Evidence that Natural Murine Soluble Interleukin 4 Receptors May Act As Transport Proteins

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Summary

The present studies were undertaken to determine whether the interleukin 4 binding proteins (IL-4BPs) previously identified in the biological fluids of mice are soluble forms of IL-4Rs. We also studied the binding properties of IL-4BPs in order to gain insight into their physiological role in vivo. Affinity-purified IL-4BPs and recombinant soluble IL-4Rs generated similar one-dimensional (Cleveland) peptide maps after digestion with either *Staphylococcus aureus* V8 protease or trypsin, indicating structural similarities. Furthermore, a rat mAb directed against the murine IL-4Rs immunoprecipitated the IL-4BPs and completely inhibited binding of ^{125}I-IL-4 to a purified preparation of IL-4BPs. Taken together these data indicate that the IL-4BPs are soluble IL-4Rs. At 4°C the IL-4BPs competitively inhibited the binding of IL-4 to membrane IL-4Rs but their ability to prevent binding of IL-4 to cells at 37°C, at the same concentrations, was significantly reduced. Kinetic binding studies of soluble IL-4BPs vs. membrane IL-4Rs disclosed important differences in their rates of dissociation from IL-4. Whereas dissociation at 4°C was slow for both, dissociation of IL-4 from IL-BPs at 37°C was considerably faster ($t_{1/2}$ of 2 min) than dissociation of IL-4 from membrane IL-4Rs ($t_{1/2}$ of ~69 min). Temperature-dependent changes in dissociation kinetics were reversible, and could not be accounted for by either inactivation of the IL-4BPs at 37°C or receptor internalization. Additional experiments also demonstrated that when IL-4BPs bind to IL-4 at 37°C, the IL-4/IL-4BPs complex can rapidly dissociate, allowing IL-4 to bind to membrane IL-4Rs. In addition, binding of IL-4 by the IL-4BPs protects IL-4 from proteolytic degradation. Taken together, these results suggest that the IL-4BPs are naturally occurring forms of soluble IL-4Rs and that some of their properties (fast dissociation kinetics and protection of IL-4 from proteolysis) are consistent with a potential role as carrier proteins for IL-4 in the circulation.

A number of hormone and cytokine receptors exist in both membrane-bound and soluble forms (e.g. insulin-like growth factor and growth hormone receptors, as well as receptors for IL-2, IL-4, IL-6, and TNF [1–11]). Soluble cytokine receptors usually represent truncated forms of the membrane-bound molecules, lacking the transmembrane and intracytoplasmic domains but retaining the ligand-binding extracytoplasmic domain (5, 9, 10, 12, 13). The roles played by soluble cytokine receptors in vivo are unclear. Possible functions include: (*a*) inhibition of cytokine activity in biological fluids (5, 9–11, 14, 15), thus confining the activity of cytokines to their site of secretion; (*b*) signaling, by interacting with receptor-associated molecules on the membranes of target cells (e.g., IL-6R) (16); and (*c*) transport of cytokines in the circulation, thus protecting their respective ligands from degradation and/or secretion, or increasing their half life in the circulation until they are delivered to target cells at distant anatomical sites (17).

Recently, we described the presence of IL-4 binding proteins (IL-4BPs)[1] in normal mouse serum and ascitic fluid (15). IL-4BPs bind IL-4 with high affinity and inhibit the activity of IL-4 in vitro (15). Mosley, et al. (9) have described cDNAs encoding both the full length IL-4R molecules and secreted, truncated forms of IL-4Rs. COS-7 cells transfected with cDNA clones encoding the latter, secreted a soluble form of the IL-4R that retained its ligand-binding capacity and, inhibited both the binding and activity of IL-4.

The purpose of the present study was to determine whether IL-4BPs are related to soluble IL-4Rs (sIL-4R) and to then investigate the binding properties of soluble IL-4BPs in order to gain insight into their potential functions in vivo as IL-4–inhibitors or as IL-4-carrier proteins. Based on one-dimensional peptide maps and reactivity with a mAb directed against the murine IL-4Rs, the IL-4BPs purified from ascitic fluid are structurally and immunologically related to sIL-4Rs and thus are likely to be their naturally-occurring form. Ki-

[1] *Abbreviation used in this paper:* IL-4BP, IL-4 binding protein.

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netic binding studies utilizing membrane IL-4Rs vs. soluble IL-4BPs indicate significant differences in their dissociation kinetics at 37°C: dissociation of IL-4 from soluble IL-4BPs is 30–40-fold faster than that from membrane IL-4Rs. In addition, kinetic and equilibrium dissociation constants (Kd) suggest a decreased affinity of soluble IL-4BPs with increased temperature (4°C vs. 37°C). Accordingly, IL-4BPs compete less effectively with membrane IL-4Rs for the binding of IL-4 at 37°C. In a two-step incubation experiment, ^{125}I–IL-4 that was previously bound by IL-4BPs could dissociate and bind to membrane IL-4Rs on HT-2 cells at 37°C but not at 4°C. Finally, binding of IL-4 by the soluble IL-4BPs resulted in acquisition of resistance of IL-4 to proteolytic degradation. Taken together, these results suggest that soluble IL-4BPs may play a role as carrier proteins for IL-4 in vivo.

Materials and Methods

Cells, Antibodies, and Reagents. The IL-2/IL-4–responsive T cell line, HT-2 (18), was maintained in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 2 mM L-glutamine, 50 μM 2-ME, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 10 U/ml recombinant human IL-2 (IL-2; Amgen, Thousand Oaks, CA) as described (19). The rat anti-mouse IL-4 antibody, 11B11 (20), was obtained by growing the 11B11 hybridoma in a VLS Cell Culture System (Amicon, Beverley, MA). The final concentrate contained >3 mg/ml of the 11B11 antibody at >90% purity, as determined by a rat Ig-specific RIA and SDS-PAGE, respectively. A rat mAb directed against the murine IL-4Rs was purchased from Genzyme (Cambridge, MA). This antibody inhibits the binding of IL-4 to both membrane and soluble IL-4Rs (21). Recombinant murine IL-4 was a generous gift from Dr. K. Grabstein (Immunex Corp., Seattle, WA) and recombinant murine soluble IL-4R (9, 22) was kindly provided by Dr. Maureen Howard (DNAX, Palo Alto, CA). *Staphylococcus aureus* strain V8 protease (type XVII), trypsin coupled to polyacrylamide beads and hydroxyapatite (type I) were purchased from Sigma Chemical Co. (St. Louis, MO); Affigel-10 was obtained from Bio Rad (Richmond, CA).

Purification of IL-4BPs. Murine ascitic fluid was chosen as the source of IL-4BPs. IL-4BPs are constitutively present in the biological fluids of normal mice, including serum and urine, but their concentration in ascitic fluid is twice that found in normal serum (15). In addition, larger volumes can be obtained. Previous estimates based on IL-4–binding activity and assuming an average mol wt of 40 kD, suggested an approximate concentration of IL-4BPs in murine ascitic fluid of 50 ng/ml (1.25×10^{-9} M). Ascites were, therefore, induced with a spontaneous IgM-secreting myeloma (MTSB) obtained from an elderly BALB/c mouse. These cells secrete IgMκ. Myeloma cells were grown in the peritoneal cavity of Pristane-primed BALB/c mice for the generation of ascites. Although the myeloma cells might secrete soluble IL-4BPs, the bulk of the IL-4BPs in ascitic fluid probably comes from serum since the concentration of IL-4BPs in serum is 50% as high as that in ascites. IL-4BPs were purified from pooled murine ascitic fluid by affinity chromatography on an IL-4–Affigel-10 column followed by removal of contaminating proteins by hydroxyapatite chromatography. Briefly, 500 ml of pooled murine ascitic fluid, was centrifuged and delipidated using 1,1,2-trichloro-trifluorethane (0.7 v/v) and was passed over an IL-4–Affigel-10 column, previously prepared by coupling 50 μg of rIL-4 to 0.5 ml Affigel-10 following the manufacturer's instructions. After extensive washing with ice-cold PBS, the bound material was eluted with 6 M deionized urea in 0.1 M Tris-HCl buffer, pH 8.0. The eluates were pooled, concentrated and equilibrated against 7.5 mM phosphate buffer, pH 7.2 using Amicon Centricon-10 devices (MW cut-off of 10 kD). The concentrated IL-4BPs preparations were further purified by incubation with hydroxyapatite equilibrated in 7.5 mM phosphate buffer, pH 7.2. Greater than 90% of the IL-4 binding activity remained in the supernatant while most of the contaminating proteins were removed. This method resulted in a $>10^5$-fold increase in specific activity (binding activity/protein) over that of the starting material. As previously reported using a different purification protocol (15), all of the renatured IL-4–binding activity recovered after SDS-PAGE was associated with molecules in the range of 30–40 kD.

Iodination of rIL-4, Recombinant Soluble IL-4R and IL-4BPs. rIL-4 was iodinated using Iodogen (Pierce Chemicals, Rockford, IL)-coated tubes and repurified over an 11B11-Sepharose column, as described by Lowenthal, et al. (23). The concentration of the labeled IL-4 preparation was determined based on its biologic activity in the HT-2 proliferation assay (24) and comparison with an unlabeled rIL-4 standard. The final ^{125}I–IL-4 preparation had a specific activity of 2×10^6 cpm/pmol. Recombinant soluble IL-4Rs or purified IL-4BPs were iodinated using the Iodogen method and were then centrifuged through Sephadex G-25 minicolumns in order to remove free iodine. For the one-dimensional peptide maps, the iodinated IL-4BPs preparation was further purified by preparative SDS-PAGE and elution of the 30–40 kD band.

One-Dimensional Peptide (Cleveland) Maps. One-dimensional peptide maps of iodinated soluble rIL-4R and purified IL-4BPs were carried out following the procedure described by Cleveland, et al. (25). Briefly, aliquots of iodinated, soluble rIL-4Rs or IL-4BPs ($2–5 \times 10^4$ cpm) in 45 μl of PBS-0.2% SDS containing 0.5 mg/ml BSA as carrier protein, were incubated for 15 min at 37°C in the presence or absence of staphylococcal V8 protease (3 μg) or immobilized trypsin (1 μg). Reactions were stopped by the addition of 50 μl of 2× Laemmli sample buffer (26) containing 5% 2-ME and boiling for 3 min. Samples were then subjected to SDS-PAGE on 15% polyacrylamide gels followed by exposure to Kodak XAR-2 films (Kodak, Rochester, NY) for 2–5 d at −70°C.

Measurement of ^{125}I–IL-4 Binding to Cells and to Soluble IL-4BPs. Binding of ^{125}I–IL-4 to HT-2 cells was measured after incubation at 4°C and centrifugation through a dibutyl/dioctyl phthalate oil mixture, as described (24). HT-2 cells possess a single class of high affinity IL-4–binding sites with a dissociation constant (Kd) of 5×10^{-11} M (23, 27, 28). Binding of ^{125}I–IL-4 to soluble IL-4BPs in ascites or purified preparations was measured using centrifugation through Sephadex G-50 minicolumns, in order to separate free from bound ^{125}I–IL-4 (15). Briefly, samples were incubated with ^{125}I–IL-4 (at 2×10^{-10} M) for 45 min at 4°C or 37°C, loaded onto 1 ml syringes containing Sephadex G-50 and centrifuged for 60 s at 600 *g*. Bound ^{125}I–IL-4 was excluded while free ^{125}I–IL-4 was retained inside the column. In both cellular and soluble binding assays, specific ^{125}I–IL-4 binding was calculated by subtracting non-specific binding (cpm bound in the presence of a 100-fold molar excess of unlabeled IL-4) from total binding (cpm bound in the absence of unlabeled IL-4).

Kinetic Binding Studies. (A) Dissociation experiments: To study the dissociation of bound ^{125}I–IL-4 from membrane IL-4Rs on HT-2 cells or from soluble IL-4BPs, cells or purified IL-4BP preparations were incubated with ^{125}I–IL-4 (100 pM) in RPMI-1640 containing 5% FCS for 60 min at 4°C or 37°C. At 0 time, a 100-fold molar excess of unlabeled IL-4 (equilibrated to the same tempera-

ture) was added and the mixtures were incubated at 4°C or 37°C. At intervals thereafter, 100 μl aliquots (containing 10^6 cells or a constant amount of IL-4BPs) were centrifuged through a dibutyl/dioctyl phthalate oil mixture (HT-2 cells) or Sephadex G-50 minicolumns (soluble IL-4BPs) and the remaining associated radioactivity (bound ^{125}I–IL-4) was measured. Dissociation rate constants (k_2) for both membrane and soluble IL-4Rs were calculated using plots of ln [HR]/[HR]o versus time, where [HR]o is the concentration of bound ligand at the initiation of the experiment (t = 0) and [HR] is the concentration of bound ligand at any given time. The dissociation rate constant, (k_2), is defined by the negative slope of the resulting curve, i.e., slope = $-k_2$.

(B) Association experiments: To study the association kinetics of IL-4 to membrane bound or soluble IL-4Rs, HT-2 cells or a purified preparation of IL-4BPs were resuspended in RPMI-5% FCS. At 0 time, ^{125}I–IL-4 was added to a final concentration of 200 or 500 pM, for cells or purified IL-4BPs, respectively. Specifically-bound ^{125}I–IL-4 was determined at different times after the addition of the labeled ligand, as described above. Association rate constants (k_1) were calculated using plots of ln [HR]eq/([HR]eq − [HR]) versus time, where [HR]eq is the concentration of ligand bound at equilibrium and [HR] is the concentration of ligand bound at any given time. The slope of the resulting curve is equal to the sum of the dissociation rate constant (k_2) and the product of the association rate constant (k_1) and the ligand concentration, i.e., slope = ($k_2 + k_1$[H]).

(C) Dissociation rate constants: Dissociation constants (Kd) based on kinetic binding data for membrane IL-4Rs on HT-2 cells and soluble IL-4BPs were calculated as the ratio of the dissociation rate constant to the association rate constant, i.e., Kd = k_2/k_1.

Protection of ^{125}I-IL-4 from Proteolysis by IL-4BPs. Constant amounts of ^{125}I–IL-4 (5×10^4 cpm) in 50 μl of PBS containing 1 mg/ml BSA as carrier protein, were incubated at 4°C or 37°C with 1 or 0.3 μg of immobilized trypsin in the presence or absence of enough purified IL-4BPs to bind >95% of the ^{125}I–IL-4 as determined by the soluble binding assay. After 15 min, 50 μl of 2× Laemmli sample buffer containing 5% 2-ME was added to each tube and the mixtures were placed in a boiling water bath for 3 min. The extent of proteolysis was then analyzed semi-quantitatively by SDS-PAGE in a 15% polyacrylamide gel followed by autoradiography.

Results

Purification of IL-4BPs from Murine Ascitic Fluid. IL-4BPs were purified from pooled mouse ascitic fluid by affinity chromatography on a rIL-4–Affigel-10 column followed by removal of contaminating proteins by hydroxyapatite chromatography. This method resulted in a >10^5-fold increase in specific activity (binding activity/protein concentration) and an approximate recovery of 15–20%. After SDS-PAGE, the IL-4–binding activity was associated with a relatively broad band of 30–40 kD (Fig. 1 *A*). The broad band may be due to heterogeneity in glycosylation or the existence of multiple forms of IL-4BPs. The IL-4BPs purified by affinity chromatography were identical to the IL-4BPs prepared using a previously published protocol (15) involving a combination of gel filtration, Blue-Sepharose affinity chromatography, chromatofocusing and hydrophobic chromatography. The IL-4–binding activity of such preparations could be completely removed by passage through an IL-4–Affigel-10 column.

Structural and Immunologic Similarities between sIL-4R and IL-4BPs. Because IL-4BPs and soluble rIL-4Rs have similar mol wt and affinities for IL-4, IL-4BPs might be natural, soluble forms of IL-4Rs. To investigate this possibility, one-dimensional (Cleveland) peptide maps (25) obtained after V8 protease or trypsin digestion of both iodinated soluble, rIL-4Rs and purified IL-4BPs, were compared. As shown in Fig. 1 *B*, proteolysis with either enzyme generated similar tyrosine-containing peptide maps from both soluble rIL-4Rs and purified IL-4BPs. Since similar peptide patterns suggest similarities in the primary structure of proteins, these results indicate that the IL-4BPs purified from ascitic fluid are structurally related to IL-4Rs and suggest that IL-4BPs might be the naturally-occurring forms of soluble IL-4Rs. This interpretation was supported by evidence that a monoclonal rat anti-murine IL-4R antibody, which reacts with both membrane and soluble IL-4Rs (21), but not normal rat IgG, immunoprecipitated IL-4BPs (Fig. 1 *A*) and completely inhibited the IL-4–binding activity of purified IL-4BP preparations (Fig. 2).

Inhibition of Binding of ^{125}I-IL-4 to Cells at 4 and 37°C. In previous experiments we observed that low concentrations of IL-4BPs were very effective in blocking the binding of ^{125}I–IL-4 to T or B cells at 4°C, but that the amounts needed to block the biological activity of IL-4 on the same cells (incubated at 37°C) were substantially higher than those required for inhibition of binding (15). Therefore, we determined whether the IL-4BPs were equally effective at blocking binding of ^{125}I–IL-4 to cells at 37°C. HT-2 cells (10^6 cells/tube) were incubated for 45 min at 4°C or 37°C with two different amounts of ^{125}I–IL-4 (10 and 50 pM) in the presence or absence of varying concentrations of purified IL-4BPs. The amount of cell-bound IL-4 was then determined. As shown in Fig. 3, some inhibition of binding of ^{125}I–IL-4 was observed at 37°C. However, the IL-4BPs competed much less effectively for binding of IL-4 to HT-2 cells at this temperature than at 4°C. The relatively poor ability of the IL-4BPs to compete for binding at the higher temperature was not due to an effect of receptor internalization at 37°C, since identical results were obtained if assays were carried out in the presence of 10 mM sodium azide and 2 mM 2-deoxyglucose in order to prevent receptor internalization. Similarly, potential inactivation of the IL-4BPs at 37°C was excluded, since we could demonstrate binding of ^{125}I–IL-4 by the IL-4BPs at 37°C in the soluble-phase binding assay and IL-4BP preparations pre-incubated at either 4°C or 37°C for 60 min were equally effective at inhibiting binding of ^{125}I–IL-4 to cells when tested at 4°C (results not shown).

Affinity of IL-4BPs for IL-4 at 4° and 37°C. To determine whether the differences in the inhibitory ability of IL-4BPs at 37°C were due to a temperature-induced decrease in the binding affinity and/or in the number of IL-4–binding sites, we performed Scatchard analyses (29) of equilibrium binding of ^{125}I–IL-4 to soluble IL-4BPs at 4°C and 37°C. In accord with previous findings, Scatchard analysis of the binding data at 4°C indicated the existence of a single class of high-affinity binding sites (Kd: 6×10^{-11} M). Although a linear curve was also obtained in the Scatchard plot at 37°C, a different

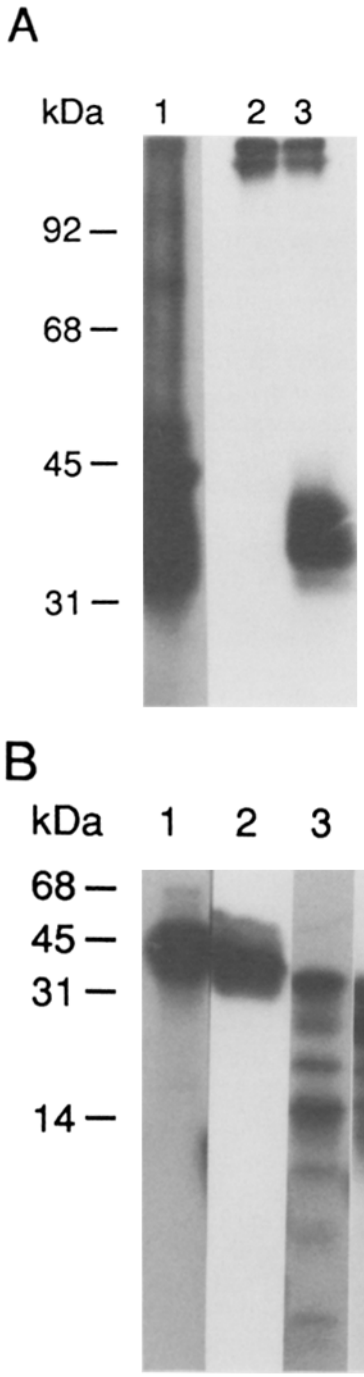

Figure 1. (*A*) SDS-PAGE of IL-4BPs and immunoprecipitation with monoclonal anti-IL4R antibody. IL-4BPs were purified from murine ascitic fluid by affinity chromatography on an rIL-4–Affigel-10 column followed by hydroxyapatite chromatography. The purified IL-4BPs were then iodinated using the Iodogen method; free ^{125}I was removed by passage through a Sephadex G-25 column and an aliquot was analyzed by SDS-PAGE (10% gel) followed by autoradiography. ^{125}I-labeled IL-4BPs were incubated with either normal rat IgG or a rat anti-mouse IL-4 mAb (5 μg) followed by incubation with a mouse anti-rat κ light chain antibody (MARK.1) coupled to Sepharose beads. The beads were washed and eluted with SDS-sample buffer. Lane *1*: IL-4BPs preparation; lane *2*: IL-4BPs plus normal rat IgG; lane *3*: IL-4BPs plus anti-IL-4R mAb. (*B*) One-dimensional (Cleveland) maps of ^{125}I-labeled soluble rIL-4R and purified IL-4BPs. Soluble rIL-4Rs and purified IL-4BPs were iodinated and digested for 15 min at 37°C in the presence of *S. aureus* V8 protease or Trypsin, as described. Resulting peptides patterns were compared after SDS-PAGE (15% gel) and autoradiography. Lanes *1*, *3*, and *5*: soluble rIL-4R; lanes *2*, *4*, and *6*: purified IL-4BPs; samples in lanes *1* and *2* were undigested, samples in lanes *3* and *4* were digested with V8 protease, and those in lanes *5* and *6* were digested with trypsin.

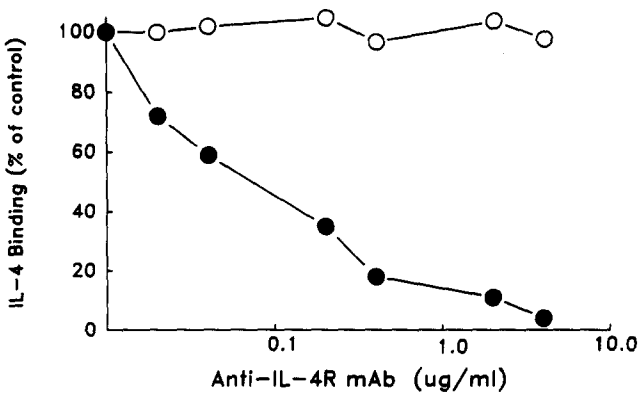

Figure 2. Inhibition of binding of ^{125}I-IL-4 to purified IL-4BPs by a monoclonal rat anti-mouse IL-4R (21). Aliquots of a purified IL-4BP preparation were preincubated for 30 min at 4°C in the absence or presence of increasing amounts of a rat anti-mouse IL-4R mAb (●) or normal rat IgG (○). ^{125}I-IL-4 was then added to a final concentration of 100 pM. After 45 mins at 4°C, specific binding was measured using a soluble phase binding assay as described. Results are expressed as percent of a control containing IL-4BPs in the absence of antibodies (control: 2,500 ± 150 cpm).

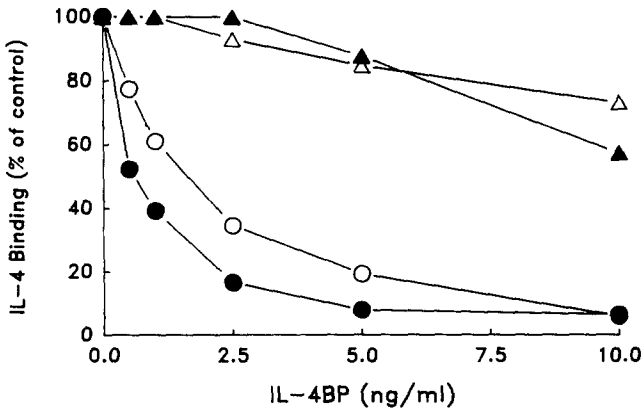

Figure 3. Inhibition of binding of ^{125}I-IL-4 to HT-2 cells by IL-4BPs at 4°C and 37°C. HT 2 cells (10^6 per tube) were incubated with ^{125}I-IL-4 at either 10 pM (●, ▲) or 50 pM (○, △) in the presence or absence of increasing concentrations (0–10 ng/ml, based on IL-4–binding activity) of a purified IL-4BP preparation. After 45 min at 4°C (●, ○) or 37°C (▲, △) the cell mixtures were centrifuged through phthalate oil in order to remove free ^{125}I-IL-4 and specific binding was determined as described in Materials and Methods.

slope suggested a threefold decrease in affinity (Kd: 2 × 10^{-10} M). A similar intercept of the y-axis by both curves indicated virtually identical numbers of binding sites, arguing against either a potential inactivation of the IL-4BPs or a reduction in the number of IL-4–binding sites (Fig. 4). The threefold reduction in the Kd of the IL-4BPs at 37°C was completely reversible if the cells were cooled to 4°C. Indeed, preparations that were pre-exposed at 37°C for 60 min had

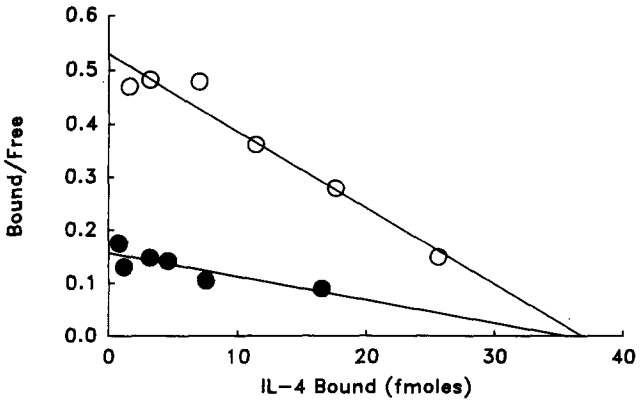

Figure 4. Scatchard analysis of binding of ^{125}I–IL-4 to IL-4BPs at 4°C and 37°C. Equilibrium binding of ^{125}I–IL-4 to purified IL-4BPs was determined in a soluble phase binding assay at both 4°C (○) and 37°C (●) using constant amounts of IL-4BPs and increasing amounts of ^{125}I–IL-4. Kd and Bmax values were (6×10^{-11} M; 36.8 fmoles) and (2×10^{-10} M; or 34.2 fmoles) at 4°C and 37°C, respectively.

Kd values at 4°C that were identical to those obtained for preparations maintained at 4°C. These results suggested that a potential temperature-induced change in conformation might have altered the binding characteristics of the IL-4BPs.

Kinetic Binding Studies. Although, as determined by equilibrium binding studies (30–32), the affinities of the p75 and p55 subunits of the IL-2R differ by only 10-fold, kinetic binding measurements for IL-2 have revealed very significant differences in the manner in which the two different subunits bind IL-2 (33, 34). Whereas the p75 (intermediate affinity) subunit has very slow association and dissociation rates, the p55 (low affinity) subunit has very rapid association/dissociation rates. The high-affinity IL-2R (p75/p55) combines the fast association of p55 and the slow dissociation of p75 (33, 34). By analogy, even though equilibrium binding analyses indicated that the IL-4BPs and membrane IL-4Rs have similar Kds, we determined whether potential differences in association/dissociation kinetics between soluble and membrane IL-4Rs at 4°C and 37°C, might explain the relatively lower inhibition by the IL-4BPs at 37°C.

Kinetic association and dissociation rates were thus calculated for both the soluble IL-4BPs (soluble-phase binding assay) and for membrane IL-4Rs (using HT-2 cells) at both temperatures. As shown in Fig. 5, dissociation of IL-4 from either the IL-4BPs or membrane IL-4Rs at 4°C was relatively slow, with $t_{1/2}$ values of ~112 and ~180 min, respectively. At 37°C, however, the dissociation of IL-4 from soluble IL-4BPs differed significantly from that of membrane IL-4Rs: the $t_{1/2}$ for the IL-4BPs was only ~2 min, whereas that of membrane IL-4Rs was ~69 min. Although association rates were relatively fast for both soluble and membrane IL-4Rs, rates were faster for the IL-4BPs. Association and dissociation plots (Fig. 5 *e* and *f*) were used to calculate the kinetic rate constants for both membrane IL-4Rs and soluble IL-4BPs, at 4°C and 37°C. These values are summarized in Table 1. Estimation of Kd values based on kinetic parameters (association/dissociation) confirmed the Kd values obtained from equilibrium-

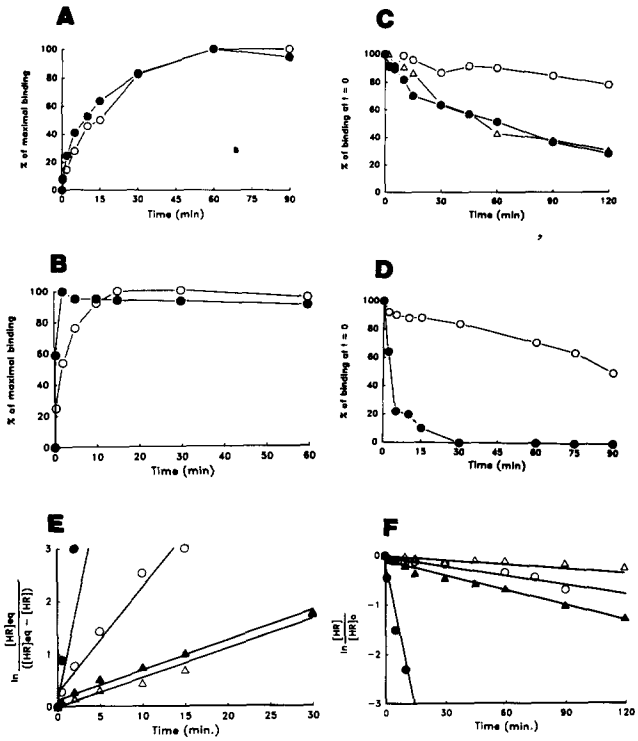

Figure 5. Kinetics of IL-4 binding to membrane and soluble IL-4Rs. (*A* and *B*) Association of ^{125}I–IL-4 to HT-2 cells (*A*) and soluble IL-4BPs (*B*) at 4°C (○) and 37°C (●). (*C* and *D*) Dissociation of ^{125}I–IL-4 from membrane (HT-2 cells, *C*) or soluble (IL-4BPs, *D*) IL-4Rs at 4°C (○) and 37°C (●). Dissociation at 37°C was also measured in the presence of 10 mM sodium azide and 2 mM 2-deoxyglucose (△). Specific binding for both the cell and soluble phase binding assays was calculated after subtraction of nonspecific binding (cpm bound in the presence of a 100-fold molar excess of unlabeled IL-4) from the Total binding. (*E* and *F*) Natural log plots of Association (*E*) and Dissociation kinetics (*F*) of ^{125}I–IL-4 binding to membrane (△, ▲) and soluble IL-4BPs (○, ●) at 4°C (△, ○) and 37°C (▲, ●). Kinetic rate constants were calculated based on these plots as described under Materials and Methods.

binding studies for both soluble and membrane IL-4Rs. Moreover, Kd values for soluble IL-4BPs obtained from kinetic data, showed a six–eight-fold higher dissociation constant at 37°C, reflecting the significantly faster dissociation rate of IL-4BPs at this temperature. Thus, the faster dissociation rate and lower affinity of soluble IL-4BPs at 37°C or 4°C might explain the differences in inhibition of binding at the two different temperatures.

Two-step Incubation of ^{125}I–IL-4 with IL-4BPs and HT-2 Cells. The different kinetic binding properties of soluble IL-4BPs and membrane IL-4Rs at 37°C have important implications for the potential physiological role of soluble IL-4BPs in vivo. Because of the fast association/dissociation rates of IL-4 from soluble IL-4BPs at 37°C, IL-4/IL-4BP complexes in vivo must be constantly dissociating and re-forming. In other words they would be transient and reversible, thereby allowing the ligand (IL-4) to dissociate from one soluble IL-4BP molecule, and become available for binding to another IL-4BP molecule or alternatively, to a membrane IL-4R. The slower dissociation kinetics of membrane IL-4Rs, combined

Table 1. *Summary of Equilibrium and Kinetic Binding Constants for Membrane and Soluble IL-4Rs at 4° and 37°C*

IL-4 Receptor	Temperature	Equilibrium dissociation constant (Kd)*	t½	Kinetic binding constants‡		k_2/k_1
				Dissociation (k_2)	Association (k_2)	
	°C	$\times 10^{-11}$ M	min	min^{-1}	$Mol^{-1} \cdot min^{-1}$	$\times 10^{-11}$ M
Membrane§	4	4	180	3.85×10^{-3}	2.57×10^{8}	1.5
	37	7	69	1.01×10^{-2}	2.34×10^{8}	4.3
Soluble‖	4	6	112	6.21×10^{-3}	3.87×10^{8}	1.6
	37	20	2.3	3.07×10^{-1}	2.34×10^{9}	13.0

* Calculated from data shown on Fig. 3.
‡ Calculated from data shown on Fig. 5.
§ HT-2 cells.
‖ Purified IL-4BP preparation.

with receptor internalization, would make IL-4 bound to membrane IL-4Rs less likely to dissociate. It should be stressed, however, that the equilibrium of this reaction would be dependent on the relative concentrations of membrane and soluble receptors and that at high enough concentrations of soluble receptors, effective inhibition of IL-4–binding to membrane IL-4Rs would occur.

To test this model, we incubated a constant amount of ^{125}I–IL-4 with an excess of IL-4BPs (2.5 and 7.5-times the amount of IL-4BPs required to saturate the IL-4) at 4°C or 37°C. Based on the soluble-phase binding assay, it was determined that all the ^{125}I–IL-4 was bound to the IL-4BPs at either temperature. The mixture was then incubated with HT-2 cells and cell-bound ^{125}I–IL-4 was determined after 45 min at 4°C or 37°C. As shown in Fig. 6, when IL-4 was exposed to IL-4BPs and then incubated with cells at 4°C, greater than 90% of the IL-4 remained associated with the soluble receptors and was unable to bind to membrane IL-4Rs. In contrast, although some inhibition was observed at 37°C (20–35%), there was still significant binding of ^{125}I–IL-4 to the cells, even when all the IL-4 was associated with IL-4BPs before the addition of the cells. The binding of IL-4 to HT-2 cells was completely inhibited at both temperatures by the anti–IL-4 mAb, 11B11 (whose binding to IL-4 is also inhibited by IL-4BPs) (data not shown), suggesting that in order for IL-4 to bind to membrane IL-4Rs on the HT-2 cells, it must first dissociate from the IL-4BPs (only free IL-4 can bind to 11B11 antibodies).

Protection of IL-4 from Proteolysis by IL-4BPs. The fact that IL-4 is able to rapidly bind to and dissociate from the IL-4BPs at 37°C, thus becoming available to bind to membrane IL-4Rs, raises the possibility that IL-4 might use IL-4BPs as "carrier

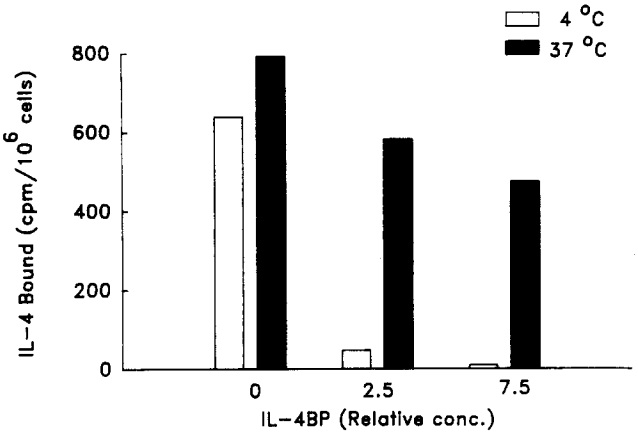

Figure 6. IL-4 bound to IL-4BPs remains available for binding to HT-2 cells at 37°C but not at 4°C. ^{125}I–IL-4 (100 pm) was incubated in the absence or presence of a 2.5- or a 7.5-fold molar excess of purified IL-4BPs for 45 min at 4°C or 37°C. It was then determined, based on the soluble phase binding assay, that >99% of the radioactivity was associated with the IL-4BPs. HT-2 cells (10^6/tube) were then added to the mixtures. The incubations were continued for 45 min at 4°C or 37°C. The amount of radioactivity associated with the cells (specific IL-4–binding) was then determined as described in Materials and Methods.

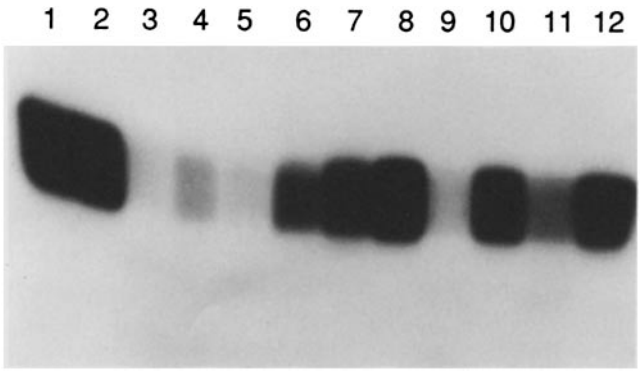

Figure 7. Protection of IL-4 from proteolytic degradation. ^{125}I–IL-4 was incubated with Trypsin at 1 or 0.3 μg/tube in the presence (lanes *2*, *4*, *6*, *8*, *10*, and *12*) or absence (lanes *1*, *3*, *5*, *7*, *9*, and *11*) of a two-fold molar excess of purified IL-4BPs in 50 μl of PBS containing 1 mg/ml BSA. After 15 min at 37°C, 21°C or 4°C, 50 μl of SDS-PAGE sample buffer was added and the mixtures were incubated for 3 min in a boiling water bath. Digestion of the samples was estimated by analysis using SDS-PAGE (15% gel) and autoradiography. Lanes *1* and *2*: undigested; lanes *3–6*, digested with 1 μg (*3–4*) or 0.3 μg trypsin (*5* and *6*) at 37°C; lanes 7, 8 digested with 1 μg trypsin at 4°C; and lanes *9–12*, digested with 1 μg (*9* and *10*) or 0.3 μg trypsin (*11* and *12*) at 21°C.

proteins" in vivo. In view of reports suggesting that α-2-macroglobulin acts as a carrier protein for IL-6 in the circulation and that it protects IL-6 from proteolytic degradation (35), we determined whether the binding of IL-4 to soluble IL-4BPs would confer protection against proteolytic degradation. Constant amounts of ^{125}I–IL-4 were preincubated in the presence or absence of a saturating amount of IL-4BPs in a solution containing BSA as a carrier protein. Two different concentrations of immobilized trypsin were then added and the mixtures were incubated at 4°C, 21°C or 37°C. The extent of proteolysis of ^{125}I–IL-4 was then ascertained by SDS-PAGE and autoradiography. As shown in Fig. 7, incubation of IL-4 with IL-4BPs in BSA and in the absence of trypsin did not result in degradation or alteration of the mobility of IL-4. When trypsin was added, the IL-4BPs partially protected IL-4 from proteolytic degradation at all temperatures tested, although this effect was more pronounced at 4°C than at 37°C. This might reflect the faster dissociation of IL-4 from the IL-4BPs at higher temperatures, allowing more IL-4 to be exposed to the protease. Nonetheless, even at 37°C there was a significative difference in the extent of degradation of IL-4 in the presence or absence of the IL-4BPs. The decreased degradation of IL-4 in the presence of the IL-4BPs was due to a decreased susceptibility of IL-4/IL-4BPs complexes to enzymatic attack and not to an inhibitory effect on trypsin itself, since the hydrolysis of a chromogenic substrate for trypsin (N-benzoyl-L-arginine ethyl ester) was unaffected by the addition of IL-4BPs (data not shown).

Discussion

Three major findings have emerged from these studies: (*a*) soluble IL-4BPs in mouse sera and ascites are structurally and antigenically similar to soluble rIL-4Rs; (*b*) the binding properties of the IL-4BPs/sIL-4Rs are consistent with a potential role as carrier proteins for IL-4 in vivo. In this regard, the dissociation of IL-4 from sIL-4Rs is rapid, whereas dissociation of IL-4 from membrane IL-4Rs is slow, suggesting that, at 37°C, IL-4 is not effectively sequestered from membrane IL-4Rs even when bound to soluble IL-4Rs (at the concentrations normally present in serum); (*c*) IL-4 is partially protected from enzymatic degradation following its binding to soluble IL-4BPs.

Several lines of evidence suggest that the IL-4BPs present in murine ascites are naturally-occurring forms of soluble IL-4Rs. Hence, Mosley, et al. (9) have reported that cDNAs encoding truncated, soluble forms of the murine IL-4Rs (sIL-4R) are expressed in several types of murine cells and Fanslow, et al. (36) have recently reported the presence of molecules in mouse serum and ascitic fluid that react with monoclonal anti–IL-4R antibodies. Since the IL-4BPs and sIL-4Rs have similar binding affinities for IL-4 (Kds: $\sim 5 \times 10^{-11}$ M) (9, 15), similar molecular weights (9, 15), and can competitively inhibit the binding and the biologic activity of IL-4 (9, 15, 22), the IL-4BPs are most likely natural forms of soluble IL-4Rs. The present data support this contention based on similar peptide maps generated after proteolytic digestion as well as antigenic cross-reactivity. Whether or not the soluble IL-4BPs originate from an alternatively-spliced mRNA encoding soluble IL-4Rs, or from proteolytic cleavage of membrane IL-4Rs remains to be determined. Indeed, both mechanisms might contribute to the presence of IL-4BPs in biological fluids. Based on the fact that the cDNA clones that encode soluble IL-4Rs contain a 114 bp insertion that results in the addition of six new amino acid residues at the C-terminus (9), it should be possible to distinguish soluble IL-4R molecules generated by alternative mRNA splicing from those shed as a result of proteolytic cleavage by comparison of their C-terminal amino acid sequences. Regardless of the mechanism involved in their generation, it will be important to determine what cell types release sIL-4Rs, the signals involved in this process and the factors regulating their generation.

An obvious question regarding the presence of soluble IL-4Rs in biological fluids is whether they are involved in the regulation of IL-4 activity in vivo. Based on evidence indicating that either purified IL-4BPs or soluble rIL-4Rs interfere with the binding and activity of IL-4, we initially suggested that soluble IL-4Rs might act in vivo by competing with cellular IL-4Rs for the binding of free IL-4 in the circulation (15). Unexpectedly, results of the present studies indicate that IL-4BPs, at concentrations normally found in serum, ($\sim$20 ng/ml based on IL-4 binding activity) are not very effective competitors of membrane IL-4Rs at physiological temperatures and that their properties are more consistent with a potential role as "carrier proteins" for IL-4 in vivo.

A comparison of the kinetics of IL-4-binding to soluble and membrane IL-4Rs at 4°C and 37°C, disclosed an important difference between both IL-4Rs forms. Thus, dissociation of IL-4 from soluble and membrane IL-4Rs was relatively slow at 4°C, whereas dissociation of IL-4 from soluble IL-4BPs followed significantly faster kinetics than that from membrane IL-4Rs at 37°C. Since dissociation of ^{125}I–IL-4 was measured after the addition of a 100-fold excess of unlabeled IL-4, the possibility existed that the apparent increase in dissociation rate could be accounted for by negative-cooperativity among soluble IL-4Rs at 37°C. However, the fact that Scatchard curves for the binding of ^{125}I–IL-4 to sIL-4Rs at 37°C were not curvilinear argues against this possibility.

The molecular mechanisms responsible for the difference between the dissociation rates of soluble and membrane IL-4Rs are not clear. It is possible that increased temperature and/or ligand binding induce conformational changes in the soluble IL-4BPs that result in the accelerated dissociation rate. These changes may not be induced in the membrane IL-4Rs because of stability conferred by anchoring in the membrane through transmembrane and intracytoplasmic domains and/or association with other membrane protein(s).

Because of the fast dissociation rate at 37°C, IL-4 molecules would be constantly associating and dissociating from sIL-4R molecules, thereby increasing their chance of binding to membrane IL-4Rs. However, the equilibrium of IL-4 between both receptor forms would remain dependent upon the relative concentrations of soluble vs. membrane IL-4Rs at any given anatomical site. Since IL-4 bound to soluble IL-4BPs can dissociate and bind to membrane IL-4Rs, this suggests that even though most of the IL-4 in the circulation

might be bound to soluble IL-4BPs, it would still be available for binding to membrane IL-4Rs, especially at those anatomical sites with high concentrations of IL-4R$^+$ cells (i.e., increased ratio of membrane IL-4Rs to sIL-4Rs). In the circulation or in nonlymphoid tissues, the lower ratio of membrane to soluble IL-4Rs would favor binding to soluble IL-4Rs. This could be a mechanism whereby active IL-4 is preferentially delivered to lymphoid organs such as lymph nodes, spleen, thymus, etc., while preventing its binding to cells at other sites. In addition, activated cells in distal sites expressing high densities of IL-4Rs would have an advantage in capturing circulating IL-4 carried by soluble IL-4Rs. Such cells could thereby utilize the IL-4 produced at distant sites. Consistent with this possibility is the observation that the binding of IL-4 by IL-4BPs protects IL-4 from proteolytic inactivation; it might also prolong the half-life of IL-4 in the circulation by preventing its clearance. These possibilities could provide a mechanism for amplifying an immune response.

One implication of the above model is that pathological conditions that result in alterations in the normal levels of soluble IL-4Rs, might lead to overall increased or decreased IL-4 activity by altering the sIL-4Rs/mIL-4Rs ratio. Hence, it will be important to correlate the changes in the serum levels of soluble IL-4Rs with the changes in the immune system observed in a variety of infectious diseases, immunodeficiencies, autoimmune and malignant diseases.

In addition to the potential role for soluble IL-4Rs in the regulation of IL-4 activity under normal and pathological conditions, soluble IL-4Rs and other soluble cytokine receptors, are also very attractive candidates for immunotherapy (37). In this regard, recombinant soluble IL-4Rs (if given in sufficiently high pharmacological concentrations) could be employed to selectively block IL-4 activity in vivo. This could be important in the treatment of allergic conditions. Therapy with soluble rIL-4Rs would have some important advantages over immunotherapy with agents such as anti-cytokine or anti-cytokine receptor antibodies. Thus, the elicitation of an antibody response against the soluble IL-4Rs would be unlikely. In addition, their smaller size might facilitate extravasation and/or penetration of tissues.

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