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Ligand-independent Homomeric and Heteromeric Complexes between Interleukin-2 or -9 Receptor Subunits and the γ Chain*

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Signaling via interleukin-2 (IL-2) and interleukin-9 receptors (IL-2R and IL-9R) involves heteromeric interactions between specific interleukin receptor subunits, which bind Janus kinase 1 (JAK1) and the JAK3 binding common γ chain (γ_c). The potential existence and roles of homomeric and heteromeric complexes before ligand binding and their modulation by ligand and JAK3 are unclear. Using computerized antibody-mediated immunofluorescence co-patching of epitope-tagged receptors at the surface of live cells, we demonstrate that IL-2Rβ, IL-9Rα, and γ_c each display a significant fraction of ligand-independent homomeric complexes (24–28% co-patching), whereas control co-patching levels with unrelated receptors are very low (7%). Heteromeric complex formation of IL2-Rβ or IL-9Rα with γ_c is also observed in the absence of ligand (15–30%). Ligand binding increases this hetero-oligomerization 2-fold but does not affect homo-oligomerization. Co-expression of IL-2Rα does not affect the hetero-oligomerization of IL-2Rβ and γ_c. Recruitment of γ_c into heterocomplexes is partly at the expense of its homo-oligomerization, suggesting that a functional role of the latter may be to keep the receptors inactive in the absence of ligand. At the same time, the preformed complexes between γ_c and IL-2Rβ or IL-9Rα promote signaling by the JAK3 A572V mutant without ligand, supporting a pathophysiological role for the constitutive oligomerization in triggering ligand-independent activation of JAK3 (and perhaps other JAK mutants) mutants identified in several human cancers.

Interleukin-2 and -9 (IL-2 and IL-9)[6] signaling pathways and receptors (IL-2R and IL-9R) are of high medical relevance in view of their important roles in the immune response and the frequent involvement of their loss or mutation in immunodeficiency and pathological autoimmune conditions (1–5). IL-2 is critically involved in regulating T cell proliferation (6). Loss of IL-2 (3), IL-2Rα (4), IL-2Rβ (5), or of the STAT5 transcription factor (2, 7) results in autoimmune diseases due to ineffective induction of anergy in peripheral T cells. IL-9 is known to induce proliferation and differentiation of mast cells as well as stimulation of murine T cell lymphomas (1). It also stimulates the proliferation of the B1 subset of B lymphocytes and of erythroid progenitors (8) and has been implicated in the induction of certain forms of asthma (9).

IL-2 and IL-9 act through binding to specific cell-surface receptors. The high affinity IL-2R is comprised of three separate chains, termed α (CD25), β (CD122), and γ (γ_c, CD132), which is a Janus tyrosine kinase 3 (JAK3)-interacting chain common to many cytokine receptors, including IL-2 and IL-9 receptors (10–15). IL-2Rα by itself has only low affinity to IL-2 and is not directly involved in signal transduction (16). On the other hand, in the absence of IL-2Rα, the IL-2Rβ/γ_c heterocomplex is sufficient to support IL-2 binding and signaling (17, 18). We, therefore, focused on the interactions of the latter subunits. In contrast, the IL-9R has only one ligand binding chain (designated IL-9Rα), which interacts with γ_c for signaling via activation of JAK3 (8, 19–21). Thus, the lack of a "modulatory" chain equivalent to IL-2Rα makes it interesting to compare the interactions between the subunits of the IL-9R and those of the IL-2R system.

Complex formation among the subunits of these receptors is an essential feature of their signaling, as they function as heteromeric complexes between the ligand binding receptor chain and the common γ_c that recruits JAK3 to the complex (20–22). Early fluorescence resonance energy transfer-based studies were conducted on IL-2R but not on IL-9R. In that study (23) the existence of preassembled heterocomplexes between α/β,

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[6] The abbreviations used are: IL, interleukin; IL-2R, IL-2 receptor; HA, influenza hemagglutinin; HBSS, Hanks' balanced salt solution; γ_c, γ chain; JAK, Janus tyrosine kinase; TpoR, thrombopoietin receptor; TβRII, type II transforming growth factor-β receptor; LHRE, lactogenic hormone response element; IRES, internal ribosome entry site; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; BSA, bovine serum albumin; STAT, signal transducers and activators of transcription; MESF, molecules of equivalent soluble fluorochrome; JAK1, Janus kinase 1.

β/γ_c, or α/γ_c of the IL-2R, which were modulated by ligand binding, was reported. However, the experiments were conducted mostly on cells expressing a high excess of IL-2Rα over the other subunits and did not explore homomeric complexes. Recent crystallographic studies on the quaternary structure of the soluble ectodomains of IL-2R subunits suggested that IL-2 first binds to IL-2Rα, enhancing IL-2 binding to IL-2Rβ, followed by recruitment of γ_c to the IL-2/IL-2Rβ complex (14, 15).

Despite the functional importance of IL-2R and IL-9R oligomerization, many aspects of the interaction between the subunits comprising these receptors and their potential modulation by ligand binding and/or JAK1 and JAK3 are still unclear, especially in their native milieu (the plasma membrane of live cells). We tackled these questions by combining computerized immunofluorescence co-patching (24, 25) to quantify both homomeric and heteromeric complex formation of IL-2R and IL-9R subunits with signaling assays in live cells. Our data demonstrate that the signaling subunits of both receptors (IL-2Rβ, IL-9Rα, and γ_c) display a subpopulation of preassembled homomeric complexes, which is not altered by ligand. On the other hand, hetero-oligomerization of both IL-2Rβ and IL-9Rα with γ_c existed before ligand binding but was significantly augmented by the relevant ligands, and IL-2Rβ/γ_c complex formation was insensitive to co-expression of IL-2R. The hetero-oligomerization of IL-2 and IL-9 receptor subunits with γ_c, even in the presence of JAK3, did not result in activation unless ligand was present, suggesting that oligomerization *per se* does not suffice for activation. However, we hypothesized that such preformed complexes could serve as scaffolds for activation of mutated JAK proteins, such as those recently described for JAK1 (26) and JAK3 (27). Indeed, we show that preformed complexes of IL-2Rβ or IL9Rα and γ_c support signaling by the constitutively active JAK3 A572V.

EXPERIMENTAL PROCEDURES

Cell Culture—BOSC23 cells (derived from HEK293T cells and expressing the gag, pol, and env proteins of the Moloney leukemia ecotropic virus (28)) and HEK293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Biological Industries Beit Haemek or Invitrogen) as described (29, 30). Ba/F3 cells (IL-3-dependent mouse pro-B cells) were maintained and transduced as described (31). BETT cells (human T-ALL cells) were maintained in RPMI medium containing 10% fetal calf serum.

Plasmids and Recombinant Virus Vectors—The human IL-2Rβ, IL-9Rα, and γ_c cDNAs were cloned in the pMX-IRES-GFP1.1 retroviral vector (32). The cDNAs encoding these proteins were cloned upstream of the internal ribosome entry site (IRES) followed by a GFP construct, such that transfection can be monitored by low level expression (at a level that does not interfere with regular immunofluorescence studies) of GFP (32, 33). Each construct was tagged extracellularly with either the 12CA5 influenza hemagglutinin (HA) tag (YPYDVPDYA) or the 9E10 Myc tag (EQKLISEEDL), with tags being introduced downstream of the predicted signal sequence cleavage site as described earlier (30). Thus, in IL-2Rβ the tags were inserted between residues Thr-31 and Ser-32, in IL-9Rα between residues Gln-26 and Gly-27 (designating the Met followed immediately by the signal sequence as 1, where the actual translation starts), and in γ_c between residues Ile-27—Leu-28. IL-2Rα cDNA (untagged) and human wild-type or JAK3 A572V mutant cDNAs were subcloned in pREX-IRES-CD4 (34). FLAG-tagged murine thrombopoietin receptor (TpoR) containing the IL-7R signal sequence (35) was cloned in pMX-IRES-GFP1.1 (30). Myc-tagged type II transforming growth factor-β (TβRII) in pcDNA3 was described (36).

Generation of Stable Cell Lines—High titer replication-defective retroviral supernatants were generated from BOSC23 cells (serving as a packaging cell line) after transfection with the retroviral bicistronic vectors encoding the constructs mentioned above as described (32). The viruses thus produced were used to transduce Ba/F3 cells by centrifugation in the presence of 4 μg/ml Polybrene (Sigma). The efficiency of infection was usually 40–50%. Populations of cells expressing the GFP marker above a predetermined level (top 10%) were isolated by FACS sorting.

Immunofluorescence Co-patching—BOSC23 cells were plated on glass coverslips in 6-well dishes (2×10^5 cells/dish). After 24 h, they were co-transfected with the different epitope-tagged IL-R subunits at various combinations using 1 μg of DNA of each construct complemented to 6 μg by empty vector. In some cases expression vectors for untagged IL-9Rα, IL-2Rβ, or IL-2Rα were also included (see Fig. 3 legend). Experiments were performed 44–48 h after transfection with calcium phosphate (Sigma). To measure receptor oligomerization, we employed antibody-mediated immunofluorescence co-patching (24, 37). Cells expressing pairs of receptors carrying different epitope tags (*e.g.* Myc-IL-9Rα together with HA-IL-9Rα) were washed twice with serum-free Dulbecco's modified Eagle's medium and incubated 30 min at 37 °C to allow digestion of serum-derived ligands. After washing twice with cold Hanks' balanced salt solution (HBSS) supplemented with 20 mM Hepes (pH 7.4) and 2% BSA (HBSS/Hepes/BSA; all from Sigma), the cells were incubated with normal goat γ globulin (Jackson ImmunoResearch Laboratories; 200 μg/ml, 30 min, 4 °C) to block nonspecific binding. This was followed by successive incubation in the cold (to avoid internalization) in HBSS/Hepes/BSA with primary anti-tag IgGs (20 μg/ml each, 45 min); that is, rabbit HA.11 against the HA tag (anti-HA) together with 9E10 mouse anti-Myc (both from Covance Research Products) followed by labeling/patching with secondary Alexa594-goat anti-rabbit and Alexa488-goat anti-mouse IgG (Invitrogen-Molecular Probes; 20 μg/ml each, 30 min, 4 °C). In some control experiments using a FLAG-tagged construct, mouse anti-FLAG IgG (M2; Sigma) was employed. The cells were washed and fixed in methanol (5 min, −20 °C) and acetone (3 min, −20 °C) and mounted in Prolong Antifade (Invitrogen-Molecular Probes). In experiments where ligands were present, human IL-9 (750 units/ml; produced in baculovirus as described in Renauld *et al.* (38)) or IL-2 (500 units/ml; Sigma) was added at 4 °C 30 min before labeling with IgGs and maintained during all subsequent incubations. For Ba/F3 cells, a similar protocol was employed, except that incubations with IgGs were carried out on cells in 100 μl of suspension. For washing, Ba/F3 cells were centrifuged for 1 min and resuspended in 100 μl of HBSS/Hepes/BSA. After antibody labeling,

the Ba/F3 cells were mounted onto poly-L-lysine-coated coverslips using cytospin centrifuge (5 min, medium speed) as described (34) followed by fixation in methanol and acetone.

Fluorescence digital images were recorded using a CCD camera as described (25). The Alexa488 (green) and Alexa594 (red) images were exported in TIFF format to Image-Pro Plus (Media Cybernetics) and subjected to quantitative analysis of the extent of co-patching using an algorithm we have recently developed (25). Briefly, the program segments the patches in a user-defined region of interest, subtracts the background, and identifies the center of each object in the green and red images. This is followed by nearest-neighbor analysis, calculating the distances from the center of green patch to that of the nearest red patch (and vice versa). Patches whose nearest neighbor is within one optical resolution unit (up to 0.2 μm) are considered colocalized (25). The % co-patching of *e.g.* green with red patches is given by dividing the number of the green patches colocalized with red patches by the total number of green patches. 20–25 cells were analyzed in each case.

Dual-luciferase Reporter Assay—HEK293 cells grown in 24-well plates were transfected using Lipofectamine 2000 (Invitrogen) with vectors encoding the IL-R ligand binding subunits and γ_c as well as wild-type or mutant JAK3 A572V where indicated (200 ng DNA/well each vector, replacing JAK3 vector with empty vector in controls). To test for STAT transcriptional activation, the STAT3-responsive or STAT5-responsive luciferase constructs (250 ng/well) were co-transfected into the cells together with 100 ng/well of a control vector (pRL-TK, expressing constitutively active Renilla luciferase; Promega) to calibrate for transfection efficiency. STAT5-mediated transcription was evaluated with the pLHRE-luc reporter gene constructs harboring tandem copies of the STAT5-inducible lactogenic hormone response element (LHRE) of the rat β-casein gene promoter, inserted upstream a luciferase gene (39). STAT3-mediated transcription was evaluated with pGL3-pap1-luc plasmid containing the luciferase gene under the control of the STAT3-inducible rat *Pap1* (pancreatitis-associated protein-1) promoter (40). After 4 h the cells were stimulated with 100 units/ml IL-2 or 100 units/ml IL-9 for 20 h and lysed with passive lysis buffer as described (31). Firefly (STAT reporter) and Renilla (control vector) luciferase activities were measured with the dual-luciferase reporter kit (Promega). To normalize for transfection efficiency, the results of firefly luciferase activity were divided by those of the respective Renilla luciferase activity.

FACS Analysis—BETT (T-ALL) cells or Ba/F3 cells expressing HA-tagged human IL-2Rβ and Myc-tagged human γ_c were washed in phosphate-buffered saline containing 0.1% fetal calf serum. 3×10^5 cells were stained by monoclonal anti-human IL-2Rβ (R&D systems) or anti-human γ_c (BD Biosciences) antibody coupled with phycoerythrin, according to manufacturer's recommendations (1 h, 4 °C). These antibodies detect both untagged endogenous receptor subunits and transduced tagged receptors and, thus, provide a means to compare cell surface levels. After washing by phosphate-buffered saline (PBS), cells were resuspended in 200 μl of PBS with 0.1% fetal calf serum, and cell surface expression of human IL-2Rβ and human γ_c was analyzed on 10^4 cells by a BD FACSCalibur^{TM} flow cytometer (BD Biosciences). An aliquot of non-stained cells was used as autofluorescence control. We employed quantitative fluorescence cytometry to measure absolute levels of receptors at the cell surface expressed as molecules of equivalent soluble fluorochrome (MESF values). To the obtained MESF values Sphero Rainbow Calibration particles were used to calibrate the flow cytometer scale. The mean/median relative fluorescence intensity channel value for each bead population was calculated using Cell Quest software. A calibration curve was constructed, and subsequent data from the analyzed samples obtained from mean/median relative fluorescence intensity were converted to MESF for each antibody. All data were corrected for autofluorescence quantified in the same way.

RESULTS

Cell-surface IL-2Rβ, IL-9Rα, and γ_c Form Ligand-independent Homomeric Complexes—In view of the proposed importance of the oligomeric state of IL-2 and IL-9 receptors for their activation, we conducted experiments to determine whether the ligand binding chains of these receptors (IL-2Rβ and IL-9Rα) form stable homo-oligomers before ligand binding or only in the presence of ligand (ligand-induced oligomerization). To detect the formation of homomeric complexes of the intact receptor chains in their natural environment (the plasma membrane of live cells), we employed a computerized digital analysis version of immunofluorescence co-patching (Ref. 25; see "Experimental Procedures"). This method, which we have formerly used to demonstrate homo- and hetero-oligomerization of several transmembrane receptors (24, 30, 41), is based on the expression at the surface of live cells of two receptors tagged with different epitope tags at their extracellular termini. One receptor is forced into micropatches by a double layer of bivalent IgGs using a fluorescent (*e.g.* green) secondary IgG. The co-expressed receptor, which carries a different epitope tag, is patched/labeled by anti-tag primary IgG from another species and secondary antibodies coupled to another fluorophore (red). Receptors in mutual oligomers are swept into mutual micropatches, which appear yellow when the two images are overlapped. To measure the homo-oligomerization of IL-2Rβ, IL-9Rα, or γ_c, we co-expressed Myc and HA-tagged versions of each protein in BOSC23 cells and subjected them to immunofluorescence co-patching studies in the absence or presence of ligand. Co-patching was determined by algorithms written for Image-Pro Plus, defining green and red patches as overlapping if their intensity peaks were separated by less than 0.2 μm (25). To evaluate the level of random co-patching (uncorrelated overlap of patches) in the same image (which depends on the surface density of the patches), a "randomized" region of interest was created by overlaying the green image of a given region of interest upon the red image of an identically sized neighboring region (see Fig. 1, *C* and *G*). This way, any co-localization reflects random overlap of patches due to their finite density at the cell surface. These randomized values can then be subtracted to obtain the actual co-patching (% co-patching). Typical images showing the co-patching between differently tagged forms of the same receptor chain (IL-9Rα or IL-2Rβ) in the absence of ligand are shown in Fig. 1. Similar experiments were conducted in the presence of IL-2 or IL-9.

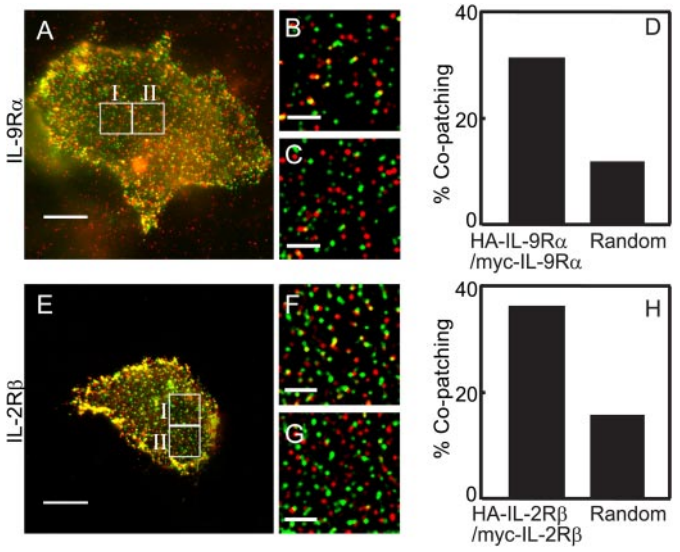

FIGURE 1. **Typical immunofluorescence co-patching images of homomeric complex formation between IL-2Rβ or IL-9Rα subunits in the absence of ligand.** BOSC23 cells were co-transfected with pairs of pMX-IRES-GFP1.1 expression vectors encoding HA- and Myc-tagged versions of IL-9Rα or IL-2Rβ. After 44–48 h, live cells were labeled consecutively in the cold by a series of antibodies to mediate patching and fluorescent labeling, as described under "Experimental Procedures." The patching/labeling protocol resulted in Alexa 594-labeled HA-tagged receptors (*red fluorescent patches*) and Alexa 488-labeled Myc-tagged receptors (*green patches*). *A* and *E*, typical images of cells subjected to co-patching. The *white borders* depict regions of interest that were analyzed for co-patching (*panels B* and *F*) and for randomized co-localization (*panels C* and *G*). *Bars*, 10 μm in *panels A* and *E* and 2 μm in *panels B*, *C*, *F*, and *G*. *B* and *F*, zoomed images of region I in *panels A* and *E*, respectively. Using an algorithm written for Image-Pro Plus, a *green* and *red patch* were defined as overlapping (co-patched) if their centers were separated by less than 0.2 μm (25). *C* and *G*, zoomed randomized images to evaluate random overlap between the *red and green patches* (control for occasional overlap due to finite density of patches). Random images were created from two adjacent regions of interest by merging the green channel of region I with the unrelated red channel of region II. *D* and *H*, computer analysis results of the specific images shown for total and randomized % co-patching of IL-2Rβ (*panel D*) or IL-9Rα (*panel H*).

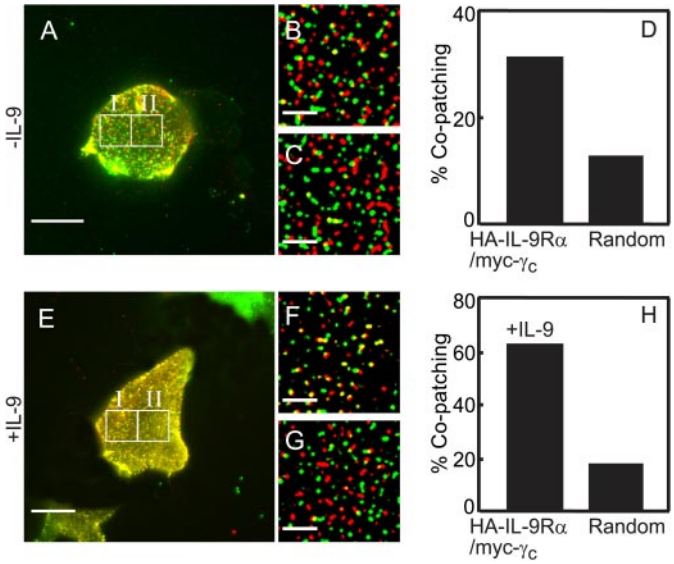

FIGURE 2. **Representative immunofluorescence co-patching images showing heterocomplex formation between IL-9Rα and γ_c.** The experiments were conducted as in Fig. 1 on cells co-expressing HA-IL-9Rα and Myc-γ_c. The IgG labeling/patching protocol resulted in HA-IL-9Rα in *red patches* and Myc-γ_c in *green patches*. *A* and *E*, typical images of cells subjected to co-patching in the absence (*panel A*) or presence (*panel E*) of IL-9. In the latter case the ligand (750 units/ml) was added for 30 min at 4 °C before antibody labeling and maintained during all subsequent incubations. The *white borders* depict regions of interest that were analyzed for co-patching (*panels B* and *F*) and for randomized co-localization (*panels C* and *G*). *Bars*, 10 μm in *panels A* and *E* and 2 μm in *panels B*, *C*, *F*, and *G*. *B* and *F*, zoomed images of region I in *panels A* and *E*, respectively, processed for co-patching by Image-Pro Plus as described in Fig. 1. *C* and *G*, zoomed randomized images to evaluate random overlap between red and green patches, constructed from the green channel of region I and the unrelated red channel of region II. *D* and *H*, computer analysis results of the specific images in the absence (*B* and *C*; shown in *panel D*) or presence (*F* and *G*; shown in *panel H*) of ligand.

Quantitative average data derived from many such experiments with IL-2Rβ, IL-9Rα, or γ_c both in the absence and presence of ligands are depicted in Fig. 3*A*. These studies demonstrate that 23–29% of each of the receptor subunits examined are already co-patched in the absence of ligand. It should be noted that if one assumes that the homo oligomers are dimeric, the percentage of homodimerization is higher than the percentage of co-patching by a factor of 3/2; as discussed by us earlier (30, 37), this occurs because homodimers containing identically tagged receptors may form but would not be swept into mutual patches. Interestingly, for all the receptor subunits examined, the homo-oligomerization did not increase significantly upon ligand binding. In control experiments (an HA-tagged IL-R chain co-expressed with an unrelated receptor-Myc-TβRII or FLAG-TpoR) the co-patching level was very low (5–10%; Fig. 3*A*).

Hetero-oligomers of IL-2Rβ or IL-9Rα with γ_c Form before Ligand Binding and Are Enhanced by Ligand—Because IL-2 and IL-9 signal transduction is mediated via hetero-oligomerization between the ligand binding chain and γ_c in the presence of ligand (8, 17–19), we proceeded to measure the hetero-oligomerization of IL-2Rβ or IL-9Rα with the common γ_c chain. BOSC23 cells were co-transfected by HA-IL-9Rα or HA-IL-2Rβ together with Myc-γ_c. The cells were then subjected to immunofluorescence co-patching studies as in Fig. 1 in the presence or absence of the respective ligands. Fig. 2 shows representative results of co-patching experiments obtained for HA-IL-9Rα heterocomplex formation with γ_c; analogous results were obtained for the association of HA-IL-2Rβ with γ_c. The average data of many such experiments are summarized in Fig. 3*A*. As shown in these figures, both IL-2Rβ and IL-9Rα had a subpopulation of receptors (15% for IL-2Rβ, 25% for IL-9Rα) residing in preformed heteromeric complexes with γ_c before ligand binding. However, unlike the situation encountered for homodimerization of the ligand binding subunits, the addition of the respective ligands enhanced the heterodimerization measured on live cells by nearly 100%, demonstrating ligand-mediated heterocomplex formation.

In view of the role IL-2Rα chain has in enhancing ligand binding to IL-2Rβ, we also examined whether overexpression of IL-2Rα enhances IL-2Rβ/γ_c hetero-oligomerization. As shown in Fig. 3*A*, no significant effects were observed either in the absence or presence of ligand, suggesting that the basic interactions between IL-2Rβ and γ_c are largely unaffected.

A large fraction of the purified γ_c ectodomain was reported to exist in homo-oligomers, which were proposed to dissociate during its association into heterocomplexes with the other IL-2R chains (15). We, therefore, examined in our system whether co-expression with untagged IL-2Rβ or IL-9Rα

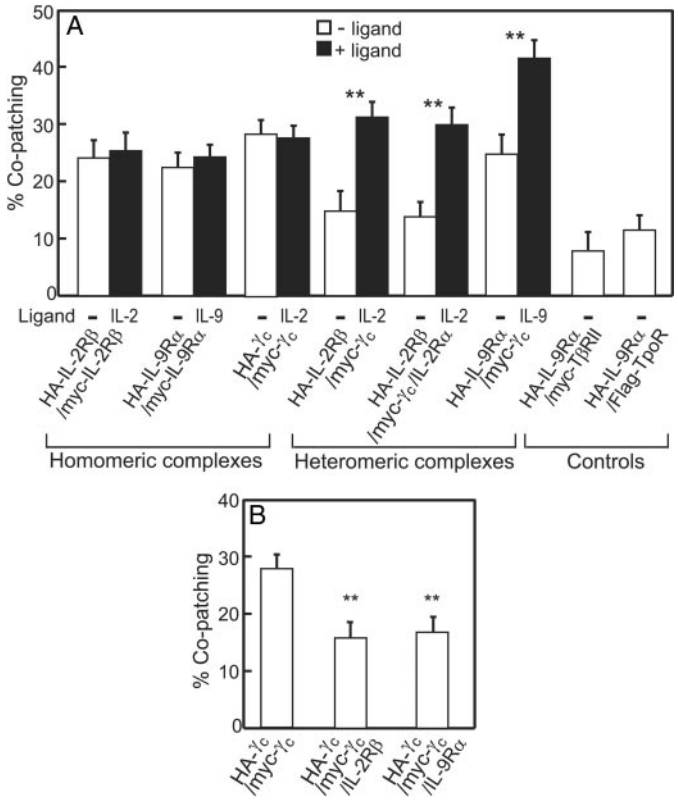

FIGURE 3. **Quantification of the co-patching experiments between the different IL-R subunits.** Co-patching experiments were conducted as in Fig. 1 (homo-oligomerization) and in Fig. 2 (heteromeric co-patching) on cells co-expressing two differently tagged versions of IL-R subunits. Regions of interest were counted on 20 cells per sample, counting the numbers of *red* (*R*), *green* (*G*), and overlapping (*yellow*, *Y*) patches by Image-Pro as described in Figs. 1 and 2. The % co-patching (% of a receptor carrying a given tag in mutual patches with a receptor carrying another tag) is given by $100 \times [Y/(Y + R)]$ or $100 \times [Y/(Y + G)]$ for the red- and green-labeled receptors, respectively. Because these values were very close, only one mean ± S.E. value is depicted for each pair. The randomized control co-patching (15–17%) for each pair was subtracted. *A*, IL-2 and IL-9 selectively enhance γ_c heteromeric co-patching with IL-2Rβ or IL-9Rα. Homomeric co-patching was measured on cells co-transfected by pairs of vectors encoding HA- and Myc-tagged versions of the same IL-R subunits. Heteromeric co-patching was measured between HA-IL-2Rβ or HA-IL-9Rα and Myc-γ_c. To measure the potential effect of IL-2Rα, expression vector encoding untagged IL-2Rα was included at a 4-fold excess over the vectors encoding HA-IL-2Rβ and Myc-γ_c (*middle pair of bars* of heteromeric complexes). Control experiments for co-patching between an IL-R subunit (*HA-IL-9R*α) and an unrelated receptor (*Myc-TβRII* or *FLAG-TpoR*) are shown on the *right*; similar results (not shown) were obtained with HA-IL-2Rβ. Incubation with ligands (IL-2 or IL-9) was as in Fig. 2. *Asterisks* indicate a significant increase in the level of co-patching upon the addition of ligand (**, $p < 2 \times 10^{-4}$, Student's *t* test). *B*, co-expression with IL-2Rβ or IL-9Rα reduces the homomeric co-patching of γ_c. HA-γ_c and Myc-γ_c were co-expressed with or without a 4-fold excess of untagged IL-2Rβ or IL-9Rα. *Asterisks* indicate a significant reduction in the homomeric co-patching of γ_c (**, $p < 3 \times 10^{-4}$).

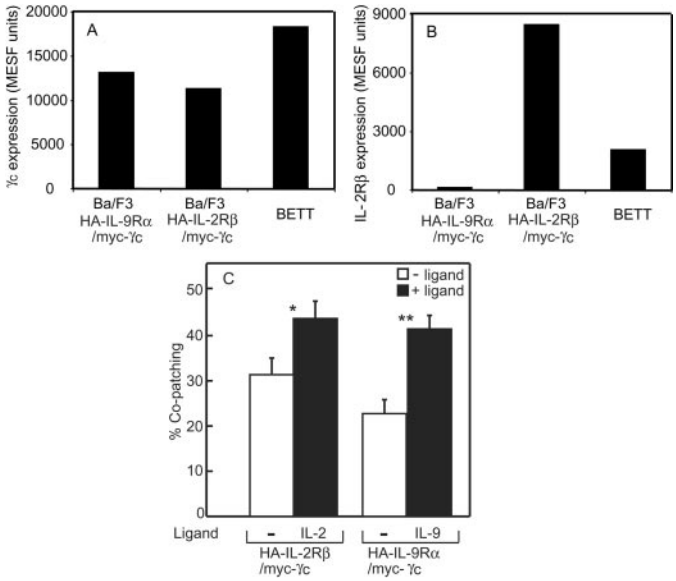

FIGURE 4. **Heteromeric co-patching of IL-2Rβ and IL-9Rα with γ_c in stably expressing Ba/F3 cells.** *A* and *B*, comparative cell surface localization of γ_c and IL2-Rβ between Ba/F3 cells stably transduced with Myc-γ_c or HA-IL2-Rβ and human T-ALL BETT cells expressing endogenous γ_c and IL-2Rβ chains. Cell surface localization of the respective chains was detected by FACS using antibodies recognizing the amino terminus region of γ_c (*A*) or of IL-2Rβ (*B*). Cell surface fluorescence was analyzed on 10^4 cells by a BD FACSCalibur™ flow cytometer (BD Biosciences). Quantitative fluorescence cytometry was employed to measure absolute levels of receptors at the cell surface expressed as *MESF* values, as described under "Experimental Procedures." *C*, co-patching experiments. Ba/F3 cells stably expressing HA-IL-2Rβ or HA-IL-9Rα together with Myc-γ_c were subjected to co-patching experiments in the presence or absence of ligands, as described in Fig. 3. *Bars*, mean ± S.E. of experiments on 20 cells in each case. *Asterisks* indicate a significant increase in the level of co-patching upon the addition of ligand (*, $p < 0.04$; **, $p < 1 \times 10^{-4}$).

reduces the level of homomeric γ_c complexes. As shown in Fig. 3*B*, co-expression with either IL-2Rβ or IL-9Rα significantly reduced the co-patching levels of HA-γ_c/Myc-γ_c (from 28 to 17–18%), suggesting that association of γ_c into heterocomplexes is indeed at the expense of its homo-oligomerization.

Oligomerization Results Are Confirmed in Hematopoietic Cells Stably Expressing Near-physiologic Levels of Tagged Receptor Subunits—The results in Fig. 3 were obtained in experiments on transiently expressing BOSC23 cells. Although the receptor expression levels are not very high due to the use of retroviral vectors, which harbor relatively weak promoters, they still represent overexpression as compared with the endogenous levels of the receptor subunits. To test whether some or all of the heteromeric complexes measured under these conditions are assembled due to overexpression, we prepared stable cell lines expressing tagged receptors. We have chosen the murine pro-B cell line Ba/F3 (42) and infected them with replication-defective retroviruses encoding the different epitope-tagged receptor chains ((33) see "Experimental Procedures"). In this manner we have created Ba/F3 cell lines stably co-expressing HA-IL-9Rα or IL-2Rβ together with Myc-tagged γ_c. These cells were subjected along with T-ALL cells that express endogenous receptors to FACS analysis for determining their cell surface levels of tagged receptors by using antibodies directed to the amino terminus of the receptors. The FACS analysis showed that for IL-2Rβ and for γ_c the tagged receptors were localized at the cell surface at levels within the same range to those normally expressed by the human T-ALL BETT cells, with γ_c being lower and IL-2Rβ being higher but within the same level of magnitude (Figs. 4, *A* and *B*). The stably transduced Ba/F3 cells were then subjected to immunofluorescence co-patching experiments in the absence or presence of ligands (IL-2 or IL-9). The results (Fig. 4*C*) were rather similar to those obtained in the transient expression experiments on BOSC23 cells, showing an even higher level of heteromeric co-patching for IL-2Rβ/γ_c and a somewhat lower (but still significant) level for IL-9Rα/γ_c co-patching. This suggests that the basic phe-

nomenon, namely a certain degree of pre-existing hetero-oligomerization, which significantly increases upon ligand binding, is reproducible in stable cell lines and is not due to excessive overexpression.

Preformed Oligomers of IL-9Rα or IL-2Rβ and γ_c Promote Signaling by Constitutively Active JAK3 A572 Mutant—Cytokine receptors lack intrinsic enzymatic activity and use cytosolic JAK proteins to phosphorylate tyrosines on the cytokine receptors themselves, STAT proteins, and additional proteins involved in signaling (43). This induces transcriptional activation of many genes (44, 45). JAK3 is recruited to interleukin receptor complexes via γ_c (20–22). We compared signaling by IL-2 and IL-9 receptor complexes in the presence and absence of JAK3 and the constitutively active JAK3 A572V mutant, which was recently described to be oncogenic and constitutively active (27). We co-transfected HEK293 cells (which are devoid of JAK3 but express the ubiquitous JAK1) with various combinations of cDNAs coding for JAK3, the ligand binding chains, and γ_c. To measure STAT transcriptional activation, two luciferase reporter constructs were included, one responsive mainly to STAT3 (pGL3-pap1-luc) and the other to STAT5 (pLHRE-luc), as well as the constitutively active Renilla luciferase plasmid pRL-TK). As shown in Fig. 5, *A* and *B*, JAK3 co-expression was necessary for transcriptional activation response to either IL-2 or IL-9; co-expression of the receptors in the absence of JAK3 was not sufficient to produce increased signaling unless JAK3 was also expressed. Signaling by IL-2 and IL-9 was marginally increased by providing extra-JAK1 to the endogenous levels in these cells, whereas under these conditions there was no detectable signaling in the absence of ligands (co-expression of specific chains, γ_c, JAK1, and JAK3 (*empty bars*, *right-most columns* of Fig. 5, *A* and *B*).

We the tested the effect of replacing JAK3 with the constitutively active A572V mutant. Like JAK2 V617F, which contains an intact FERM domain and which requires interaction with cytokine receptors for signaling, JAK3 A572V is predicted to interact with γ_c. In Fig. 5, *C* and *D*, we show that co-expression of JAK3 A572V with specific chains and JAK1 leads to significant constitutive signaling both in the case of IL-2Rβ and γ_c and IL-9Rα and γ_c complexes. Interestingly, preformed heteromeric complexes are required for constitutive signaling by JAK3 A572V, as no transcriptional activity was detected when only specific chains or only γ_c were expressed. These data suggest that preformed heteromeric oligomerization might provide an important support for mutated JAKs to induce signaling in the absence of ligand.

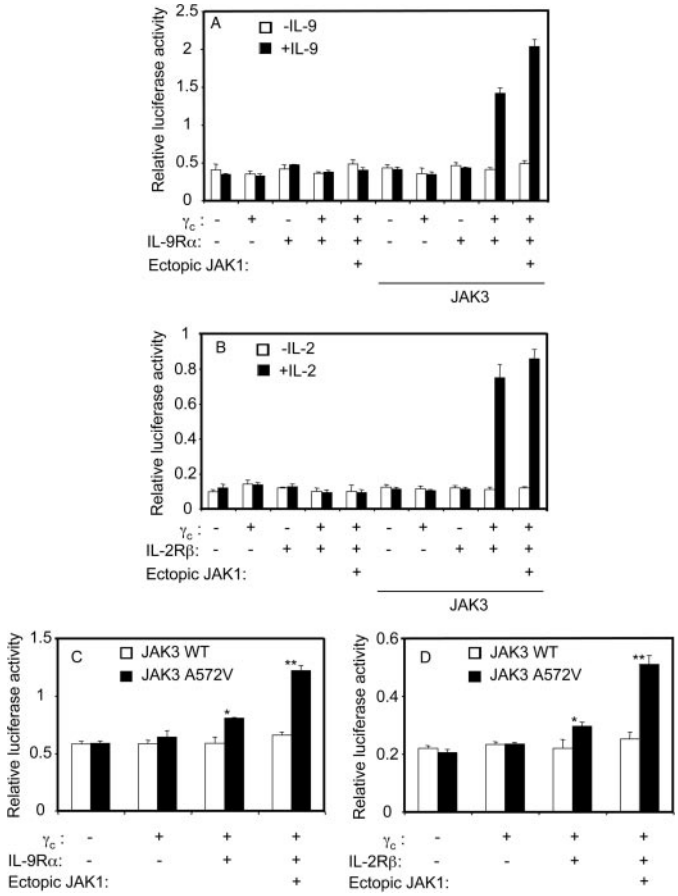

FIGURE 5. **Luciferase transcriptional activation assay demonstrating that JAK3 expression is required for IL-2 and IL-9 response and that preformed heteromeric complexes promote constitutive signaling by JAK3 A572V.** HEK293 cells, which do not express endogenous JAK3, were co-transfected with pMX-IRES-GFP1.1 vectors encoding HA-tagged IL-2Rβ or IL-9Rα in various combinations with HA-γ_c, JAK3, JAK1, JAK3 A572V, the STAT-responsive luciferase reporter vectors pGL3-pap1-luc (for STAT3 in the case of IL-9Rα, *panel C*), pLHRE-luc (for STAT5 in the case of IL-2Rβ, *panel D*), and the pRL-TK constitutively active Renilla luciferase vector. *A* and *B*, 4 h post-transcription, the cells were incubated overnight with ligands (*solid bars*) or with vehicle control (*empty bars*) and subjected to luciferase assays ("Experimental Procedures"). Results are the mean ± variation of duplicate samples, which are representative of three independent experiments. *C* and *D*, HEK293 cells were transfected with cDNA coding for HA-tagged IL-2Rβ or IL-9Rα in various combinations with HA-γ_c along with cDNAs coding for JAK3 (*empty bars*), JAK3 A572V (*solid bars*), and when indicated the cDNA coding for JAK1. Shown are the means ± S.E. of two experiments, each performed in duplicate. C, *, $p < 0.02$; **, $p < 3 \times 10^{-4}$. *D*, *, $p < 0.04$; **, $p < 3 \times 10^{-4}$ (indicate a significant increase in the luciferase activity).

DISCUSSION

In the studies reported here we investigated homomeric and heteromeric complex formation among IL-2 or IL-9 receptor chains situated at the plasma membrane of intact cells. Our key finding is that all the IL-R subunits examined (IL-2Rβ, IL-9Rα, and γ_c) display a fraction of homomeric complexes at the cell surface independent of ligand binding. Heteromeric complexes between IL-9Rα or IL-2Rβ and γ_c are also formed in the absence of ligand, but unlike the insensitivity of the homomeric complexes to ligand, heteromeric complex formation was increased by 100% after ligand binding. The latter findings in transiently transfected BOSC23 cells were also validated in Ba/F3 cells stably expressing near-physiologic levels of tagged IL-R subunits. Importantly, the need for ligand binding to activate even the preformed heteromeric complexes of IL-2Rα or IL-9Rβ with γ_c suggests that the oligomeric interfaces between IL-9Rα or IL-2Rβ and γ_c must differ between the unliganded and liganded receptor complexes. Although strictly inactive, we propose that the preformed heteromeric complexes may act to prime signaling by low levels of ligands. This is reminiscent of the case of the erythropoietin receptor, where preformed dimers were also detected by immunofluorescence co-patching (30, 37) and by fluorescence resonance energy transfer (46). These preformed dimers require a conformational change for

activation (47) in order to impose a productive dimeric interface necessary to activate JAK2.

The immunofluorescence co-patching studies on homo-oligomerization (Figs. 1 and 3) demonstrate that significant fractions of IL-2Rβ, IL-9Rα, or γ_c reside in homomeric complexes before ligand binding. The co-patching observed for the homomeric interactions of each chain was rather similar (23–29%; Figs. 1 and 3). As discussed earlier (30, 37), the % co-patching is identical to the percentage of dimerization in the case of heterodimers (where each interacting chain is necessarily labeled by a different tag) but not in the case of homo-oligomers. Thus, for a pure homodimeric population, the statistical prediction is for 66.6% (2/3) co-patching, as homodimers containing identically tagged chains may also form but would not be swept into mutual patches with similar chains carrying the other tag. For a homodimer, the fraction of "same tag" complexes is ⅓, leaving ⅔ as differently tagged dimers. Thus, 23–29% co-patching would actually reflect a proportion of 37–44% of receptor chains in homodimers. This level is significant, especially because it is obtained after the subtraction of the randomized co-localization of patches, which is enabled by the new computerized approach.

Importantly, for all the above receptor chains, the homomeric co-patching levels were not affected by ligands (IL-2 or IL-9), demonstrating that the homomeric complexes are ligand-independent. This situation is different from that reported for the epidermal growth factor receptor, where dimerization is ligand-dependent (48). However, it is in accord with earlier reports by us and by others, where the unliganded erythropoietin receptors were found to form homodimers at high efficiency (30, 47, 49, 51). Moreover, our present finding that a significant fraction of γ_c is in homo-oligomers (Fig. 3) is in accord with a recent report that the purified ectodomain of γ_c forms homo-oligomers, which may dissociate and incorporate into heteromeric signaling complexes upon incubation with the other IL-2 receptor chains in the presence of ligand (15). Indeed, co-expression with IL-2Rβ or IL-9Rα reduced the homo-oligomerization of γ_c (Fig. 3*B*), suggesting that the formation of heteromeric signaling complexes is at the expense of γ_c homo-oligomerization. Because heterocomplex formation is selectively increased in the presence of ligand (see below), γ_c homo-oligomerization may serve a physiological role by reducing the potential for activation in the absence of ligand.

A different picture was observed for the heteromeric association of IL-2Rβ and IL-9Rα with γ_c. Although detectable levels of heteromeric complexes were also observed before ligand binding both in transiently expressing BOSC23 cells and in stably expressing Ba/F3 cells (Figs. 2–4), the effect of ligand was strikingly different, inducing a significant increase (2-fold) in the percentage of heteromeric co-patching. This suggests that the binding of IL-2 or IL-9 to their respective ligand-binding subunits results in ligand-mediated increase in the association of these subunits with γ_c. These findings, obtained on full-length receptor chains situated at the plasma membrane of live cells, are in agreement with crystallographic studies on the quaternary structure of IL-2 in complex with IL-2Rα, IL-2Rβ, and γ_c (14, 15), where a large IL-2Rβ/γ_c interface was proposed to undergo stabilization following ligand binding. They are also in agreement with fluorescence resonance energy transfer studies (23) which reported the existence of some IL-2Rβ/γ_c complexes before ligand binding and their modulation in the presence of IL-2. Interestingly, the crystallographic studies on heterocomplexes comprised of the IL-2R subunits (14, 15) showed that IL-2Rα lacks contact with IL-2Rβ or γ_c, in accord with our demonstration (Fig. 3) that overexpression of IL-2Rα fails to affect IL-2Rβ/γ_c heteromeric complex formation. The insensitivity of IL-R homomeric complex formation to IL-2 or IL-9 binding contrasts with the significant augmentation mediated by the ligands in the formation of heteromeric complexes with γ_c. This implies that ligand binding alters the balance between homomeric and heteromeric complexes containing γ_c in favor of the latter. This notion is in accord with the reported shift of the ectodomain of γ_c from homotrimers into IL-2-containing quaternary complexes with the other receptor in the presence of ligand (15). Moreover, formation of IL-2Rβ/γ_c or IL-9Rα/γ_c heterocomplexes, even in the presence of JAK3, is not sufficient for signaling, which still requires ligand binding. This suggests that the oligomeric interfaces within the heteromeric complex differ between the unliganded and ligand-activated complexes. Furthermore, the requirement for JAK3 for signaling indicates that the ligand-induced conformation in the quaternary complex may be important for the proper positioning of JAK1 and JAK3 for cross-phosphorylation.

Recently, several point mutations have been identified in Janus kinases, especially in pseudokinase domains, which render them constitutively active for signaling. Such mutations have been genetically linked to different malignancies, *i.e.* myeloproliferative syndromes for JAK2 mutants (V617F and exon 12 mutations) (52–56), adult T-acute lymphoblastic leukemia (JAK1 mutations) (26), and acute megakaryocytic leukemia (JAK3 mutations) (27). JAK2 V617F, the model for such mutant JAKs, and homologous mutants of JAK1 and Tyk2 are constitutively active when overexpressed (57). However, at low physiologic levels of expression, dimerization of a cytokine receptor before ligand binding (preformed dimers) was shown to be necessary for activation of JAK2 V617F (50). Our results show that subunits of IL-2R and IL-9R exhibit ligand-independent homomeric complexes as well as both preformed and ligand-induced heteromeric complexes. We tested whether, in an analogous manner with the described effect of preformed erythropoietin receptor dimers on JAK2 V617F (50), signaling by the constitutively active JAK3 A572V mutant is promoted by homomeric γ_c complexes or by heteromeric complexes between IL-2Rβ or IL9Rα and γ_c. These data suggest that although preformed heteromeric complexes are strictly inactive in the presence of ligand in the case of wild-type JAK proteins, they promote signaling by JAK3 A572V. We suggest that preformed heteromeric complexes of IL9-Rα and γ_c in megakaryocytic progenitors may explain the paradox where JAK3, the unique Janus kinase specific for γ_c, triggers megakaryocytic leukemia (27).

In contrast, homo-oligomerization of JAK3 A572V by γ_c homomeric complexes does not induce signaling, emphasizing the need for JAK1 and the cytosolic tyrosine residues of the other receptor chains for STAT signaling. These results point to a novel difference between the activation and signaling

mechanisms of JAK3 A572V and mutants of the other three JAKs. Previously, it has been noted that introduction of a phenylalanine at the homologous V617 position activated all JAKs except JAK3 (57).

In conclusion our study provides evidence that in the absence of ligand, homomeric and heteromeric complexes are formed between the subunits of the IL-2 and Il-9 receptor complexes. Homomeric complexes of γ_c are formed at the expense of heteromeric complexes with IL-2Rβ and IL-9Rα. All preformed complexes are strictly inactive in the absence of ligand. We also show that signaling by an oncogenic mutant of JAK3 (A572V) is promoted by preformed heteromeric complexes of IL-2Rβ or IL9Rα and γ_c. We suggest that preformed oligomers of cytokine receptors might have a more general role in supporting ligand-independent oncogenic signaling by constitutively active forms of Janus kinases.

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