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Detection of an Interleukin-1 Intracellular Receptor Antagonist mRNA Variant

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At least two versions of interleukin-1 receptor antagonist generated by alternative splicing are found intracellularly, but their functions remain poorly characterized. During studies aimed at characterizing the expression of these transcripts in human articular cartilage, we detected a variant cDNA species that contained an additional 171 nucleotides within the type I interleukin-1 intracellular receptor antagonist cDNA which interrupted the coding region. This mRNA variant was also found to be expressed in keratinocytes. Translation likely initiates at an alternate methionine codon than that utilized for the previously reported interleukin-1 intracellular receptor antagonist isoforms, suggesting that this mRNA variant encodes a novel polypeptide that may play a role in interleukin-1 signaling.

Interleukin-1 (IL-1) is a prototype proinflammatory cytokine capable of regulating the metabolism of a wide variety of cell types during infection, inflammation, or autoimmune phenomena (1). The two forms of IL-1, IL-1α and β, bind to the type I IL-1 cell surface receptor and provoke alterations in intracellular signaling that lead to changes in gene expression, culminating in many instances in tissue destruction. Binding of IL-1 to its receptor can be attenuated by the competitive inhibition of the secreted form of the IL-1 receptor antagonist (IL-1 SRA), thus providing a mechanism for titrating the cellular response of cells to IL-1 (2). The signaling mechanisms responsible for propagating the effects of IL-1 binding to its receptor are poorly understood, although recent work has uncovered several proteins that associate with the IL-1 type I receptor, including a serine/threonine kinase complexed with IL-1 receptor accessory protein, and phosphatidylinositol 3-kinase (3,4). Furthermore, the transcription factor NFκB, which normally resides in the cytoplasm as an inactive species in complex with its inhibitor IκB, is activated in response to IL-1, and migrates to the nucleus where it affects transcription of multiple target genes (5).

The intracellular signaling triggered by IL-1 may involve biochemical pathways that bypass the prerequisite of IL-1 binding to its cell surface receptor. The fact that IL-1 does not possess a recognizable signal sequence suggests that at least a subset of IL-1 molecules may function intracellularly, although alternative pathways for secretion have been proposed (6). Furthermore, the finding that intracellular forms of the IL-1 receptor antagonist (IL-1 IRAs) exist raise questions concerning possible intracellular signaling routes that involve mature or unprocessed IL-1 (7,8). The function of these intracellular forms may involve IL-1 receptor antagonism following cell trauma or death, inhibition of internalized IL-1, or modifying the response of cells to IL-1 (7–9). Alternative RNA splicing produces at least two non-secreted forms of the IL-1 RA, designated as type I and type II, which have no signal peptide. Furthermore, mRNAs encoding the intracellular forms are controlled by a distinct promoter, leading to a pattern of expression distinct from that of the IL-1 SRA (10,11). The fact that the IL-1 SRA, in contrast to the IL-1 IRAs , is an acute-phase protein, clearly demonstrates the dual regulation of expression for the intracellular and secreted isoforms (12). Reinforcing the notion of an intracellular or "intracrine" mode of action for IL-1 is the provocative finding that the N-terminal propiece of IL-1α (IL-1 NTP), encompassing amino acids 1-112, gains entry into the nucleus by virtue of a nuclear localization sequence and can act as a transforming oncoprotein in certain cell types (13). Interestingly, only precursor IL-1α but not mature IL-1α (which lacks the IL-1 NTP) or precursor IL-1α harboring a mutation in the nuclear localization sequence is able to modulate endothelial cell migration

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in an IL-1 RA-insensitive manner, further suggesting an intracellular and possibly intranuclear mode of action for IL-1 (14).

Chondrocytes, which are known to express IL-1 (15), are also extremely responsive to IL-1, as demonstrated by dramatic changes in articular chondrocyte gene expression following exposure to IL-1 (16), and by studies implicating IL-1 as a culprit in the development of the debilitating joint disease osteoarthritis (17). During our studies on IL-1 signaling in human articular chondrocytes, we isolated a variant IL-1 IRA cDNA that previously has not been reported. We also demonstrated the existence of this mRNA species in cultured human keratinocytes.

MATERIALS AND METHODS

RNA isolation. Articular cartilage was resected from osteoarthritic knee joints at the time of total knee arthroplasty, and the chondrocytes were released from the cartilage matrix by overnight digestion with 0.1% clostridal collagenase in Dulbecco's modified Eagle medium. Total chondrocyte RNA was isolated by the acid guanidinium-thiocyanate-phenol procedure (18), using the RNA STAT-60 reagent (Tel-Test, Inc.). Human keratinocytes were isolated from foreskin, cultured in KGM media (Clonetics) containing calcium to promote differentiation, and total RNA prepared by the same procedure used for chondrocytes.

RT-PCR and DNA sequencing. Reverse transcriptase (RT) reactions and polymerase chain reactions (PCR) were performed essentially as described previously (19), except that a ribonuclease inhibitor (RNasin, Promega) was included in the reverse transcription step. RNA was primed with an antisense oligonucleotide specific for IL-1 RA exon 7 in the common region, (5′ → 3′) CTTCGTCAGGCATATTGG (nt 719-736 in the cDNA shown in figure 1). The exon-specific oligonucleotide primers employed in PCR (the numbers indicate the corresponding region in the cDNA) were (all in the 5′ → 3′ direction): Exon1: (sense) CAGAAGACCTCCTGTCCTATG, nt 95-115; Exon 2: (sense) GCTGACTTGTATGAAGAAGGAGGTGG; Exon 3: (sense) ATCATCAAAGCCAAGAAGGC, nt 153-172; Exon 3: (antisense) TCTTGTGAATCCTTTCTGAGG, nt 290-310; Exon 7: (antisense) CTTCCATCGCTGTGCAGAGGAACC, nt 674-697.

PCR products were subcloned into the pCR2.1 vector by TA cloning (Invitrogen) and sequenced using the Applied Biosystems Taq Dye-Deoxy Terminator Cycle Sequencing kit. DNA sequences were resolved on an ABI 377 Prism automated DNA sequencer.

Southern blot analysis. PCR products were electrophoresed in a 1% agarose gel and transferred to Duralon nylon membrane (Stratagene). A cDNA probe corresponding to exon 3 was generated by PCR using the two exon 3-specific primers described above, labeled by random priming with digoxigenin-dUTP, and hybridized to nylon-bound DNA by standard conditions at high stringency (20). Hybridized probe remaining bound to the filter after washing was detected immunologically with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) followed by a mixture of nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS AND DISCUSSION

Studies aimed at detecting by RT-PCR the previously described type I IL-1 IRA transcript in human articular cartilage revealed a variant form of this mRNA consisting of an extra 171 nt inserted immediately after exon 1 (previously referred to as icI, see refs. 7 and 8) of the type I IL-1 IRA (figure 1). The insertion interrupts the coding region of the type I IL-1 IRA species after the first nucleotide of the fourth codon, generating a stop codon after only six amino acids. In the revised scheme for the exons in the IL-1 RA mRNA, exon 1 is found in all isoforms of the IL-1 IRA, exon 2 is present only in the typeII IL-1 IRA, and exon 3 is associated with a novel mRNA variant (figure 2) (7,8). Exons 4-7 comprise the so-called common region which is shared by all isoforms of the IL-1 RA, including the secreted form (although an internal splice-acceptor site results in the intracellular forms lacking a region at the 5′ end of exon 4 that codes for the first 21 amino acids of the signal peptide in the IL-1 SRA). Therefore, the entire IL-1 mRNA is comprised of at least seven exons. Sequencing of PCR-generated genomic clones revealed that exon 3 is preceded by a polypyrimidine tract followed by an AG dinucleotide, which is characteristic of splice-acceptor junctions. To verify that this newly described exon, which we have labeled as exon 3, is present in the IL-1 RA gene, we performed PCR on human genomic DNA with primer pairs specific for exons $\frac{1}{3}$ or $\frac{2}{3}$. A 3500 bp PCR product was obtained when primers specific for exons 1 and 3 were employed, while a 1300 bp product was observed when primers specific for exons 2 and 3 were used (data not shown). During preparation of this manuscript, the full genomic sequence for the IL-1 RA gene became available (genbank accession #U65590), confirming the sequence and location of exon 3 (nt 19631-19801). Using RT-PCR with human articular chondrocyte-derived RNA as template and oligonucleotide primers specific for exons 3 and 7, which is in the common region, the expected 545 bp exon was obtained (data not shown), demonstrating that exon 3 is expressed and fused to exons 4-7 in articular cartilage.

Type I and type II IL-1 IRA are constitutively expressed in epithelial cells such as keratinocytes, in contrast to other cell types such as fibroblasts and various blood cell types, where treatment by various agents such as bacterial lipopolysaccharide is required to induce IL-1 IRA mRNA (7,8,10,11,21). To test for the presence of the exon 3-containing IL-1 IRA species in keratinocytes, we performed RT-PCR using primers specific for exons 1 and 7. We reasoned that if keratinocytes expressed IL-1 IRA mRNA moieties that both contained and lacked exon 3, then at least two DNA products should be observed. As shown in figure 3A, the major ethidium bromide-stained DNA product of 432 bp corresponds to the previously reported type I IL-1 IRA, in which exon 1 is fused to exons 4-7 (lane 1). However, a second DNA product, approximately 1-3% of the major product, can be observed in lane 1 at 603 bp, which agrees with the expected size product if the structure of the mRNA included exon 3 fused to both exons 1 and exons 4-7. This hypothesis is borne

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1    GGGCAGCTCCACCCTGGGAGGGACTGTGGCCCAGGTACTGCCCGGGTGCTACTTTATGGGCAGCAGCTCAG

                                                          M  A  L  G↓ G  L
72   TTGAGTTAGAGTCTGGAAGACCTCAGAAGACCTCCTGTCCTATGAGGCCCTCCCCATGGCTTTAGGGGGAT

     *
143  TATAAAACTAATCATCAAAGCCAAGAAGGCAAGAGCAAGCATGTACCGCTGAAAACACAAGATAACTGCAT

214  AAGTAATGACTTTCAGTGCAGATTCATAGCTAACCCATAAACTGCTGGGGCAAAAATCATCTTGGAAGGCT

                          ↓                                M  Q  A  F
285  CTGAACCTCAGAAAGGATTCACAAGACGATCTGCCGACCCTCTGGGAGAAAATCCAGCAAGATGCAAGCCT

       R ↓I  W  D  V  N  Q  K  T  F  Y  L  R  N  N  Q  L  V  A  G  Y  L  Q
356  TCAGAATCTGGGATGTTAACCAGAAGACCTTCTATCTGAGGAACAACCAACTAGTTGCTGGATACTTGCAA

     G  P  N  V  N  L  E  E↓ K  I  D  V  V  P  I  E  P  H  A  L  F  L  G  I
427  GGACCAAATGTCAATTTAGAAGAAAAGATAGATGTGGTACCCATTGAGCCTCATGCTCTGTTCTTGGGAAT

      H  G  G  K  M  C  L  S  C  V  K  S  G  D  E  T  R  L  Q  L  E ↓A  V  N
498  CCATGGAGGGAAGATGTGCCTGTCCTGTGTCAAGTCTGGTGATGAGACCAGACTCCAGCTGGAGGCAGTTA

       I  T  D  L  S  E  N  R  K  Q  D  K  R  F  A  F  I  R  S  D  S  G  P
569  ACATCACTGACCTGAGCGAGAACAGAAAGCAGGACAAGCGCTTCGCCTTCATCCGCTCAGACAGTGGCCCC

     T  T  S  F  E  S  A  A  C  P  G  W  F  L  C  T  A  M  E  A  D  Q  P  V
640  ACCACCAGTTTTGAGTCTGCCGCCTGCCCCGGTTGGTTCCTCTGCACAGCGATGGAAGCTGACCAGCCCGT

      S  L  T  N  M  P  D  E  G  V  M  V  T  K  F  Y  F  Q  E  D  E  *
711  CAGCCTCACCAATATGCCTGACGAAGGCGTCATGGTCACCAAATTCTACTTCCAGGAGGACGAGTAG
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FIG. 1. DNA sequence for exon 3-containing IL-1 IRA cDNA. The cDNA sequence is compiled from refs. 7, 10 and this paper, and represents the alternatively-spliced isoform containing exons 1, 3, 4_i, and 5-7. For simplicity, the long 3′ UTR is not included (2). Two discontiguous predicted protein sequences are displayed above the cDNA sequence. The hexapeptide is encoded by the cDNA sequence that includes the initiating methionine codon utilized for the type I and type II IL-1 IRA mRNAs and is interrupted by an in frame stop codon contributed by exon 3 (7,8). The longer amino acid sequence is the predicted polypeptide sequence for this exon 3-containing mRNA variant, which would use an alternate initiation codon supplied by exon 4. The underlined sequence corresponds to exon 3. Downward arrows indicate exon-intron boundaries (obtained from the GenBank entry having accession number U65590). This cDNA sequence has been deposited in the GenBank with accession number AF043143.

out by the results shown in lane 3, in which a specific DNA product of 545 bp is observed if oligonucleotide primers specific for exons 3 and 7 are employed. To verify that the faint DNA product observed in lane 1 corresponds to exon 3-containing IL-1 IRA cDNA, we performed a Southern blot analysis of the DNA products shown in lanes 1 and 3 of figure 3A with a PCR-generated cDNA probe encompassing only exon 3. Figure 3B shows that the probe hybridizes to the 545 bp DNA product amplified with an exon 3-specific primer (lane 1 in figure 3B, which corresponds to lane 3 in figure 3A) as well as the 603 bp DNA product that includes exon 3 inserted between exons 1 and exons 4-7 (lane 2). However, the prominent 432 bp product from

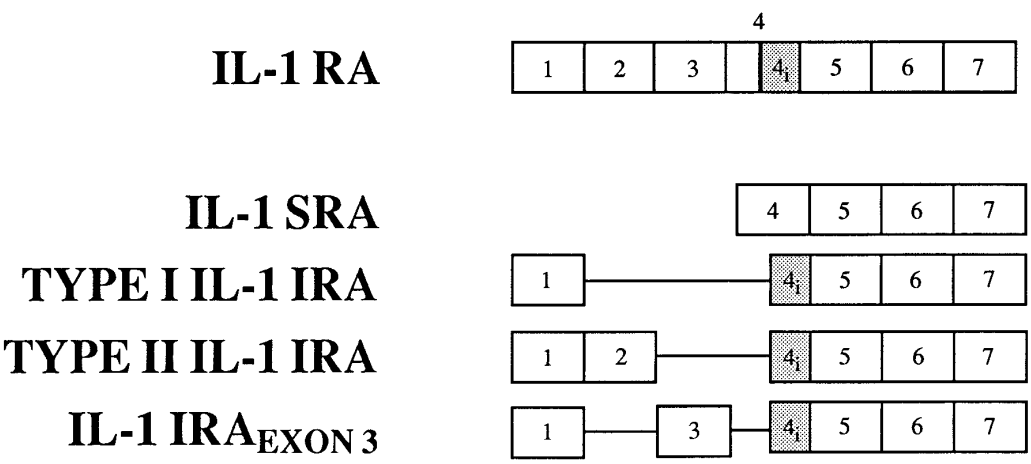

FIG. 2. Exon structure of different IL-1 RA variants. The seven known exons comprising the IL-1 RA gene are displayed on the top line, with the alternatively spliced secreted and intracellular isoforms of the IL-1 RA depicted below. The size of the boxes is not proportional to the length in bp of each exon. Exon 4_i indicates the truncated fourth exon produced as a result of an internal splice acceptor site.

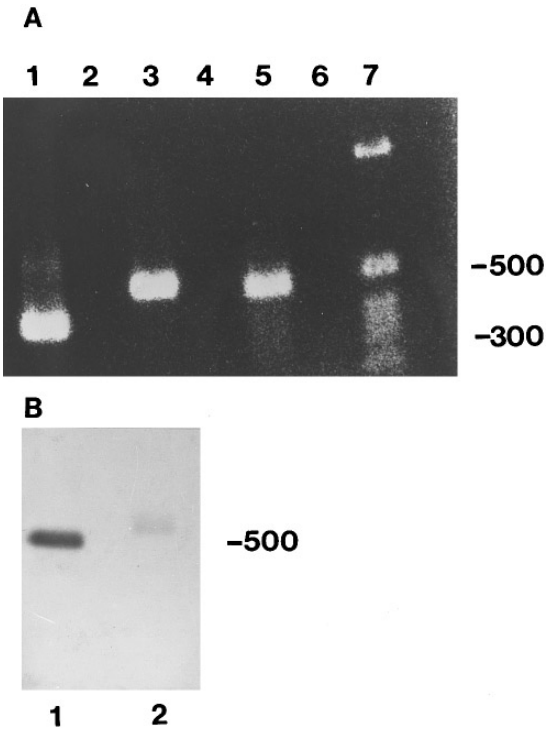

FIG. 3. Detection by agarose gel electrophoresis (A) and Southern blot analysis (B) of the IL-1 IRA transcript that includes exon 3 sequences. RT-PCR was performed as described (see Materials and Methods) employing human keratinocyte RNA, and products electrophoresed in a 1% agarose gel (A). Lanes 1, 3, and 5 correspond to reactions containing RT, while lanes 2, 4, and 6 lack RT. Lanes 1 and 2 include oligonucleotide primers specific for exons 1 and 7, while lanes 3 and 4 correspond to RT-PCR reactions employing DNA primers unique for exons 3 and 7. Lanes 5 and 6 are control RT-PCR reactions utilizing primers specific for a widely expressed gene, IQGAP1 (product size is 513 bp). DNA molecular weight standards were electrophoresed in lane 7. Panel B depicts the results of a DNA hybridization to membrane-bound DNA, utilizing a cDNA probe specific for exon 3. PCR products employing primers specific for exon 3 and 7 (identical to those visualized in lane 3, figure 3A) were electrophoresed in lane 1, while primers unique for exons 1 and 7 were used to generate the PCR products in lane 2 (identical to those observed in lane 1, figure 3A).

the RT-PCR reaction employing primers corresponding to exons 1 and 7 (lane 1 in figure 3A), which represented IL-1 IRA mRNA lacking exon 3, did not hybridize to the exon 3-specific cDNA probe (figure 3B, lane 2). Therefore, we can conclude that keratinocytes spontaneously express an alternatively spliced form of the IL-1 IRA mRNA that contains the newly described exon 3 fused to exons 1 and exons 4-7.

The structure of the protein encoded by the exon 3-containing species is unknown, and several hypotheses can be advanced to predict the actual coding region. If the initiating methionine codon used for the two IL-1 IRA forms previously described is also recognized by the translation apparatus for the alternatively spliced variant possessing exon 3, then a peptide of only six amino acids would be synthesized, which seems unlikely. We hypothesize that the longest open reading frame in the cDNA, beginning at nt 289, 19 nt upstream of the common region shared by all IL-1 RA isoforms, and extending to the end of the common region, most likely includes the protein coding region. Within this ORF, the first methionine is at nt 346, thus beginning at amino acid 13 of the common region. The predicted protein would contain 143 amino acids with a calculated molecular weight of 16.1 kD. This putative protein would resemble the known IL-1 IRA isoforms except that it would lack either 16 or 37 amino acids at its N-terminus when compared with the type I and type II IL-1 IRAs, respectively. Alternatively, protein translation could begin at a non-AUG codon such as CUG, as has been demonstrated for a handful of proteins, including c-myc (22), the leukocyte tyrosine kinase (23), and the p50 Bag-1 isoform (24). However, there is no CUG codon upstream and in frame with the first methionine codon in the common region.

Two theoretical considerations pertaining to the first hypothesis deserve mention. The first involves the optimal consensus sequence surrounding initiating methionine codons, and the second relates to methionine codons upstream of the translation initiation site. The most favorable sequence for initiation of translation contains a purine at the -3 position (with +1 denoting the first nucleotide of the methionine codon) and a purine, most often G, at the +4 position (25). However, the proposed translation initiation site contains a pyrimidine rather than a purine at the +4 position. The sequence surrounding the initiating methionine codon used for both the type I and II IL-1 IRA mRNAs is also not optimal, with a pyrimidine at the −3 position (7,8). In a survey of vertebrate mRNAs, 31% of the sequences possessed a pyrimidine at the +4 position, demonstrating the flexibility of the consensus translation initiation sequence (25).

Our hypothesis predicts that there would be five methionine codons upstream of the proposed initiating methionine codon in the 5′ untranslated region (UTR). According to the scanning model, initiation of translation by ribosomes usually begins at the first AUG codon encountered beginning from the 5′ end of the mRNA, although deviations from the consensus initiation sequence or stable RNA secondary structure may reprogram initiation at a downstream methionine codon (26). Only the methionine codon immediately upstream of the proposed initiating AUG codon in the exon 3-containing mRNA is located within a sequence possessing purines at the critical -4 and +3 positions, but a stop codon exists after only seven amino acids of coding region (figure 1A).

Interestingly, although approximately 90% of vertebrate mRNAs initiate translation at the most 5′ AUG, the translation initiation site for the type I and type II IL-1 IRA transcripts is positioned downstream of two

methionine codons (7,8), and both murine and human type I IL-1 receptor mRNAs commence translation downstream of AUG codons (27,28). Furthermore, analysis of translation initiation, which involves multiple parameters, has revealed that a subset of vertebrate transcripts such as growth factor and oncoprotein mRNAs typically exhibit 5′ UTRs containing methionine codons; as a result, these transcripts are translated inefficiently, which may be vital for preventing unrestricted cell growth (26). This phenomenon is well-illustrated by the T cell growth factor IL-15 mRNA, which normally contains 10 AUG codons upstream of the initiating methionine codon, and translation proceeds poorly (29,30). However, a naturally-occurring IL-15 fusion message in a T-cell leukemia cell line removes eight of 10 upstream methionine codons, with a concomitant stimulation of translation efficiency (30). A further parallel exists with IL-15 in that alternative splicing introduces a premature stop codon, resulting in a nonsecreted isoform, with translation beginning downstream at a suboptimal translation initiation site (31).

We speculate that the exon 3-containing IL-1 IRA may also be translated at low levels due to the upstream methionine codons. The function of this putative protein is unclear, as are possible regulatory mechanisms that might modulate the levels of this mRNA species in relation to the other IL-1 RA isoforms. The loss of the first 12 amino acids from the common region may affect IL-1 RA activity or result in shuttling of the polypeptide to a different intracellular compartment. In vitro translation experiments will be performed to address the issue of protein coding capacity for the exon 3-containing IL-1 IRA mRNA, with the goal of understanding the function of the putative protein.

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