Human/mouse interleukin-1 receptor/receptor accessory protein interactions in IL-1β-induced NFκB activation

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Abstract We examined whether functional heterologous complexes between human IL-1RI (hIL-1RI) and murine IL-1R accessory protein (mIL-1RAcP) can be formed, utilizing human fibroblast HEK 293 cells and murine fibroblast C127 cells, non-transfected or stably transfected with hIL-1RI (C127-hIL-1RI), respectively. In non-transfected C127 cells, IL-1β signalled through the mIL-1RI-mIL-1RAcP complex and activated NFκB p50/p65 heterodimers. In C127-hIL-1RI cells, IL-1β signalled through the hIL-1RI and activated both p65/p65 and p50/p65 NFκB complexes, where only the activation of NFκB p65/p65 was dependent on mIL-1RAcP. Thus, clearly both homologous and heterologous IL-1RI-IL-1RAcP interactions support NFκB translocation, but with differences in signalling pattern.

Key words: Human interleukin-1 receptor type I; Mouse interleukin-1 receptor accessory protein; Nuclear factor κB; Heteroduplex

1. Introduction

Interleukin-1 (IL-1) binds to two subtypes of IL-1 receptors, the 80 kDa type I IL-1R (IL-1RI) and the 68 kDa type II IL-1R (IL-1RII) [1,2]. IL-1RI is a signalling receptor while IL-1RII is a decoy receptor for IL-1 [3,4]. After binding to IL-1RI, IL-1 activates intracellular signalling cascades including nuclear translocation of nuclear factor κB (NFκB) [5–8].

An IL-1R accessory protein (IL-1RAcP) has been described, which does not bind IL-1 itself, but associates with and increases the affinity of IL-1RI for its agonists [9]. The role of IL-1RAcP in IL-1RI mediated signalling is inferred from experiments in which IL-1β mediated NFκB translocation is restored upon IL-1RAcP cDNA transfer into an IL-1 non-responsive cell line containing an IL-1RI [10]. These mouse thymoma EL-4 D6/76 cells express the murine IL-1RI (as evidenced by RT-PCR results and binding of IL-1β) but lack the IL-1RAcP [11,12].

In the present study we have addressed the importance of IL-1RAcP in IL-1 mediated NFκB activation in an endogenously IL-1 responsive murine fibroblast cell line, C127. Moreover, this cell line has also been stably transfected with human IL-1RI (C127-hIL-1RI), to be able to compare the IL-1β binding and signalling through homologous and heterologous complexes of mIL-1RAcP and murine or human IL-1RI, respectively. We also examined heterologous hIL-1RI-mIL-1RAcP complex formations in human HEK 293 cells.

2. Materials and methods

2.1. Materials

Recombinant human IL-1β was a gift from Glaxo, Geneva, Switzerland. The monoclonal antibodies (mAbs) 4C5 (anti-mIL-1RAcP, rIgG2a), 35F5 (anti-mIL-1RI, rIgG1) and 4E2 (anti-mIL-1RII, rIgG2a) were kindly provided by Dr. R.A. Chizzonite, Hoffmann La Roche, Nutley, NJ, USA [9,13]. The anti-hIL-1RI antibody (P2), the human IL-1RI cDNA provided in the pDC303 expression vector and the hIL-1RI stably transfected murine fibroblast C127 (C127-hIL-1RI) were generous gifts by Dr. J.E. Sims, Immunex. HEK 293 and C127 cell lines were from ATCC. All reagents for cell culturing were purchased from Life Technologies. ^{125}I-rhIL-1β (2000 Ci/mmol) was bought from NEN and γ-^{32}P-ATP (5000 Ci/mmol) was from Amersham. All oligonucleotides were bought from Medprobe. Restriction enzymes and Pfu DNA polymerase were purchased from NBL and Stratagene, respectively. Antibodies directed against p65, p50 and c-Rel were from Santa-Cruz Biotechnology.

2.2. IL-1RAcP expression plasmids

The coding region of the murine IL-1RAcP cDNA was generated by RT-PCR amplification, using cDNA of the murine hypothalamic GT1-7 cell line [9]. The final PCR product was purified, cut and ligated into the *Not*I/*Xba*I site of the pcDNA1.neo vector (Invitrogen). The sequence of the cloned mIL-1RAcP was verified by restriction enzyme digestion and partial nucleotide sequencing.

2.3. Cell culture and transfection

C127 and 293 cells were cultured in modified Eagle medium, 10% (v/v) fetal calf serum, 100 μg/ml penicillin G and 100 μg/ml streptomycin. C127-hIL-1RI cells were selected with 250 μg/ml geneticin. The 293 cells were transiently transfected with various IL-1R expression plasmids by the calcium phosphate precipitation method [14]. Transfected cells were harvested 48–72 h later.

2.4. Electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared using a modified extraction method [15]. Cells were lysed in 10 mM Tris pH 7.3, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.5 mM β-mercaptoethanol and 0.4% (v/v) NP40. Nuclear pellet was then lysed in 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and the protein concentration was measured by the Bradford dye binding assay [16]. A double strand oligonucleotide (5′-TGACAGAGGGGACTTTCC-GAGAGGA-3′) was labeled with γ-^{32}P-ATP using T4 polynucleotide kinase and was separated from unincorporated labeled nucleotides by spin column purification (Chromaspin-10, Clontech). 5 μg nuclear proteins, 10^5 cpm of the labeled oligonucleotide, 2 μg poly(dIdC) (Pharmacia) and 1×binding buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 6% (v/v) glycerol) were incubated for 30 min at room temperature and subsequently electrophoresed through a 6% (w/v) polyacrylamide gel. The gel was dried and exposed to medical X-ray film. Competition experiments were performed by inclusion of a 50-fold molar excess of unlabeled probe. Supershift assays were per-

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Abbreviations: 293-hIL-1RI/mIL-1RAcP, 293 cells transfected with hIL-1RI and mIL-1RAcP cDNAs; P2, anti-hIL-1RI; 4C5, anti-mIL-1RAcP; 35F5, anti-mIL-1RI; 4E2, anti-mIL-1RII; C127-hIL-1RI, C127 cells transfected with human IL-1RI; HEK, human embryonic kidney; IL-1RI, IL-1 receptor type I; IL-1RAcP, IL-1R accessory protein; IL-1, interleukin-1; m, murine; h, human; NFκB, nuclear factor κB

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formed by including 1 μg of antisera against the individual NFκB/rel proteins (p65, p50, c-Rel) in the incubation mixture.

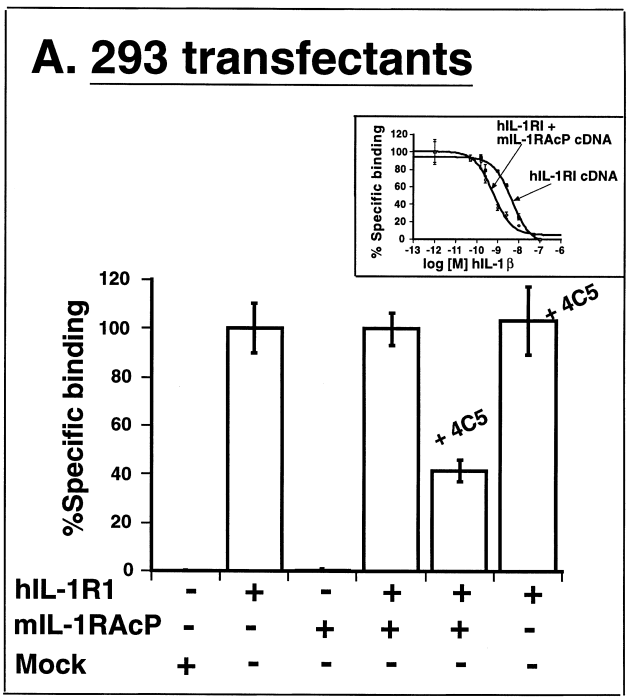
2.5. Displacement binding studies with ^{125}I-rhIL-1β

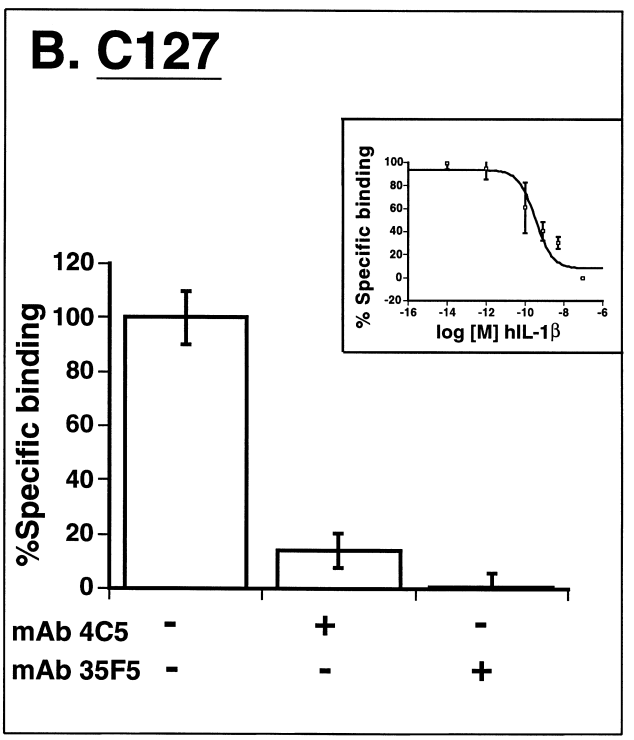
Confluent cells were resuspended in binding buffer (RPMI 1640 buffered with 25 mM HEPES pH 7.2, 1% (w/v) BSA, 0.1% (w/v) sodium azide). Displacement binding studies were carried out at room temperature for 1 h in a total volume of 100 μl containing 1.5×10^6 C127 cells or $1–3\times10^5$ C127-hIL-1R1 cells or transiently transfected 293 cells, incubated with 50 pM ^{125}I-rhIL-1β and different concentrations (1 pM–100 nM) either of rhIL-1β or of antibodies to different members of the IL-1R, IL-1RAcP complexes (see figures for concentrations). The binding data were analyzed using non-linear regression with the Kaleidagraph program.

3. Results

3.1. Human IL-1RI forms a complex with murine IL-1RAcP in transiently and stably transfected cells

To examine the ability of hIL-1RI and mIL-1RAcP to form a rhIL-1β binding heterologous complex, binding studies were performed with transiently and stably transfected cell lines.





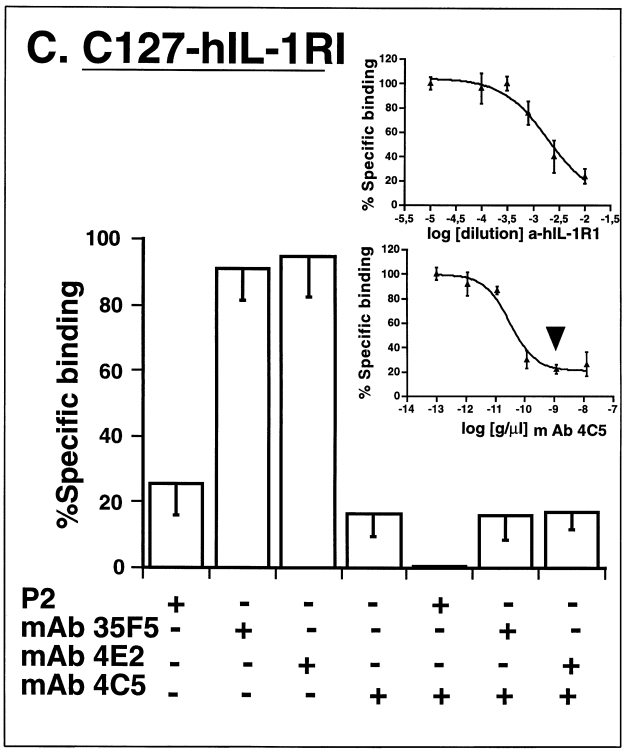


Fig. 1. Displacement of ^{125}I-rhIL-1β from transfected HEK 293, C127 and C127/hIL-1RI cells by rhIL-1β and different species and subtype selective anti-IL-1R and anti-IL-1RAcP antibodies. a: Displacement of ^{125}I-rhIL-1β (50 pM) by rhIL-1β (displacement curve) or by mAb 4C5 (anti-mIL-1RAcP) (10 μg/ml) (graph). Binding experiments were carried out with human fibroblast HEK 293 cells, transiently transfected with human IL-1RI cDNA, murine IL-1RAcP cDNA, human IL-1RI and murine IL-1RAcP cDNA, or mock cDNA, respectively. 100% specific binding was defined as the difference of bound ^{125}I-rhIL-1β in the absence and presence of 100 nM unlabelled rhIL-1β. Each incubation was performed in triplicate (displacement curve) or duplicate (graph) (±S.D.). The figure is representative of two experiments. b: Displacement of ^{125}I-rhIL-1β (50 pM) from murine fibroblast C127 cells by rhIL-1β (displacement curve), or by the mAb 4C5 (10 μg/ml) or mAb 35F5 (anti-mIL-1RI) (6.5 μg/ml) (graph). 100% specific binding was defined as the difference of bound ^{125}I-rhIL-1β in the absence and presence of 100 nM unlabelled rhIL-1β. Each incubation was performed in triplicate (±S.D.). The figure is representative of two experiments. c: Displacement of ^{125}I-rhIL-1β (50 pM) by P2 antiserum (anti-hIL-1RI) or mAb 4C5 (displacement curves) and the same antibodies (1000× dilution and 10 μg/ml, respectively) and in addition mAb 35F5 (6.5 μg/ml) and mAb 4E2 (anti-mIL-1RII) (1.1 μg/ml) (graph). Binding experiments were carried out with C127-hIL-1RI cells. 100% specific binding was defined as the difference of bound ^{125}I-rhIL-1β in the absence and presence of 100 nM unlabelled rhIL-1β. Each incubation was performed at least in duplicate and performed twice or more (displacement curves), and in triplicate with the experiment performed twice (graph) (±S.D.). The arrowhead indicates the concentration of the mAb 4C5 which was used in the IL-1 signalling experiments.

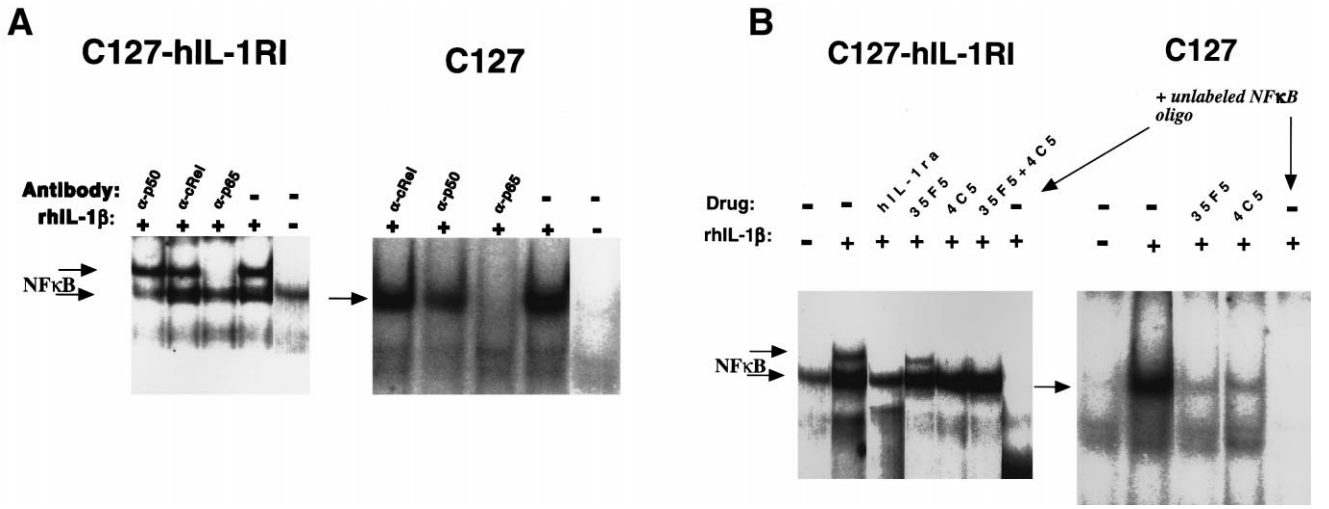


Fig. 2. IL-1β induced NFκB nuclear translocation in murine C127 and C127-hIL-1RI cells as assayed by EMSA. a: C127 and C127-hIL-1RI were treated with rhIL-1β (10 ng/ml, 15 min) and nuclear extracts were prepared. The identity of the NFκB proteins was determined by including monoclonal antibodies directed to the p65, p50 and c-Rel subunits of the NFκB/Rel family in the binding reaction. The resulting NFκB complexes which bound to the ^{32}P-labeled NFκB DNA probe were subsequently examined by gel electrophoresis and subsequent autoradiography. The arrows indicate NFκB complexes. b: C127 and C127-hIL-1RI were treated with either rhIL-1ra (2.5 μg/ml), mAb 4E2 (anti-mIL-1RII) (1.1 μg/ml), mAb 35F5 (anti-mIL-1RI) (6.5 μg/ml), mAb 4C5 (anti-mIL-1RAcP) (10 μg/ml) or a combination of mAbs 35F5 and 4C5, for 15 min prior to the addition of rhIL-1β (10 ng/ml, 15 min). Nuclear extracts were prepared and the presence of NFκB complexes was examined by gel electrophoresis and subsequent autoradiography. The arrows indicate the two rhIL-1β induced NFκB complexes.

HEK 293 cotransfected with the cDNAs encoding human IL-1RI and murine IL-1RAcP; 293-hIL-1RI/mIL-1RAcP bound ^{125}I-rhIL-1β with higher affinity (IC_{50} = 0.6 nM) compared to 293 cells transfected with only the cDNA encoding human IL-1RI, 293-hIL-1RI (IC_{50} = 5 nM) (Fig. 1A). On the other hand, HEK 293 cells transfected with the cDNA encoding murine IL-1RAcP, 293-mIL-1RAcP, did not show any detectable specific binding of ^{125}I-rhIL-1β. The mAb 4C5 (anti-mIL-1RAcP) displaced approximately 60% of the specifically bound ^{125}I-rhIL-1β to 293-hIL-1RI/mIL-1RAcP cells, whereas it did not displace any of the specifically bound ^{125}I-rhIL-1β to 293-hIL-1RI cells (Fig. 1A).

In C127 cells, 80 ± 10% of the specific binding of ^{125}I-rhIL-1β was displaced by mAb 4C5 and 100% of the specific binding of ^{125}I-rhIL-1β by the mAb 35F5 (anti-mIL-1RI) (Fig. 1B). From C127-hIL-1RI cells, which express the human IL-1RI in addition to the endogenously occurring murine IL-1RI, both antiserum P2 (anti-hIL-1RI) and mAb 4C5 displaced the specific binding of ^{125}I-rhIL-1β in a dose dependent manner, with 80% of maximal displacement (Fig. 1C). When combined, these antibodies displaced all specifically bound ^{125}I-rhIL-1β (Fig. 1C). In contrast, neither an excess of mAb 35F5 nor mAb 4E2 (anti-mIL-1RII) displaced more than 10% of the specific binding of ^{125}I-rhIL-1β (Fig. 1C).

3.2. IL-1β induced nuclear translocation of NFκB in C127-hIL-1RI cells

To test the functionality of the heterologous hIL-1R1 and mIL-1RAcP receptor complex formed in stably transfected murine C127 fibroblasts upon expression of the hIL-1RI, the IL-1β induced nuclear translocation of NFκB was assessed by EMSA. Incubation of C127 cells with rhIL-1β (10 ng/ml) for 15 min induced the DNA binding activity of a NFκB complex, which contained both p65 and p50 immunoreactive proteins (Fig. 2A). In C127-hIL-1RI cells rhIL-1β (10 ng/ml, 15 min) induced an additional p65 homodimeric (p65/p65) NFκB complex besides the heterodimeric p50/p65 NFκB complex whose activation was observed in C127 cells (Fig. 2A).

To further dissect the involvement of mIL-1RAcP in the rhIL-1β induced NFκB activation in C127 and in C127-hIL-1RI cells, we used species and subtype selective IL-1RAcP and IL-1R antibodies in an immunoneutralizing approach. In C127 cells, the rhIL-1β induced nuclear translocation of NFκB was blocked by treating the cells with either mAb 35F5 or mAb 4C5 (Fig. 2B). In C127-hIL-1RI cells, mAb 35F5 no longer affected the IL-1β induced NFκB translocation. The mAb 4C5, however, blocked the translocation of the slower migrating NFκB dimer (p65/p65) while leaving the faster migrating NFκB dimer (p50/p65) unaffected (Fig. 2B). Upon preincubation of C127-hIL-1RI cells with either rhIL-1 receptor antagonist (rhIL-1ra) or a combination of mAb 4C5, mAb 35F5 and P2 antiserum, all the rhIL-1β induced NFκB activation was inhibited (Fig. 2B and data not shown).

4. Discussion

The objective of the present study was to investigate whether heterologous complexes between hIL-1RI and mIL-1RAcP could form, and to compare the role of IL-1RAcP in IL-1β-induced NFκB activation in homologous and heterologous IL-1RI complexes, respectively.

Transfection of HEK 293 cells with the hIL-1RI cDNA gave rise to high specific binding of ^{125}I-rhIL-1β. Transfection with the mIL-1RAcP cDNA did not give rise to any additional ^{125}I-rhIL-1β binding sites while the cotransfection with hIL-1RI and mIL-RAcP cDNAs enhanced the affinity of rhIL-1β binding eightfold compared to HEK 293 cells transfected with the hIL-1RI cDNA alone. This is consistent with another study which found that cotransfection of mIL-1RI

and mIL-1RAcP increased the affinity of IL-1 binding fivefold as compared to free mIL-1R1 bound IL-1 [9]. Moreover, the binding of ^{125}I-rhIL-1β to the heterologous interacting mIL-1RAcP and hIL-1RI was partially blocked by the mAb 4C5 (anti-mIL-1RAcP) while the same mAb had no effect on ^{125}I-rhIL-1β binding to HEK 293 cells that are only transfected with hIL-1RI. These data clearly indicate a functional heterologous interaction between hIL-1RI and mIL-1RAcP at the level of ligand binding.

Functional consequences of heterologous complex formations between mIL-1RAcP and hIL-1RI were further examined using murine C127 fibroblasts, stably transfected with the hIL-1RI cDNA. In non-transfected C127 cells, the total number of ^{125}I-rhIL-1β binding sites is very low so that upon transfection with the hIL-1RI cDNA, whose expression is driven by the very strong CMV promoter, the predominant IL-1RI receptor is hIL-1RI in C127-hIL-1RI cells. This is clearly demonstrated by the ability of antisera P2 (anti-hIL-1RI) to displace 75 ± 10% of the specific binding of ^{125}I-rhIL-1β (due to lack of availability we could not use higher concentration of the P2 antisera, but it is possible that an even higher degree of inhibition could be reached). In C127-hIL-1RI cells, the hIL-1RI forms heterologous complex with the endogenous mIL-1RAcP since an excess of the mAb 4C5 can block 80 ± 10% of the specific ^{125}I-rhIL-1β binding. Combination of antiserum P2 and mAb 4C5 blocked all detectable specific binding. Thus we have shown using either transfection of a human cell line (HEK 293) with cDNAs encoding hIL-1RI and mIL-1RAcP, or in a murine cell line stably transfected with hIL-1RI cDNA, that heterologous complex between human IL-1RI and murine IL-1RAcP can be formed and that binding of ligand to these complexes could be inhibited by the mAb 4C5, and by the P2 antiserum, respectively.

In the non-transfected C127 cells, despite the low abundance of mIL-1RI, there are signalling complexes formed between mIL-1RI and mIL-1RAcP as evidenced by rhIL-1β stimulated NFκB nuclear translocation. Under these conditions, when mIL-1RI and mIL-1RAcP mediate the signal, the NFκB complex that is translocated to the nucleus is a p50/p65 heterodimer. This is in agreement with a recent study which found that IL-1 induced p50/p65 activation in EL-4 cells stably transfected with mIL-1RAcP cDNA [17]. When the same rhIL-1β concentration and incubation time are used with the C127-hIL-1RI cells, where now the majority of the rhIL-1β binding sites is the hIL-1RI-mIL-1RAcP heterologous complex, the type of NFκB activation is different, namely both p50/p65 and p65/p65 dimers are translocated to the nucleus. Hence, human IL-1RI is obviously a signalling receptor in the C127-hIL-1RI cells, since a new dimer of NFκB, a p65/p65 homodimer, is also translocated. The immunoneutralization experiments further support this view. In the C127 cells, the IL-1β induced nuclear translocation of p50/p65 dimers can be blocked either by the mAb 35F5 (anti-mIL-1RI) or by the mAb 4C5 (anti-mIL-1RAcP), suggesting that mIL-1RI cannot signal alone but must be in complex with mIL-1RAcP to elicit IL-1β induced NFκB nuclear translocation. This result is consistent with the studies of Wesche and colleagues [10,11]; they found that in an IL-1 non-responsive cell line expressing IL-1RI but not IL-1RAcP, IL-1 responsiveness was restored upon transfecting the cells with mIL-1RAcP cDNA [10].

In C127-hIL-1RI cells the signalling must occur predominantly via the hIL-1RI, which is in large excess, since the mAb 35F5 does not inhibit the translocation of either the p50/p65 or the p65/p65 NFκB complex. There is probably such an excess of hIL-1RI that it outweighs the mIL-1RAcP so that not all hIL-1RI is in complex with the mIL-1RAcP. This uncomplexed hIL-1RI alone may be responsible for the IL-1β induced translocation of the NFκB p50/p65 dimer, since mAb 4C5 did not block this translocation, while the same antibody blocked the hIL-1RI mediated nuclear translocation of NFκB p65/p65 dimers. It is possible that the hIL-1RI mediated NFκB p50/p65 activation is mediated via the lipid ceramide, as IL-1RI alone has been shown to mediate IL-1β activation of the neutral sphingomyelinase in an IL-1RAcP deficient cell line [17]. However, whether the rhIL-1β induced activation of the p50/p65 NFκB dimer, mediated through the hIL-1RI alone, arose as an artifact of the hIL-1RI over-expression system is impossible to tell from these experiments. Our experiments suggest, though, that hIL-1RI can signal either alone (p50/p65) or in complex with mIL-RAcP (p65/p65) in the hIL-1RI cDNA transfected murine C127 cells. Thus, in addition to enhanced rhIL-1β binding affinity for the human IL-1RI, the heterologous complexing with the murine IL-1RAcP also yields signalling complexes.

In summary, we have presented data supporting a necessary role of IL-1RAcP in mediating IL-1β induced NFκB activation through homologous IL-1RI complexes in murine fibroblast C127 cells. Moreover, human IL-1RI and murine IL-1RAcP could form functional rhIL-1β binding complexes which transduce an altered rhIL-1β-signalled NFκB activation compared to that mediated by homologous mIL-1RI/mIL-1RAcP complexes.

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