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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 Signalosome

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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 recombinant 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-1c-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-1c-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 peptide 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

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Caco-2 inhibited IL-1-induced IL-8 production (20). Intracellular expression of iCL-1Ra 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 line for proteins binding to IL-1Ra isoforms using a yeast two-hybrid screen. The third component of the COP9 signalosome (CSN3) was identified as a cytoplasmic protein that interacted specifically with iCL-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 Isc115, p105, c-Jun, and p53 (24). We characterized the binding of iCL-1Ra1 to CSN3 and observed that this intracellular isoform of IL-1Ra specifically inhibited the activity of CSN-associated kinases. Moreover, we observed that iCL-1Ra1 inhibited IL-1α-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 iCL-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 iCL-1Ra1 and iCL-1Ra3 were created by PCR cloning of the protein coding regions into the pEG202 vector producing an N-terminal LexA-iCL-1Ra fusion protein. The EGY strain of Saccharomyces cerevisiae, containing both Leu2 and lacZ reporter systems, was used as a recipient strain. The EGY48-EG202-iCL-1Ra1 strain was created following transformation of EGY48 with pEG202-iCL-1Ra1. A HeLa cell cDNA library, cloned in the pJG45 vector, was generated in the laboratory of Dr. Brent. The HeLa pJG45 cDNA library was introduced into EGY48-EG202-iCL-1Ra1 using standard yeast transformation methods. Transformants were recovered on glucose, Ura−His+Trp+ plates. The colonies were scarred 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 × 105 cells were plated on eight large (10 cm) galactose Ura−His+Trp+Leu+ plates to select for colonies that complemented the Leu auxotrophic mutation. After 4 days of growth at 30°C, ~1000 Leu+ colonies grew. Each colony was transferred to glucose Ura−His+Trp+ master plates and then replica plated onto glucose Ura−His+Trp−Leu+ plates, galactose Ura−His+Trp− plates, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) Ura−His+Trp− plates. Of the original 1000 colonies, ~350 showed galactose-dependent growth on Leu−medium and strong galactose-dependent blue color on X-Gal. Plasmid DNA from each of the selected clones was harvested by introduction into Escherichia coli KC8 cells and selecting transformants on Trp−ampicillin plates. The selected library plasmids were further classified by restriction patterns on 1.8% agarose TBE (Tris/boric acid/EDTA) gels after digestion with EcoRI, Xhol, and either AatII or HaeIII. To confirm the specificity of interaction, the yeast two-hybrid screen was repeated by reintroducing prey plasmids from each restriction enzyme class map into EGY48 containing non-specific bait plasmids cloned into pEG202-EG202-iCL-1Ra1, or pEG202-EG202-iCL-1Ra3. Fifty iCL-1Ra1-interacting proteins were identified following this screening procedure and their specificity was reconfirmed by a mating assay between EGY188 cells containing the cDNAs encoding the interacting proteins and EGY48-EG202-iCL-1Ra1 cells. The identity of the iCL-1Ra1-interacting proteins was assessed by sequencing the cDNAs and comparing their nucleotide sequences or in silico translated amino acids to the public genomic and protein sequences in GenBank and Swiss-Prot databases. Two proteins were identified that strongly interacted with iCL-1Ra1. 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).

Glycerol gradients, and Western and Far Western blots to characterize binding of iCL-1Ra1 to CSN3

Glycerol 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 krpm (SW40, 12-tube; 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 isoform. The blots were incubated with rIL-1Ra isoforms (1 μg/ml). After three washings 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, iCL-1Ra1, or iCL-1Ra3. When the OD600 of the cultured bacteria reached 0.7, they were induced with 100 μM of isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C with shaking. The cultured bacteria were harvested in PBS with 100 μM PMSF, then lysed by sonication and cleared of cellular debris by centrifugation. The supernatant was incubated with glutathione-Sepharose 500 μl/5 mg total protein overnight at 4°C. The beads were washed with PBS in a total volume of 35 ml and eluted with 10 ml of glutathione buffer (50 mM Tris-HCl, pH 8.0, 100 mM glutathione) for 4 h at 4°C. The eluted fusion proteins were then concentrated against PBS using Centricon Plus-20 (Millipore) with a 10,000 NMWL membrane. The proteins were then rebound to fresh glutathione-Sepharose, washed with PBS in a total volume of 10 ml, and resuspended in a 50% slurry.

HeLa cell lysates

HeLa cells were grown in DMEM with 10% FCS, penicillin (1 U/ml) and streptomycin (1 μg/ml) until confluency. The cells were scraped into PBS with a protease inhibitor mixture (Roche). The cells were frozen and thawed three times, 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

Expression of the three 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 lystate 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 iCL-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 IL-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 IL-1Ra isoforms were immunoprecipitated 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 a CSN3 polyclonal Ab bound 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 dialysed in PBS 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 isoforms 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 IgB, p53, or c-Jun in a kinase buffer (30 mM Tris-HCl, pH 7.8, 10 mM KCl, 0.5 mM DTT, 5 mM 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 × 104/well in MEM (Biological Industries). In a gradient in which the CSN was not included, icIL-1Ra1 using glycerol gradient centrifugation and Far Western blots. The results of a yeast two-hybrid screen identified the third component of the CSN as a specific interacting protein with icIL-1Ra1.

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-1α (R&D Systems), IL-6 or IL-8 (BD Biosciences). The lower limits of sensitivities for these ELISAs were: IL-6, 4.70 pg/ml; IL-8, 3.10 pg/ml; and IL-1α, 150 pg/ml. All ELISA readings were measured in triplicate for each supernatant and lysate 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, 1/600; 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 (1/40,000 dilution; Jackson ImmunoResearch Laboratories), and the secondary Abs for IL-1Ra and GAPDH were peroxidase-conjugated bovine anti-goat IgG (1/100,000 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 computerized densitometer (Molecular Dynamics). The percentage of increase or decrease was calculated by using the formula: [(activity of control siRNA – activity of siRNA for CSN3 or icIL-1Ra1)/activity 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 co-sediment 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 specific interaction; icIL-1Ra1 bound specifically to ∆CSN3 (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 lysate. After separation by
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). 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, 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, B).

FIGURE 1. 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 was collected, protein was precipitated with TCA, and then immunoblotted with recombinant icIL-1Ra1 (2 μg/ml). After washing, the blots were probed with a mAb to IL-1Ra. icIL-1Ra1 bound to the CSN in a glutathione pull-down experiment. 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 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).

FIGURE 3. Coimmunoprecipitation of the CSN with icIL-1Ra1. Recombinant preparations of icIL-1Ra1, icIL-1Ra3, or sIL-1Ra were incubated with HeLa cell lysates containing intact CSN. Immunoprecipitation was conducted using specific Abs to IL-1Ra or CSN1 coupled to Sepharose beads. The flow-through and Sepharose-bound fractions were boiled in SDS buffer and separated on SDS-PAGE. The presence of IL-1Ra or the CSN was detected by Western blot analyses using Abs reactive with all isoforms of IL-1Ra or specific to CSN1. The first column in the set of blots represents proteins in the eluate (free) and the second column proteins bound to the Sepharose column (bound). The data are compiled from different gels in two separate experiments.
IL-8 production through inhibition of p38 MAPK and NF-

A laboratory recently observed that icIL-1Ra1 transfected into Caco-2 intestinal epithelial cells inhibited IL-1-induced IL-6 and IL-8 production (Fig. 6). These results indicate 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-1β 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 100–1000-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.

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 production 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.
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 proteasome (36). The CSN is a positive regulator of E3 ubiquitin ligases and promotes removal of Nedd8 (deneddylation) from the proteasome (36). The CSN is a positive regulator of E3 ubiquitin ligases and promotes removal of Nedd8 (deneddylation) from the proteasome (36). The CSN is a positive regulator of E3 ubiquitin ligases and promotes removal of Nedd8 (deneddylation) from the proteasome (36). The CSN is a positive regulator of E3 ubiquitin ligases and promotes removal of Nedd8 (deneddylation) from the proteasome (36).

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-1Ra, inhibited the crude CSN-associated kinase in phosphorylation of cxK, 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 signalosome 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 IkB (42). PKD also modulates the activities of several kinase signal transduction pathways, including activation of NF-κB-dependent genes by acting downstream of IkB 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 S3, a regulatory subunit of the 26 S proteasome (24). CSN3 was identified by a yeast two-hybrid screen to interact specifically with IkB kinase γ, a nonenzymatic regulatory protein in the IkB kinase complex (50). Furthermore, CSN3 appeared to function as a negative regulator of TNF-α-induced but not IL-1-induced NF-κB activation in the human embryonic kidney 293 cell line.
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 upstream 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 with 10 ng/ml IL-1α. A–D. Data are expressed as described for Fig. 7. D. The icIL-1Ra1 protein levels were decreased ~80% or more after transfection with the icIL-1Ra1 siRNA (lanes 4 and 5). p < 0.03 for IL-6 (A), p < 0.01 for IL-8 (B), and p < 0.001 for icIL-1Ra1 (C) in cells transfected with icIL-1Ra1 siRNA in comparison to cells transfected with the control siRNA.

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References


