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Rat CINC (Cytokine-Induced Neutrophil Chemoattractant) Is the Homolog of the Human GRO Proteins but Is Encoded by a Single Gene

John Zagorski and Joseph E. DeLarco

Department of Health Sciences, Monsanto Corporate Research, Monsanto Company, St. Louis, MO 63167

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SUMMARY Human GRO and rat CINC proteins are members of the pro-inflammatory "chemokine" superfamily of chemotactic cytokines which includes IL-8. We have used Northern and Southern blot analyses to compare the expression of CINC-related and GRO transripts in human cells and to compare the hybridization patterns of CINC and GRO probes in human and rat genomic DNA. Our data indicate that rat CINC is encoded by a single gene unlike GRO-alpha, -beta and -gamma which are encoded by three distinct genes and that they are the nearest homologs to each other from their respective species.

We have been studying a molecule termed "CINC" (Cytokine-Induced Neutrophil Chemoattractant. CINC is an 8 kdal polypeptide originally identified in the conditioned media of interleukin-1ß stimulated rat glomerular epithelial cells and subsequently purified from NRK52E rat epithelioid cells (1-3). CINC is a member of a rapidly expanding superfamily of peptides often referred to as "chemokines" or "intercrines" (4). Over 40 proteins or cDNA's in this superfamily have been identified to date including 10 human sequences closely related to CINC. Like CINC, many of these proteins are chemotactic for leukocytes (5-8). The most similar human molecules to CINC are the protein products of the three GRO genes (9-12). The percent similarities between CINC and GRO-alpha, -beta and -gamma are 70%, 72% and 70%, respectively, including signal sequences.

Although the GRO proteins are the most similar known molecules to CINC in terms of sequence homologies, there have been no attempts at identifying human genes more similar to CINC than the GRO's. Also, there have been no studies aimed at

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identifying additional CINC genes. We have used northern and Southern hybridization analyses to determine the human counterpart of CINC. By the criteria of cross-hybridization we have shown that the three GRO genes are the most homologous sequences to CINC in the human and show that CINC is encoded by a single copy gene in the rat genome.

MATERIALS AND METHODS

Materials. Recombinant human IL-1ß expressed in E. coli was produced at Monsanto. Lipopolysaccharide (LPS) was obtained from Sigma (#L3755). TNFα was purchased from IBI (#01-164). Rat cell line NRK52E was cloned from a mixed population of the the Normal Rat Kidney (NRK) line (13). Human melanoma MEL was the gift of Dr. Lawrence Helson. Human fibrosarcoma 8387 is a part of our laboratory collection. Human Caco-2 and T-84 epithelial cells were obtained from ATCC. Human dermal fibroblasts were purchased from Clonetics. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone). Cytokines were added to confluent monolayers on 100mm tissue culture dishes at 1nM. LPS was used at 100ug/ml.

Preparation of probes. A 204bp partial CINC cDNA was amplified using a cloned cDNA as template (Wittwer et al., in prep.). Amplification reactions contained 0.5µg each of primers HMC3 and HMC20 (5'-TGAGCTGCGCTGTCAGTGCCTGCAGA; 5'-TTACTTGGGGACACCTTTT AGCATCTTTTGGAC, nucleotides +87-+112 and +254 -+292 in ref.14), 1µg DNA, 0.2mM each dNTP and 2.5 units Taq Polymerase (Promega) per 100 µl. 35 cycles were done: 94°C, 30 sec., 50°C, 30 sec., 72°C, 30 sec. A 450bp partial GROα cDNA was amplified from MEL RNA using primers α26 and α27 (5'-GGTGGATCCTCGCCAGCTCTTCCGCT-3'; 5'-GGAGATCTCTTCTGTTCCTATAAGGG-3', nucleotides +1-+18 and +404 - +421 in ref.11). Amplification was done using the Perkin-Elmer RT-PCR kit for 35 cycles: 94°C, 1 min., 60°C, 1 min. For hybridizations, a 250 nucleotide HaeIII fragment was excised from the partial cDNA. A 500bp partial IL-8 cDNA was amplified from IL-1ß-stimulated 8387 RNA using primers JZ140 and JZ141 (5'-CTCCATAAGGCACAAACTTTCAGAC; 5'-CAGACCCACACAATACATGAAGTGT, nucleotides +1-+25 and +473-+497 in ref.15) as described for GROα. Probes (25-50ng) were random-primed with 100µCi ^{32}P-dCTP (NEN, 6000Ci/mmol) using the Boehringer-Mannheim kit.

Northern hybridizations. RNA from cell monolayers on petri dishes was isolated by the acid-phenol method (16). RNA was fractionated in 1.5% agarose gels containing 6% formaldehyde, 10mM sodium phosphate pH7 and transfered to nitrocellulose menbranes (MSI) by capillary transfer. Hybridizations were done using 50 ml of 50% formamide, 5X SSC (1X = 150mM sodium chloride, 15mM sodium citrate), 1X Denhardt's solution (0.02% each BSA, polyvinyl pyrolidone, ficoll), 0.1mg/ml sheared herring sperm DNA, 0.1% SDS plus 12.5 ml of 50% dextran sulfate at 42°C. Washes were done to a final stringency of 0.1X SSC at 50°C. Membranes were stripped of probe for re-use by exposure to boiling 0.1X SSC, 0.1% SDS for 15 min. and exposed to X-ray film for several days to assess probe removal.

Southern hybridizations. Confluent monolayers of NRK52E and human fibroblasts were lysed with 10 ml/150mm^2 flask of lysis

buffer: 100nM sodium chloride, 10mM EDTA, 10mM Tris pH8, 5% SDS, 0.2mg/ml Proteinase K, overnight at 37°C. Lysates were extracted three times with tris-saturated phenol pH7.5 and once with chloroform before ethanol precipitation. Digests of genomic DNA were electrophoresed through 0.8% agarose gels in 1X TBE buffer and transfered to Nytran membranes (Schleicher and Schuell) by capillary transfer using 20X SSC buffer. Hybridizations were done at 65°C in 5X SSPE (180mM sodium chloride, 10mM sodium phosphate, 1mM EDTA, pH7), 10X Denhardt's solution, 0.1mg/ml herring sperm DNA, 2% SDS. Membranes were washed at 60°C with decreasing concentrations of SSC.

RESULTS AND DISCUSSION

We decided to test whether CINC-related transcripts could be detected in human cells. NRK52E cells were used as a control. Each human cell type was treated with IL-1ß, LPS or TNFα for one or eight hours. Rat cells were treated for one hour only. As shown in Figure 1 (top panels), the CINC cDNA probe detected

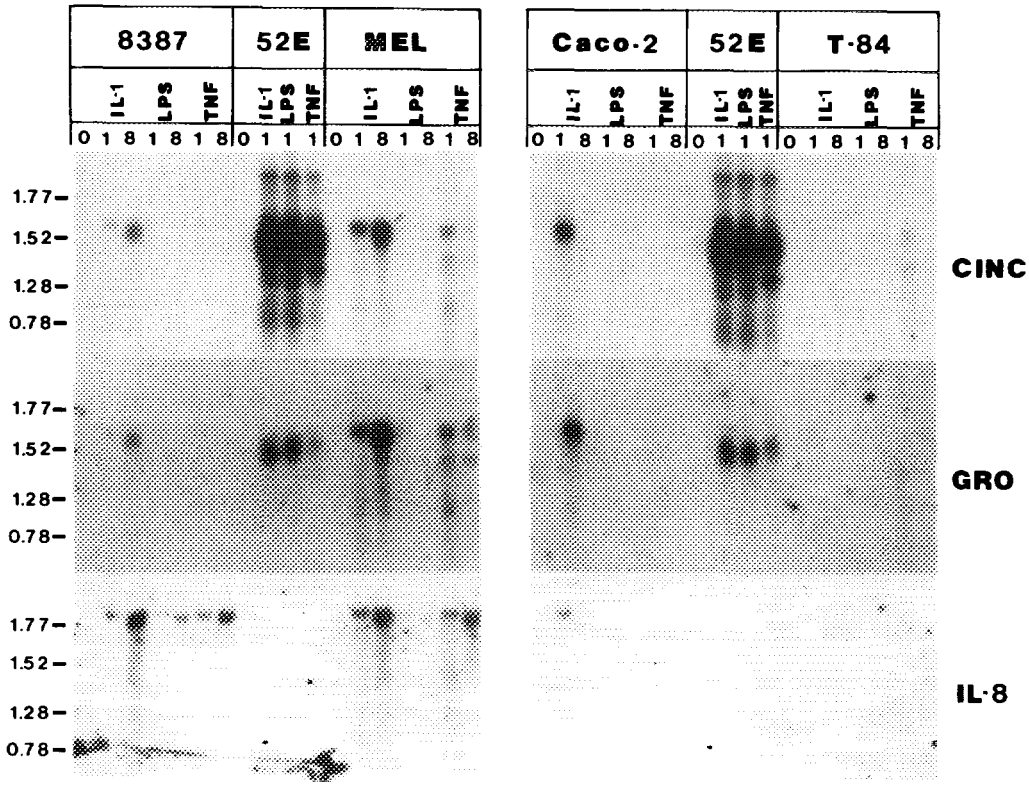

Fig.1. Expression of CINC-related, GRO and IL-8 transcripts in cytokine stimulated human cells. RNA from cytokine stimulated cells was loaded at 5µg total RNA per lane. Cell sources and inducers are listed above the panels. Molecular size markers to the left of the panels are in kilobases. Probes used are listed to the right of the panels. Times of exposure to cytokines are zero ("0"), 1 hour ("1") and 8 hours ("8").

abundant transcripts in the NRK52E samples obtained with all three inducers. Untreated cells contained no message. The pattern of expression of CINC in human cells was varied. MEL cells expressed the CINC-related transcripts with all three inducers, particularly with IL-1 . Both 8387 and Caco-2 also expressed CINC-related transcripts in response to IL-1ß although the kinetics of expression with Caco-2 were more rapid and transient than with 8387 and MEL. The responsiveness of the cells and the kinetics of induction of GRO transcripts (middle panel) was nearly identical to the results obtained with the CINC probe. In contrast, the patterns of expression for the related chemokine IL-8 (bottom panel) were very different than those observed for CINC or GRO.

To more definately determine the human homolog of CINC we used Southern blot analysis of human and rat genomic DNA probed with CINC and GRO cDNA's. To optimize hybridization conditions, we began by probing a Southern blot of DNA from eight animal species and yeast (a "ZooBlot"; Clontech Labs). Figure 2 shows the results of hybridization using a CINC cDNA probe. The left panel shows the results of washing at low stringency (2X SSC @ 60°C) to preserve interspecies cross-hybridization. All animal DNA's yielded strong signals. In most lanes, the number of bands

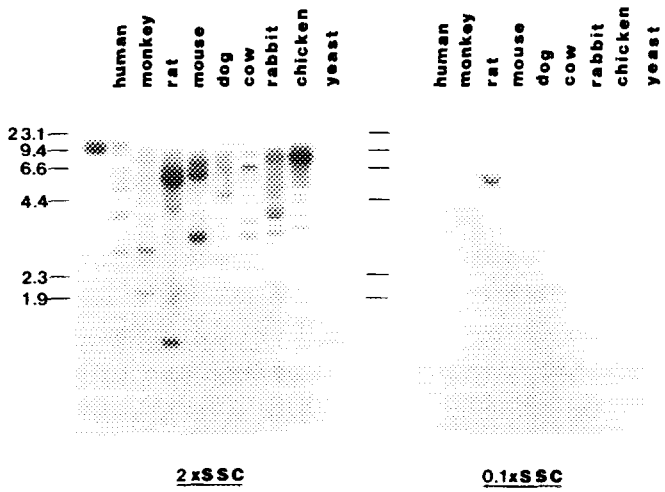

Fig. 2. Detection of CINC genes in diverse species. A ZooBlot was probed with a CINC cDNA and sequentially washed at low stringency (2X SSC), autoradiographed, washed at high stringency (0.1X SSC) and re-autoradiographed. Molecular size markers to the left of the figure are a HindIII digest of lambda DNA expressed in kilobasepairs.

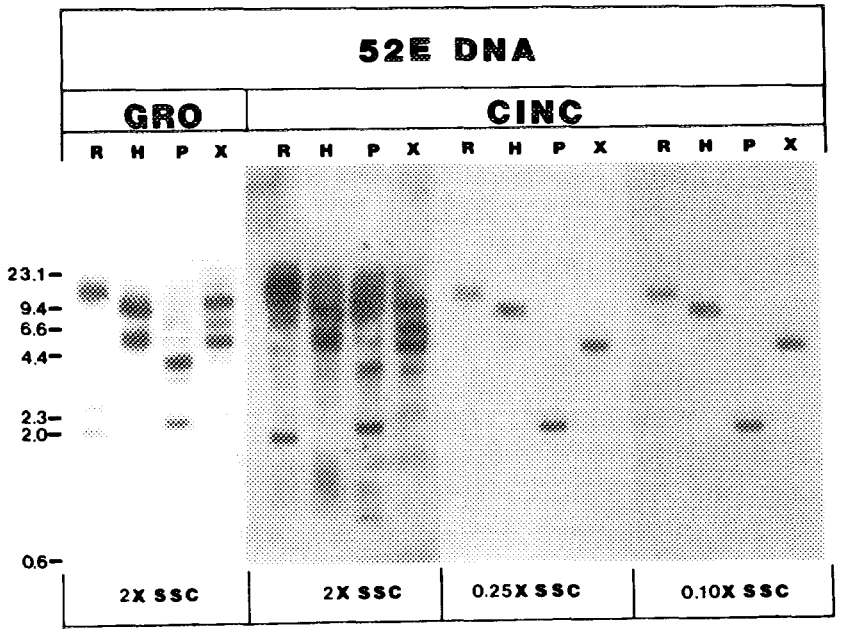

Fig. 3. Analysis of CINC and GRO genes in rat DNA. All panels are 20µg/lane NRK52E genomic DNA digested with either EcoRI ("R"), HindIII ("H"), PstI ("P") or XbaI ("X"). Probes used are listed above the enzyme designations. Wash stringencies are listed below the panels and were conducted at 60°C.

obtained suggested that CINC was encoded by a multigene family as is the situation with GRO (11). Following a higher stringency wash (right panel 0.1X SSC @ 60°C) CINC was detected only in rat DNA.

To extend the ZooBlot results we prepared Southern blots of rat and human DNA digested with four restriction enzymes (EcoRI, HindIII, PstI, XbaI) and hybridized with CINC and GRO cDNA probes. As shown in Figure 3, hybridization of rat genomic DNA at low stringency (2X SSC) with both CINC and GRO probes resulted in identical patterns suggestive of a gene family. However, at higher stringencies (0.25X or 0.1X SSC) the pattern of hybridization of the CINC probe to rat DNA clearly identifies a single gene. All hybridization of the GRO probe to rat DNA was lost at high stringency (0.1X SSC; not shown). When human DNA was used as the target (Figure 4), both probes detected nearly identical patterns at low (2X SSC) and high (0.25X or 0.1X SSC) strigency that are indicative of a multi-gene family.

The growing size of the chemokine superfamily makes assignments of inter-species homologs among the proteins confusing. Although the three GRO proteins are the most similar

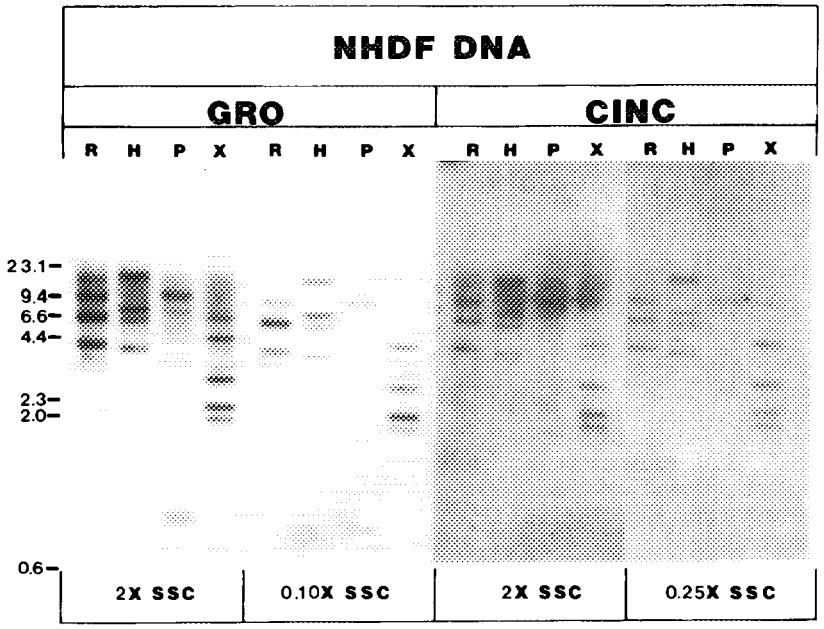

Fig. 4. Analysis of CINC and GRO genes in human DNA. All panels are digests of human fibroblast DNA. Enzyme, probe and washing designations are identical to Fig. 3.

known human proteins to rat CINC, the fact that only one CINC protein but three GRO cDNA's have been identified suggested to us either that additional rat CINC genes existed or that humans had a single copy gene more homologous to CINC than are the GRO's. Neither of these possibilities is consistant with our results. Instead, our results support the assumption that CINC and the GRO's should be considered homologs but create the dilemma of explaining why CINC/GRO has diverged from a single copy gene in the rat into a gene family in humans.

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