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Intracellular IL-1 Receptor Antagonist Type 1 Inhibits IL-1-Induced Cytokine Production in Keratinocytes through Binding to the Third Component of the COP9 Signalosome1

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The IL-1 receptor antagonist (IL-1Ra) exists in four isoforms, three of which lack signal peptides and are primarily intracellular proteins. The biologic roles of the intracellular isoforms of IL-1Ra have remained unknown. The objective of these studies was to determine whether the major intracellular isoform of IL-1Ra 18-kDa type 1 (icIL-1Ra1), mediated unique functions inside cells. A yeast two-hybrid screen with HeLa cell lysates revealed specific binding of icIL-1Ra1, and not of the other IL-1Ra isoforms, to the third component of the COP9 signalosome complex (CSN3). This binding was confirmed by Far Western blot analysis, sedimentation on a glycerol gradient, glutathione pull-down experiments, and coimmunoprecipitation. In addition to binding specifically to CSN3, icIL-1Ra1 inhibited phosphorylation of p53, c-Jun, and IκB by the crude CSN-associated kinase and of p53 by recombiant protein kinase CK2 and protein kinase D, both associated with CSN3. The biologic relevance of the interaction between icIL-1Ra1 and CSN3 was demonstrated in the keratinocyte cell lines KB and A431, both possessing abundant CSN3. A431 cells exhibited high levels of icIL-1Ra1 but lacked both detectable IL-1α-induced IL-6 and IL-8 production and phosphorylation of p38 MAPK. KB cells displayed the opposite pattern which was reversed after transfection with icIL-1Ra1 mRNA. Inhibition of CSN3 or of icIL-1Ra1 production through gene knockdown with specific small interfering RNA in A431 cells each led to an inhibition of IL-1α-induced IL-6 and IL-8 production. Thus, icIL-1Ra1 exhibits unique anti-inflammatory properties inside cells through binding to CSN3 with subsequent inhibition of the p38 MAPK signal transduction pathway. The Journal of Immunology, 2005, 174: 3608–3616.

The IL-1 receptor antagonist (IL-1Ra) is a naturally occurring structural variant of IL-1 that binds to IL-1Rs but fails to stimulate target cells (1, 2). IL-1Ra binds avidly to the biologically active type 1 IL-1R (IL-1R1) but does not permit the association of the IL-1R accessory protein with this complex, a necessary step in the induction of signal transduction pathways (3). Thus, IL-1Ra functions as a competitive inhibitor of receptor binding of IL-1 and is biologically important in regulation of the inflammatory effects of IL-1.

Four isoforms of the IL-1Ra molecule have been described; one secreted (sIL-1Ra) and three intracellular (icIL-1Ra types 1, 2, and 3). The originally described 17-kDa sIL-1Ra possesses a leader peptide and is released from cells by the-secretory pathway as a variably glycosylated protein of 22–25 kDa (4, 5). The icIL-1Ra1 molecule is created by a transcriptional splice from an alternate upstream exon into the leader region for sIL-1Ra (6). icIL-1Ra1 possesses seven additional N-terminal residues in comparison to sIL-1Ra, but lacks a signal sequence and is synthesized in the cytoplasm as a nonglycosylated protein of 18 kDa. icIL-1Ra2 also results from another alternative first exon and encodes for a predicted protein of 25 kDa (7). Although this mRNA has been localized in vivo, it may not be translated. Lastly, icIL-1Ra3 is a truncated variant of 16 kDa that may be created by alternative translational initiation primarily from the sIL-1Ra mRNA (8, 9), or by an alternative transcriptional splice (10).

The role in biology of sIL-1Ra appears to be as an inhibitor of IL-1 in the microenvironment of cells (11). sIL-1Ra is produced primarily by monocytes and macrophages but may be synthesized by almost any cell that produces IL-1. In addition, sIL-1Ra is produced in large amounts by the liver as an acute phase protein and may also function as a systemic anti-inflammatory protein (12–14).

The biologic roles of the intracellular isoforms of IL-1Ra have remained unclear (11). icIL-1Ra1 is a major constitutive product of keratinocytes and other epithelial cells, particularly those lining the entire gastrointestinal tract. Although most icIL-1Ra1 remains in the cytoplasm of cells, this intracellular isoform may be released from keratinocytes and from epithelial cells lining the upper or lower respiratory tract under some conditions (15–17). icIL-1Ra1 binds to IL-1R1 equally as avidly as sIL-1Ra (18); therefore both isoforms may function as extracellular receptor antagonists of IL-1. However, icIL-1Ra1 may carry out additional functions inside cells not involving receptor binding. In ovarian cancer epithelial cells, the presence of icIL-1Ra1 was associated with an inhibition of IL-1-induced production of the chemokines GRO and IL-8, presumably by altering mRNA stability (19). Similarly, expression of icIL-1Ra1 in the human intestinal epithelial cell line...
Caco-2 inhibited IL-1-induced IL-8 production (20). Intracellular expression of icIL-1Ra1 was also described to decrease NF-κB activity, although possible effects on other signal transduction pathways were not explored in this study (21).

We hypothesized that the intracellular isoforms of IL-1Ra may carry out unique functions inside cells through binding to other cytoplasmic proteins. To investigate this possibility, we examined a HeLa cell library for proteins binding to IL-1Ra isoforms using a yeast two-hybrid screen. The third component of the COP9 signaling complex (CSN3) was identified as a cytoplasmic protein that interacted specifically with icIL-1Ra1. The CSN was originally described in Arabidopsis as a repressor of photomorphogenesis and is a 450-kDa complex containing eight subunits (22). The CSN possesses structural similarities with the 26 S proteasome lid complex, is found in the cytoplasm and nucleus of all mammalian cells, and appears to function as an interface between signal transduction pathways and ubiquitin-dependent proteolysis (23). Most importantly, the CSN demonstrates an associated kinase activity that phosphorylates many proteins involved in signal transduction including IκBα, p105, c-Jun, and p53 (24). We characterized the binding of icIL-1Ra1 to CSN3 and observed that this intracellular isoform of IL-1Ra specifically inhibited the activity of CSN-associated kinases. Moreover, we observed that icIL-1Ra1 inhibited IL-1β-induced IL-6 and IL-8 production in keratinocyte cell lines through binding to CSN3 with subsequent inhibition of phosphorylation of p38 MAPK.

Materials and Methods

Yeast two-hybrid screens with icIL-1Ra isoforms

Vectors, yeast, and strains of bacteria were a gift from Dr. R. Brent, Massachusetts General Hospital (Boston, MA). All methods and protocols for performance of the yeast two-hybrid interaction trap were adapted from published procedures (25). Bait plasmids (DNA binding domain fusion proteins) for icIL-1Ra1 and icIL-1Ra3 were created by PCR cloning of the protein coding regions into the pEG202 vector producing an N-terminal LexA-icIL-1Ra fusion protein. The EGY strain of Saccharomyces cerevisiae, containing both Leu2 and lacZ reporter systems, was used as a reporter strain. The EGY48-pEG202-icIL-1Ra1 strain was created following transformation of EGY48 with pEG202-icIL-1Ra1. A HeLa cell cDNA library, cloned in the pG45 vector, was generated in the laboratory of Dr. Brent. The HeLa pG45 cDNA library was introduced into EGY48-pEG202-icIL-1Ra1 using standard yeast transformation methods. Transformants were recovered on glucose, Ura’ His’ Trp’ plates. The colonies were scraped and resuspended in ~20 ml of 65% glycerol, 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and the cells were stored in 1-ml aliquots at −80°C. For selection of interaction clones, ~2 × 106 cells were plated on plates with 8% agar, 1.5 g/L galactose, 0.1% Tween, the blots were reprobed with a mAb specific for all isoforms of IL-1Ra. One of these proteins was CSN3, a member of the CSN complex (24). The second interacting protein was encoded by the neuroblastoma-associated gene and was not further characterized (26).

Glyceraldehyde gradient centrifugation, and Western and Far Western blot analyses were conducted as recently described (27). Briefly, to determine binding between IL-1Ra isoforms and the CSN, 30 μg of a purified IL-1Ra isoform and 40 μg of isolated CSN, in a final volume of 300 μl containing 30 mM Tris-HCl, pH 7.8, 10 mM KCl, and 0.5 mM DTE were incubated for 60 min at 37°C. Subsequently, the mixture was loaded onto 10–30% glycerol gradient and centrifuged for 24 h at 27 k rpm (SW40, 12-ml tubes; Beckman Instruments). After centrifugation the glycerol gradients were fractionated (0.6 ml per fraction) from the bottom (high density) to the top (low density). Proteins in fractions from the gradients performed with the CSN, and from the control gradients performed without CSN, were precipitated by TCA, then separated by SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose membranes were first probed with a mAb specific for all isoforms of IL-1Ra (28), then stripped and reprobed with anti-CSN2 Abs to identify the intact CSN complex (24).

Western and Far Western blot analyses were conducted by separating a truncated fraction of CSN3, ΔCSN3 (111–403) lacking the N-terminal 110 amino acids, and CSN5 by SDS-PAGE, then blotting to nitrocellulose. This recombinant fragment of CSN3 was used because of initial difficulty in expressing a stable recombinant CSN3. The blots were incubated with rIL-1Ra isoforms (1 μg/ml). After three washes with PBS containing 0.1% Tween, the blots were reprobed with a mAb specific for all isoforms of IL-1Ra and developed by the ECL technique (Amersham).

Expression of rIL-1Ra isoforms

All three isoforms of IL-1Ra were produced as GST fusion proteins. E. coli BL21 was transformed with pGEX-5x-1 containing cDNAs for sIL-1Ra, icIL-1Ra1, or icIL-1Ra3. When the OD660 of the cultured bacteria reached 0.7, they were induced with 100 μM of isopropyl-β-D-thiogalactopyranoside for 60 min at 37°C. Subsequently, the mixture was loaded onto a 10–30% glycerol gradient and centrifuged for 24 h at 27 k rpm (SW40, 12-ml tubes; Beckman Instruments). After centrifugation the glycerol gradients were fractionated (0.6 ml per fraction) from the bottom (high density) to the top (low density). Proteins in fractions from the gradients performed with the CSN, and from the control gradients performed without CSN, were precipitated by TCA, then separated by SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose membranes were first probed with a mAb specific for all isoforms of IL-1Ra and developed by the ECL technique (Amersham).

HeLa cell lysates

HeLa cells were grown in DMEM with 10% FCS, penicillin (1 U/ml) and streptomycin (1 μg/ml) until confluence. The cells were scraped into PBS with a protease inhibitor mixture (Roche). The cells were frozen and thawed for three cycles, then passed through a 5/8-inch 25-gauge needle. Cellular debris was removed by centrifugation and the supernatant was harvested. The total protein concentration was determined using a protein dye assay (Bio-Rad).

Glutathione pull-down assay

The expression of the rIL-1Ra isoforms as GST fusion proteins coupled to glutathione-Sepharose beads was conducted as earlier described with 30 μl of each slurry used for all binding experiments. HeLa lysate protein was added in the amounts of 0, 150, 300, 450, and 600 μg to the slurries containing the GST fusion protein glutathione-Sepharose beads and incubated for 4 h at 4°C with rocking, followed by washing five times with 1 ml of PBS. The samples were then boiled with 20 μl of sample buffer and run on 10% Bis-Tris gels under denaturing conditions using the Novex NuPAGE buffer system (Invitrogen Life Technologies), followed by transfer to polyvinylidene fluoride membranes (Millipore). Immunoblotting was performed with rabbit anti-CSN4 Ab (Affinity) followed by donkey anti-rabbit serum (Santa Cruz Biotechnology) using an ECL detection system. An anti-CSN4 Ab was used in these experiments because it was more avid than the anti-CSN3 Ab and it better detected a denatured CSN component as representative of the intact complex that had bound icIL-1Ra1. GST alone attached to glutathione beads was added to HeLa lysate as a negative control. HeLa lysate added to each isoform of IL-1Ra as GST fusion proteins coupled to beads was used as a positive control. In each of these controls, the immunoblot was probed with an Ab reactive against all isoforms of IL-1Ra.
Coimmunoprecipitation

The expression of recombinant proteins was conducted as described earlier through washing the protein glutathione-Sepharose beads with PBS. Instead of glutathione elution, the washed beads were resuspended in 1.5 ml of PBS. To cleave the rIL-1Ra proteins, Factor Xa (New England Biolabs) was added to the slurry at 1 μg/50 μl protein and incubated overnight at 4°C. The supernatant was harvested and IL-1Ra protein concentrations were determined by an ELISA that was specific for all isoforms (28). For the coimmunoprecipitation experiments, 1 μg of each of the three rIL-1Ra isoforms were preincubated with 20 μg of total protein from HeLa lysates for 12 h at 4°C. Immunoprecipitation was conducted using a Pierce Seize X kit according to the manufacturer’s instructions, with Abs to IL-1Ra or to CSN2 coupled to Sepharose used to precipitate the bound proteins. An anti-CSN1 Ab was used rather than anti-CSN3 because of higher avidity and a greater ability to remove all of the CSN complex from solution when coupled to Sepharose. The flow-through fractions from a column separation, as well as the Sepharose with bound proteins, were concentrated and boiled in SDS buffer before SDS-PAGE. All IL-1Ra isoforms were immunoprecipitated with a monoclonal anti-IL-1Ra Ab bound to Sepharose, and the immunoblots were probed with both the anti-IL-1Ra Ab and the anti-CSN1 Ab.

In vitro kinase assay

The CSN with associated kinases was isolated from human RBC as previously described (24). The CSN kinase reactions were performed at 37°C for 1 h and contained 8 μCi of [γ-32P]ATP, 100 ng of isolated CSN, increasing amounts of a purified IL-1Ra isoform, and 1 μg of IκB, p53, or c-Jun in a kinase buffer (30 mM Tris-HCl, pH 7.8, 10 mM KCl, 0.5 mM DTT, 5 μM MgCl2) in a final volume of 20 μl. The reactions were stopped with 4× protein sample buffer and separated on 12.5% SDS-PAGE. To determine the effects of icIL-1Ra1 on activity of kinases known to be associated with the CSN, 25 μg/ml p53 was incubated with purified CSN, recombinant protein kinase CK2 (CK2, formerly casein kinase II), and protein kinase D (PKD; both from Calbiochem) in the presence or absence of 60 μg/ml icIL-1Ra1 (29). The gels were dried and autoradiographed. Experiments were repeated three times with similar results.

Culture and transfection of keratinocyte cell lines

The human keratinocyte cell lines A431 and HaCaT (compliments of Dr. D. Norris, University of Colorado Health Sciences Center, Denver, CO) were cultured in DMEM supplemented with 10% FCS (HyClone Laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin (complete MEM) in a humidified atmosphere of 5% CO2 to 95% air at 37°C. Human KB cells (American Type Culture Collection) were cultured in MEM under similar conditions. Cells were harvested with trypsin/EDTA and all experiments were conducted in six-well tissue culture plates (BD Biosciences). For the transient transfection studies, KB or A431 cells were plated at a density of 2 × 10^5 and the experiments were performed when cells were 80–90% confluent. KB cells were allowed to culture for 3 days before transfection with Lipofectamine 2000 reagent (Invitrogen Life Technologies) following the manufacturer’s recommendations and according to our recently published studies with minor modifications (30). After culture with DNA:lipofectamine, the medium was removed and the cells were washed once with MEM before stimulating with 10 ng/ml human IL-1α in complete MEM for an additional 24 h. Control KB cells were also transfected with the corresponding empty pHook-3 vector under identical conditions. Supernatants and lysates were collected for cytokine measurements and Western blot analyses.

To inhibit sequence-specific target mRNA without inducing IFN response in A431 or KB cells, the cells were transfected with a mixture of chemically synthesized human small interfering (si)RNAs (31, 32). Control siGLO RISC-free control siRNA (100 nM, a random control containing a fluorescent dye), 100 nM siGenome SMART-pool CSN3 siRNA (NM_003655), and 100 nM siGenome SMART-pool icIL-1Ra1 siRNA (NM_173842) (Dharmacon) were used with 10 μl of lipofectamine reagent. The siRNA:lipofectamine mixture was removed after 24 h and the cells were incubated for another 48 h in complete MEM. The medium was again removed, the cells were washed once with MEM, and then stimulated with 10 ng/ml human IL-1α in complete MEM for an additional 24 h. Supernatants and lysates were collected for cytokine measurements and Western blot analyses. The efficiency of transfection was measured by FACS analysis before the stimulation with IL-1α and ranged between 80 and 87%.

ELISA

Supernatants were collected from the keratinocyte cell lines stimulated with 10 ng/ml IL-1α for 24 h; in some experiments, the cells were transiently transfected as previously described. Cytokine levels in the supernatants were measured using ELISA for IL-1Ra (R&D Systems), IL-6 or IL-8 (BD Biosciences). The lower limits of sensitivities for these ELISA were: IL-6, 4.70 pg/ml; IL-8, 3.10 pg/ml; and IL-1Ra, 150 pg/ml. All ELISA readings were measured in triplicate for each supernatant and lystate sample. The data were normalized to total protein concentration in cell lysates when transfections were performed with siRNA, to correct for possible variations in cell numbers.

Western blot analysis on lysates of keratinocyte cell lines

Keratinocyte cell lines were cultured in the presence of 10 ng/ml IL-1α for 24 h. Lysates were prepared by harvesting the cells in 200 μl of ice-cold lysis buffer containing 50 mM Tris, 0.1% SDS, 150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 25 mM Na glycerophosphate, protease inhibitors (10 μg/ml aprotinin, 1 mM PMSF and 10 μg/ml leupeptin), and phosphatase inhibitors (10 mM Na pyrophosphate and 2 mM sodium orthovanadate, pH 7.2) for 5 min followed by centrifugation (14,000 × g at 4°C for 10 min). Total protein levels in the cell lysates were measured using a Bio-Rad protein assay.

Lysates (15 μg of protein) were examined by SDS-PAGE, as described recently (30) The blots were incubated overnight at 4°C with primary Abs to CSN3 (polyclonal rabbit Ab, 1/2000 dilution; Affinity), IL-1Ra (goat Ab, 1/1000; R&D Systems), and GAPDH (goat Ab, 1600; R&D Systems). After washing, a species-matched peroxidase-conjugated secondary Ab was incubated for 1 h at room temperature. The secondary Abs used for CSN3 were peroxidase-conjugated goat anti-rabbit IgG (1/10,000 dilution; Jackson ImmunoResearch Laboratories), and the secondary Abs for IL-1Ra and GAPDH were peroxidase-conjugated bovine anti-goat IgG (1/10,000 dilution; Santa Cruz Biotechnology). Western blot analyses for total and phosphorylated p38 MAPK were performed as recently described (30). Immunoreactive bands were visualized by using a Supersignal West Pico Chemiluminescent substrate kit (Pierce).

Densitometric analysis was performed by scanning radiographs with a computing densitometer (Molecular Dynamics). The percentage of increase or decrease was calculated by using the formula: [(area of control siRNA — area of siRNA for CSN3 or icIL-1Ra1)/area of control siRNA] × 100.

Statistical analyses

A two-tailed Student’s t test was used to analyze the data from the transfection experiments. In all cases, the data were expressed as mean ± SEM with values of p < 0.05 considered significant. Where error bars cannot be seen, they were smaller than the thickness of the line.

Results

In vitro interaction between icIL-1Ra1 and CSN3

The results of a yeast two-hybrid screen identified the third component of the CSN as a specific interacting protein with icIL-1Ra1. To support this finding, binding studies were performed with icIL-1Ra1 using glycerol gradient centrifugation and Far Western blots. In glycerol gradients, recombinant icIL-1Ra1 was observed to cosediment with the intact CSN, as identified with anti-CSN2 Abs (Fig. 1A). In a gradient in which the CSN was not included, icIL-1Ra1 was confined to the top of the gradient in the least dense fractions. Analysis by Far Western blot using all recombinant subunits of the CSN indicated a similar result; icIL-1Ra1 bound specifically to ΔCSN3 (111–403) but not to CSN5 (Fig. 1B). Furthermore, icIL-1Ra1 failed to interact with any other CSN component, and icIL-1Ra3 did not interact with CSN3 (data not shown). These data confirm the results of the yeast two-hybrid screen and indicate that the N-terminal 110 residues of CSN3 were not required for binding of icIL-1Ra1.

Specificity of binding of icIL-1Ra1 to CSN3

To further explore the specificity of the interaction between icIL-1Ra1 and intact CSN, glutathione pull-down experiments were performed. In these studies, GST fusion proteins of the three IL-1Ra isoforms coupled to glutathione-Sepharose beads were incubated with increasing amounts of HeLa lystate. After separation by
icIL-1Ra1 binds to the third component of the CSN. A, Glycerol gradient. Recombinant icIL-1Ra1 (30 μg) was incubated for 1 h at 37°C with or without isolated CSN (40 μg) and the samples were loaded on a 10–30% glycerol gradient. After centrifugation, fractions (600 μl) were collected, protein was precipitated with TCA, and then immunoblotted. The blots were probed with anti-CSN2 or anti-IL-1Ra Abs; icIL-1Ra1 bound to the CSN. B, Far Western blot analysis. Recombinant ΔCSN3 (111–403) and CSN5 (~1 μg of each) were immobilized on nitrocellulose and incubated with recombinant icIL-1Ra1 (2 μg/ml). After washing, the blots were probed with a mAb to IL-1Ra. icIL-1Ra1 bound to ΔCSN3 but not to CSN5. These experiments were repeated twice.

FIGURE 2. icIL-1Ra1 binds to the CSN in a glutathione pull-down experiment. GST fusion proteins of icIL-1Ra1, icIL-1Ra3, and sIL-1Ra were coupled to Sepharose beads and incubated with increasing amounts of the CSN in HeLa cell lysates. The proteins were separated from the beads by boiling, then electrophoresed on SDS-PAGE; bound CSN was detected with an Ab to CSN4. The results indicate that only icIL-1Ra1 bound to the CSN, as icIL-1Ra3 and sIL-1Ra exhibited no binding even at a concentration 4-fold above that at which binding of icIL-1Ra1 was observed (Fig. 2).

To further explore this interaction in vivo, binding of icIL-1Ra1 to the CSN was next examined using a coimmunoprecipitated system in which rIL-1Ra proteins were incubated with HeLa lysates containing intact CSN. The coprecipitated proteins were recognized using specific Abs in Western blot analyses. The mixture of each IL-1Ra isoform and the HeLa lysate was precipitated with an Ab recognizing all three isoforms of IL-1Ra or with the anti-CSN1 Ab coupled to Sepharose beads. Using the anti-IL-1Ra column, all three isoforms of IL-1Ra were precipitated; however, only the reaction mixture containing icIL-1Ra1 exhibited coprecipitation of CSN1 (Fig. 3). In addition, precipitation with the anti-CSN1 Ab revealed coprecipitation only of icIL-1Ra1, not of icIL-1Ra3 or sIL-1Ra. This result indicates that icIL-1Ra1 is bound to CSN3 in vivo in HeLa cells.

Effect of icIL-1Ra1 on activity of CSN-associated kinases

The results of initial studies indicated that none of the three isoforms of IL-1Ra were phosphorylated by the CSN-associated kinase using CSN purified from human erythrocytes (Fig. 4A). However, icIL-1Ra1 inhibited phosphorylation of various substrates by the CSN-associated kinase. Phosphorylation of c-Jun was almost completely inhibited by icIL-1Ra1, whereas under the same conditions sIL-1Ra exhibited no effect and icIL-1Ra3 showed little impact (Fig. 4A). Increasing concentrations of icIL-1Ra1 were added to a constant amount of IkB, p53, or c-Jun, all substrates of the CSN-associated kinase. The phosphorylation of all three substrates was inhibited in a dose-responsive fashion by icIL-1Ra1, although at different concentrations (Fig. 4B). Two protein kinases recently described to be associated with the CSN are CK2 and PKD (29). The results of in vitro kinase assays indicated that icIL-1Ra1 inhibited phosphorylation of p53 by recombinant CK2 and PKD; however, icIL-1Ra1 itself was not phosphorylated by these two protein kinases (data not shown).

IL-6 and IL-8 production by keratinocyte cell lines

To investigate whether the interaction of icIL-1Ra1 with CSN3 was important for a cell function, we examined IL-1α-induced IL-6 and IL-8 production in keratinocyte cell lines that varied in the amounts of constitutive icIL-1Ra1 in cell lysates. A431 cells and HaCaT cells exhibited very low levels of IL-1α-induced IL-6 and IL-8 production with high amounts of icIL-1Ra1 found in the cell lysates (Fig. 5, A and B). KB cells displayed the opposite pattern with abundant IL-6 and IL-8 production after stimulation with IL-1α in the virtual absence of icIL-1Ra1 in the cells (Fig. 5, A and B).
IL-8 production through inhibition of p38 MAPK and NF-
Caco-2 intestinal epithelial cells inhibited IL-1-induced IL-6 and
Our laboratory recently observed that icIL-1Ra1 transfected into
Inhibition of phosphorylation of p38 MAPK
seen with A431 and HaCaT cells that endogenously make
1Ra incubated with isolated CSN and 1
[32P]ATP with the reactions separated by SDS-PAGE. A, Coomassie stain (left) and autoradiography (right) of 1.3 μg of icIL-1Ra1, icIL-1Ra3, or siIL-
1Ra incubated with isolated CSN and 1 μg of c-Jun. B, Coomassie stain (left) and autoradiography (right) of increasing amounts of icIL-1Ra1 incubated with CSN and constant amounts (50 μg/ml) of IxB, p53, or c-Jun. Representative result of three separate experiments.
A–D), The levels of IL-6 protein in the supernatants (but not that of IL-8), and of icIL-1Ra1 protein in the lysates, were paralleled by mRNA levels in the cells (data not shown). The amounts of IL-1Ra measured in the cell supernatants in these experiments were: A431 cells, 1,2–15.5 ng/ml; HaCaT cells, 1.1–1.7 ng/ml; and KB cells, undetectable. All three cell lines contained equivalent amounts of CSN3 (Fig. 3F).
However, transfection of KB cells with icIL-1Ra1 reversed this pattern in which culture with IL-1α now resulted in very low levels of IL-6 and IL-8 production (Fig. 6). These results indicate that generation of high levels of icIL-1Ra1 in KB cells inhibited IL-
1α-induced production of IL-6 and IL-8, similar to the responses seen with A431 and HaCaT cells that endogenously make icIL-1Ra1.
Inhibition of phosphorylation of p38 MAPK
Our laboratory recently observed that icIL-1Ra1 transfected into Caco-2 intestinal epithelial cells inhibited IL-1-induced IL-6 and IL-8 production through inhibition of p38 MAPK and NF-κB pathways (30). To investigate the possible effects of differential icIL-
1Ra1 expression on the p38 MAPK pathway in keratinocyte cell lines, we performed Western blot analyses on A431 and KB cells. The A431 cells, expressing high levels of icIL-1Ra1, contained no detectable phosphorylated p38 MAPK over the first 30 min after stimulation with IL-1α (Fig. 5E). In contrast, KB cells expressed very little icIL-1Ra1 and demonstrated abundant phosphorylated p38 MAPK under the same conditions (Fig. 5E). The levels of total p38 MAPK remained constant in both cell lines. In addition, transfection of KB cells with icIL-1Ra1 led to decreased phosphorylation of p38 MAPK after stimulation with IL-1α (data not shown). These results suggest that the inhibitory effects of icIL-1Ra1 on IL-1α-induced IL-6 and IL-8 production in keratinocytes may be mediated, at least in part, through the p38 MAPK pathway, similar to Caco-2 cells.
Effects of siRNA to CSN3 and icIL-Ra1
To further explore the interaction between CSN3 and icIL-1Ra1, we used transfection of A431 cells with mixtures of siRNA to give specific posttranscriptional silencing of production of these intracellular proteins. Transfection of A431 cells with CSN3 siRNA led to a marked increase in the production of IL-6 and IL-8 in response to stimulation with IL-1α in comparison to cells transfected with a random siRNA and cultured with IL-1α (Fig. 7, A and B). The levels of icIL-1Ra1 production were not altered by this transfection (Fig. 7C) whereas the levels of CSN3 in the cells were reduced by 80% or greater (Fig. 7D). The levels of IL-1Ra in the supernatants in this experiment, as measured by ELISA, were 2.6–10.5 ng/ml.
An identical result was observed after transfection of A431 cells with icIL-1Ra1 siRNA. An increase in the levels of IL-1α-induced IL-6 and IL-8 produced were observed in comparison to cells transfected with the control siRNA (Fig. 8, A and B). Transfection with the icIL-1Ra1 siRNA led to a marked inhibition of icIL-1Ra1 protein production as determined by ELISA (Fig. 8C) or Western blot analysis, whereas the levels of CSN3 in the cells were not altered (Fig. 8D). The levels of IL-1Ra in the supernatants in this experiment were 4.3–10.8 ng/ml.
These results indicate that depletion of CSN3 or icIL-1Ra1 levels in A431 cells with specific siRNA led to enhanced production of IL-6 and IL-8 after IL-1α stimulation. However, the robust levels of IL-1α-induced IL-6 and IL-8 production in KB cells were not altered significantly after transfection with siRNA for CSN3 or icIL-1Ra1 (data not shown). Furthermore, the absence of CSN3 in KB cells, induced by the specific siRNA, did not further enhance the measured responses stimulated by IL-1α, suggesting that the observed inhibition in A431 cells required icIL-1Ra1 in the presence of CSN3 but was not due to CSN3 alone.
Discussion
The data presented in this study indicate that the major intracellular isoform of IL-1Ra, icIL-1Ra1, binds specifically to the third component of the CSN and inhibits activity of CSN-associated kinases. The interaction between icIL-1Ra1 and CSN3 was demonstrated to be important in cell function through experiments in keratinocyte cell lines in which icIL-1Ra1, in the presence of CSN3, blocked IL-1α-induced IL-6 and IL-8 production. Furthermore, these intracellular effects of icIL-1Ra1 may be mediated through inhibition of phosphorylation of p38 MAPK.
We have recently observed that expression of icIL-1Ra1 in human Caco-2 intestinal epithelial cells, normally lacking this protein, decreased by 50% the IL-1α-induced production of IL-6 and IL-8 through inhibition of the p38 MAPK and NF-κB pathways (30). Secretion of the leaderless icIL-1Ra1 occurs under certain conditions and is dependent on activation of the P2X7 receptor, identical with the mechanism for release of IL-1β (33). However, in the experiments with Caco-2 cells as well as in the studies with keratinocyte cell lines reported herein, the observed effects of icIL-
1Ra1 were not mediated through release from the cells and competing with IL-1 for binding to the membrane-bound IL-1 receptors. In the present experiments, the medium was removed and the cells were washed before culture with the stimulatory IL-1α. Up to 11 ng/ml IL-1Ra was released by the cells during the 24 h incubation with 10 ng/ml IL-1α. Fifty percent inhibition of IL-1 stimulation of cells requires 100–to 1000-fold excess amounts of IL-
1Ra (34). Thus, insufficient amounts of IL-1Ra were released by the cells in our experiments to account for the inhibition of IL-1-induced responses. Further support for the conclusion that icIL-
1Ra1 truly was acting inside the cells was found in the restoration of IL-1α-induced IL-6 and IL-8 production in KB cells treated with siRNA for CSN3, in which icIL-1Ra1 production and release of small amounts of protein remained intact.
It has been hypothesized that the CSN may provide a platform to connect cytoplasmic proteins, including molecules involved in signal transduction pathways, with ubiquitin-dependent protein degradation by the 26 S proteasome (23). One suggested mechanism is that the CSN replaces the lid of the 26 S proteasome, allowing ubiquitinated proteins to be channeled directly into the proteasome (35). The CSN is a positive regulator of E3 ubiquitin ligases and promotes removal of Nedd8 (deneddylation) from the cullin component (Cul1) of SCF ubiquitin ligases (36). The CSN may inhibit SCF ubiquitin ligase activity by targeting p27 proteolysis, thus negatively regulating the cell cycle at the G1 phase by promoting deneddylation of cullin component (37). Recent studies in Arabidopsis indicate that CSN3 deficiency may predispose to the intracellular accumulation of mult ubiquitinintiated proteins, suggesting that CSN3 in the intact complex may regulate proteasome-mediated protein degradation (38).

The other major function of the CSN is phosphorylation of various intracellular proteins by the activity of CSN-associated protein kinases. The icIL-1Ra1 molecule, and not any other isoform of IL-1Ra1, inhibited the crude CSN-associated kinase in phosphorylation of IkB, c-Jun, and p53. Like JNK, this kinase has previously been shown to phosphorylate c-Jun at Ser63 and Ser73 (24). icIL-1Ra1 also inhibited phosphorylation of p53 by recombinant CK2 and PKD, two serine/threonine kinases associated with the CSN through binding to CSN3 (29). The effect of icIL-1Ra1 on phosphorylation of p38 MAPK was not examined. In a recent study, inositol 1,3,4-triphosphate 5/6-kinase (a serine/threonine kinase) copurified with the COP9 signalsosome from calf brain (39). This kinase was shown to associate specifically with CSN1, both free and assembled in the intact CSN, and not with any other CSN component. Thus, three CSN-associated kinases have been described to date, with one binding to CSN1 and two binding to CSN3.

Three possibilities exist to explain the mechanism of icIL-1Ra1 inhibition of phosphorylation of various substrates by the CSN-associated kinases CK2 and PKD. The icIL-1Ra1 molecule could compete with the kinases for binding to CSN3, could sterically block kinase targets from coming into proximity with the enzyme, or could directly inhibit kinase activity. Our results indicate that the third possibility exists as icIL-1Ra1 inhibited in vitro activity of the recombinant CSN-associated kinases CK2 and PKD. However, it is not known whether icIL-1Ra1, in addition to inhibiting kinase activity of free and CSN-associated CK2 and PKD, also competes with these kinases for binding to CSN3.

Inhibition of CK2 and PKD by icIL-1Ra1 may affect some of the multiple activities of these protein kinases inside cells. CK2 may play an important role in regulating growth of normal and malignant cells as well as in promoting cell survival (40). CK2 forms a stress-induced complex with p38 MAPK, leading to activation of CK2 and phosphorylation of p53 (41). In addition, CK2 may mediate TNF-α- and IL-1-induced phosphorylation of IκB (42). PKD also modulates the activities of several kinase signal transduction pathways, including activation of NF-κB-dependent genes by acting downstream of IκB degradation (43, 44). The involvement of p38 MAPK and NF-κB pathways in cytokine induction of IL-6 and IL-8 production have been well described in multiple cell types, including keratinocytes, acting either through enhancement of transcription or stabilization of mRNA (45–49).

The results of our recent studies with Caco-2 cells suggest that one of the effects of icIL-1Ra1 could be inhibition of the p38 MAPK and NF-κB pathways (30).

CSN3, the CSN component interacting with icIL-1Ra1, is a 48 kDa protein that exhibits ~30% identity to the C-terminal region of S5, a regulatory subunit of the 26 S proteasome (24). CSN3 was identified by a yeast two-hybrid screen to interact specifically with IκB kinase γ, a nonenzymatic regulatory protein in the IκB kinase complex (50). Furthermore, CSN3 appeared to function as a negative regulator of TNF-induced but not IL-1-induced NF-κB activation in the human embryonic kidney 293 cell line. We could not

**FIGURE 5.** The levels of IL-1α-induced IL-6 and IL-8 production in keratinocyte cell lines were inversely related to the levels of icIL-1Ra1 in the cells. Unstimulated cells (control, □) and the cells after stimulation (■) with IL-1α are represented. A431, HaCaT, and KB cells were stimulated with 10 ng/ml IL-1α for 24 h with IL-6 (A) and IL-8 (B) production levels measured in the supernatants by ELISA; these data are expressed as nanograms per milliliter. C, The levels of icIL-1Ra1 in cell lysates were determined by ELISA with the data expressed as nanograms per milligrams of total protein. D, The levels of CSN3, icIL-1Ra1, and GAPDH in cell lysates also were determined by Western blot analysis. A431 cells (lane 1), HaCaT cells (lane 2), and KB cells (lane 3) are shown. The level of icIL-1Ra1 in KB cells was 90–95% less compared with A431 and HaCaT cells. E, Western blots of the levels of total and phosphorylated p38 MAPK in A431 and KB cells at serial times through 30 min after stimulation with 10 ng/ml IL-1α. A–C, The data are expressed as mean ± SEM from triplicates in one representative experiment of three performed, p < 0.001 for IL-6 (A) or IL-8 (B) produced by KB cells stimulated with IL-1α in comparison to unstimulated cells.
determine the possible effects of icIL-1Ra1 on TNF-induced effects in the keratinocyte cell lines used in our experiments because these cells failed to respond to TNF (data not shown).

Interactions between other cytoplasmic molecules and components of the CSN have been described. The most well characterized component of the CSN is CSN5, or Jun activation-domain binding protein 1 (JAB1), which binds many molecules including c-Jun, p53, p27, and Bcl3 (23). Jun is stabilized by its interaction with CSN5, and AP-1 is stimulated by interaction of CSN5 with the integrin LFA-1 (51). The specific hepatocyte growth factor hepatopoietin interacts with CSN5 leading to increased phosphorylation of c-Jun and enhanced AP-1 activity (52). In contrast, the cytokine migration inhibitory factor also interacts with CSN5 in the cell cytosol but inhibits CSN5-induced AP-1 activity without altering induction of NF-κB (53).

We propose the following scenario for the mechanism of anti-inflammatory effects of icIL-1Ra1 in keratinocytes (Fig. 9). IL-1 binding to IL-1R1 stimulates intracellular responses resulting in activation of the p38 MAPK and NF-κB pathways and to enhanced production of IL-6 and IL-8. Both sIL-1Ra and icIL-1Ra1 inhibit the stimulatory effects of IL-1 by competing for binding to cell surface IL-1R1. In addition, the interaction of icIL-1Ra1 with CSN3 in the cell cytosol may block the activities of necessary kinases in signal transduction pathways involved in IL-1-induced IL-6 and IL-8 production. The role of CSN3 in this proposed mechanism would be to bring icIL-1Ra1 into physical proximity with the appropriate kinases, resulting in their inhibition. Multiple kinases may

**FIGURE 6.** Inhibition of IL-1α-induced IL-6 and IL-8 production in KB cells after transfection with icIL-1Ra1. A, IL-1α-induced IL-6 and IL-8 in supernatants (ng/ml) of KB cells transfected with an empty vector (■) or with a vector containing cDNA for icIL-1Ra1 (▲). B, icIL-1Ra1 levels in lysates (ng/mg of total protein) of IL-1α-induced KB cells transfected with an empty vector (■) or a vector containing cDNA for icIL-1Ra1 (▲). A and B, The data represent mean ± SEM based on triplicates from one representative experiment of three performed. p < 0.001 for IL-6 (A), IL-8 (A), and icIL-1Ra1 (B) after transfection with a vector containing icIL-1Ra1 cDNA in comparison to an empty vector.

**FIGURE 7.** IL-1α-induced IL-6 and IL-8 production is enhanced in A431 cells expressing decreased levels of CSN3. A431 cells were transfected with specific siRNA for CSN3, then the medium was replaced before a 24 h culture with 10 ng/ml IL-1α. IL-6 (A) and IL-8 (B) levels (ng/ml) in cell supernatants. C, icIL-1Ra1 in cell lysates expressed as nanograms per milligram of total protein. D, Western blot analysis of cell lysates is shown. The levels of CSN3 were decreased ~80% after treatment with siRNA. A–C, Data are expressed as mean ± SEM based on triplicates in one representative experiment of three performed. p < 0.001 for levels of IL-1α-induced IL-6 (A) and IL-8 (B) production after transfection with CSN3 siRNA in comparison to transfection with the control siRNA.
be involved in phosphorylation of p38 MAPK and the relative roles of CK2 and PKD are not known. icIL-1Ra1 may indirectly, rather than directly, inhibit p38 MAPK phosphorylation by possibly blocking up-stream kinases in this pathway. Nevertheless, our results indicate that the major intracellular isoform of IL-1Ra may exhibit unique anti-inflammatory properties inside cells through a novel mechanism involving binding to CSN3, augmenting the IL-1R1 blocking activities of IL-1Ra in the cell microenvironment.

FIGURE 8. IL-1α-induced IL-6 and IL-8 production is enhanced in A431 cells expressing decreased levels of icIL-1Ra1. A431 cells were transfected with specific siRNA for icIL-1Ra1, then the medium was replaced before a 24 h culture and interleukin-1 receptor antagonist during differentiation of cultured keratinocytes. Exp. Cell Res. 217:355.


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