCCTACCTCCAGCTACGTTGTGTTGAACACAACTGATC	2960
2961	CTCCAGGTGCTTGTGGTGCAGGAACCGGGACAGAGCAGCTGCTGCTTCTCCTACCTCCAGCTACGTTGTGTTGAACACAACTGATC	3040
3041	CAGTCCCTCTCCTCTCCTCTCCTCTCCTTAGGACAGTCCCGGCTCTGGAATCATGTTCTTCTCACTCATGGTAGCC	3120
3121	AGCTAAGAAAGCTGCAAAACCGGACGAAACAGAGCAGCTGAACCCCTGGCCATCTGACATCACCGCACAGGAGAA	
3122	AGGGTCATTAGACACAGCCAAGTCGTCAAAGGCCCTTTCTTGTACGGGGCCCCCGCCCCGGGAGCTTGACACTGA	
	TTTCATGCTATTGTTGGGAGAAAGCAATTGTTCTTGGAAATTTGTATGTTGTAGGAATCTTGAAGAGTGTGGTTTCT	
	TCTGATGGGGAGAAAGGCAAAATATTTTAATATTTTGTATTTACCTTTATAACATGAATCTCAGGGGTGAAGAACA	
	GTTTGATAAATTTCTGAATTCAGGCACTTTGTGCTATATGAGGACCATATATTTAAGCTTTTGTGTCAGTAAGAACA	
	TGTAAAGCAATTCAGTGTTGGACGAAACAGGCTCTCGTATTTAGTCAAGGTGTCTCCATCTCTATCAGTGCAGGGAC	
	ATGCAGTTTCTGTGGGCAGGGTAGGACCTGCATCATTTGGAGCCCAAGAGGGCCAGCTGGCCAGGGCTCACCGCT	
	CAGTATGCACTCCAGCTCCCGTCACAAATGGTTAGTAGCAACGCTCTGGGTTGAACGCCAGGCGTGGTTATA	
	TTATTGAGGATGCTTTGCACATGTGCCATGCTGTGTTAGGACTGTGCCCCAGGGCCCGGACTTGAAGCTAGAGCTGCG	
	AGAAGAGCTCCTGGCATCCATGGTGGCATGCTGCCGCCACCCAGTTTCTCCATTGGAAGACAAGGGAATGAGAAGACTGC	
	TGTGTATGTGATTTGTGGAACCTTGGTTGTGATCTGGTATGCCATAGGATGTGAGCAATATCACTGGTTAAAGTAAAGCC	
	TATTTTTCAGAT	3132

Figure 1. Nucleotide and amino acid sequence for rat p38 MAPK. TGY dual phosphorylation site is underlined

88% to *Xenopus* Mpk2 and 50.3% to yeast HOG1p. Figure 2 shows a dendrogram with the amino acid similarities among the p38 MAPK family members. At the nucleotide level, there is 95.1% identity in the coding region of rat and mouse p38 while the 3' UTRs showed an identity of 88.5%.

Genes detected by SSH are upregulated by prolactin.

The ability of the SSH method to enrich for differentially expressed sequences was confirmed by Southern blotting. The filter containing the unsubtracted and subtracted samples was probed for the 450 bp MAP kinase fragment. The unsubtracted sample was prepared by PCR amplification of the tester cDNA that was ligated to both SSH adaptors. Figure 3 shows that unsubtracted sample (lane 2) contained an undetectable amount of the 450 bp product whereas the same product was greatly enriched in the subtracted sample (lane 1).

Northern analysis confirmed that the three SSH products were derived from the prolactin-sensitive mRNAs. Northern blots hybridized with the 330 bp fragment hybridized to a 5 kb mRNA which was upregulated 5-fold (corrected for RNA loading) after 1h of prolactin stimulation (Figure 4a). The 600 bp fragment corresponded to a 2 kb transcript which was upregulated 2-fold (corrected for RNA loading). (Figure 4b). Since both of these products had no matches in the GenBank database, they potentially represent portions of novel prolactin-inducible genes.

Northern blot analyses with the p38 MAP kinase probe showed a single transcript at the predicted 3.1 kb which was upregulated an average of 1.5-fold in four independent experiments. A representative autoradiogram of p38 MAPK expression and the RNA loading control together with their quantitation is shown in Figure 5.

A time course experiment showed that p38 MAPK gene expression was increased as early as 30 min after PRL addition, with maximal expression at 4h (2.5-fold increase) (Figure 6).

DISCUSSION

In the past decade, a number of methods have been introduced that aim at identifying differences in gene expression between two types of mRNA populations. Among the newer techniques are serial analysis of gene expression (SAGE) (19), representational difference analysis (RDA) (20) and differential display (21). Recently, another new method called suppression subtractive hybridization has been introduced (1).

Figure 4. Northern blot analysis of mRNA from Nb2 cells. Lane 1: untreated cells. Lane 2: 1h prolactin treated cells. a. Filter hybridized with 330bp fragment. b. Filter hybridized with 600bp fragment.

Figure 5. Northern blot analysis of total RNA from Nb2 cells. a. Filter hybridized with p38 MAPK and ribosomal RNA probe. Lane 1: untreated cells. Lane 2: 1h prolactin treated cells. Each lane contains 30µg of RNA. b. Densitometric analysis of MAPK gene expression. The MAPK/RNA loading control is expressed as the mean with SD of four independent experiments where the control value was set as 1.0. ($p < 0.0001$)

Figure 6. Northern blot analysis of p38 MAPK gene expression in Nb2 cells at different time points. The cells were treated with prolactin for ½, 1, 2 and 4h. a. Filter hybridized to the p38 MAPK probe. b. Ethidium bromide staining of the mRNA gel to show equal RNA loading.

In this study, we have demonstrated that SSH is a reliable and relatively rapid method for identifying differentially expressed genes. It has the added advantage of requiring only a small amount of the starting material (2 µg of poly(A)⁺ RNA). It provides a high level of enrichment of differentially expressed transcripts as shown by Southern analysis of p38 cDNA abundance before and after subtraction. The sensitivity of the SSH also proved to be excellent since it was possible to recover genes that were upregulated at more modest levels: p38 MAP kinase - 1.5-fold, and two other gene transcripts: 2 kb mRNA - 2-fold and 5 kb mRNA - 5-fold. Our study therefore identified three differentially expressed genes, and although two had no match with any entries in the GenBank database, they may represent novel genes involved in the early response to prolactin's mitogenic signals.

The cDNA corresponding to the identifiable differentially expressed SSH fragment was fully cloned

and encodes the rat p38 MAP kinase. The protein product is a serine/threonine kinase that is highly conserved across species. The p38 group of MAP kinases is distinct from ERK MAP kinases which are also known to be affected by prolactin. All of the members of p38 group, including the rat p38 cloned here, have a characteristic dual phosphorylation site TGY in the subdomain VIII (14). p38 MAP kinase is predominantly activated by a variety of cellular stresses such as ultraviolet light, ionizing radiation, osmotic shock and cytokines, and to a lesser degree by growth factors. It has been implicated in regulating inflammatory responses, including cytokine secretion and apoptosis (22).

The data in this study shows that prolactin modestly affects the gene expression of p38 MAP kinase. However, further work is necessary in order to show that p38 MAP kinase is involved in the prolactin signal transduction pathway. It may be relevant to this study that the p38 MAP kinase cascade is activated in response to osmotic shock (23) and prolactin is known to be a major osmoregulatory hormone in lower vertebrates.

There may be a long-term regulatory mechanism where the elevated levels of mRNAs encoding kinases would increase the respective protein levels, which in turn would amplify the signal transduction efficiency of the cascade. Since MAP kinase enzymatic activity dramatically increases upon phosphorylation (24), even the modest mRNA upregulation detected here could lead to significant signal amplification.

This study demonstrates that SSH can successfully be used to identify changes in gene expression between two contrasting mRNA populations. SSH has provided preliminary evidence to suggest that prolactin may stimulate the p38 MAP kinase gene expression and additional experiments including extensive time course studies of mRNA expression as well as protein level and activity are therefore warranted.

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