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Real-time Kinetic Measurements of the Interactions between Lactogenic Hormones and Prolactin-Receptor Extracellular Domains from Several Species Support the Model of Hormone-induced Transient Receptor Dimerization*

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Interactions of recombinant soluble prolactin receptors-extracellular domains (PRLR-ECDs) from rabbit, rat, and cow and human growth hormone receptor ECD with immobilized human growth hormone, several prolactins, and bovine placental lactogen were studied utilizing surface plasmon resonance. This method enables real-time kinetic measurements of the interactions and calculations of kinetic constants and of the stoichiometry of interaction, even in cases where only transient interactions occur. In contrast to gel filtration or crystallographic studies, where in most cases the interaction of PRLR-ECDs with various lactogenic hormones indicated formation of 1:1 complexes, our surface plasmon resonance experiments indicated in all cases the transient formation of a 2:1 complex. In most of the interactions the 2:1 complex was very unstable and underwent rapid dissociation to a 1:1 complex. This situation was particularly characteristic of homologous interactions involving hormone and receptor from the same species and was mainly attributed to increased dissociation constants. We suggest that as in the case of growth hormone PRLR activation occurs via hormone-induced transient homodimerization of the receptor, lasting only a few seconds, and that this may be sufficient to initiate the biological signal. Once the signal is initiated, the receptor dimer is no longer required. Its rapid dissociation to a 1:1 complex or to its components may even be advantageous in that it permits activation of additional receptors.

Prolactin (PRL)[1] and growth hormone (GH) receptors (R) belong to the large cytokine receptor family, which is characterized by a conserved 200–240-amino acid sequence in the extracellular domain (ECD) (1–3). Four positionally conserved cysteine residues that form two disulfide bridges and a WS*X*WS motif are found, respectively, in the amino- and carboxyl-terminal portions of the ECD. Accumulating evidence indicates that ligand-induced homodimerization, heterodimerization, or oligomerization of the receptor is a common step that leads to the activation of the cytoplasmic, receptor-associated Jak tyrosine kinases (4, 5). However, despite the structural homology of the ligands, characterized by a bundle of four α-helices (6), and the ECDs of their receptors, the mechanism and stoichiometry of receptor oligomerization does not seem to follow identical or even similar patterns (7–9).

The most extensively studied interaction to date has been that between human (h) GH and hGHR-ECD. Using a slightly truncated, soluble, nonglycosylated hGHR-ECD (amino acids 1–238), de Vos and co-workers (10) crystallized the hormone-receptor complex, proving the simultaneous binding of the hormone to two hGHR-ECDs and structurally identified the different hGH sites involved in the interactions. Results of these studies substantiated those of Wells and co-workers (11–13), who were the first to suggest a hormone-induced sequential receptor homodimerization in which hGH binds one molecule of hGHR-ECD through site I, and then a second through site II, and offered a paradigm of ligand-induced receptor oligomerization in the cytokine receptor family (14). These results were further confirmed by us using full-size (amino acids 1–246) hGHR-ECD (15). Similar bovine (b) GH-induced homodimerization using recombinant soluble bGHR-ECD was also reported (16). Indirect evidence reported by us (17, 18) suggested that receptor dimerization or oligomerization also occurs in PRLR as an initial step in hormone-induced signal transduction. Thus, to evaluate whether the same paradigm of hormone-receptor interaction applies to PRL, we prepared soluble recombinant PRLR-ECDs from rabbit (rb) (19, 20), bovine (b) (21), and rat (r) (22) and studied their interactions with several PRLs and related lactogenic hormones in binding and gel filtration experiments. Results of those studies revealed a highly heterogeneous picture. Whereas with bPRLR-ECD, only 1:1 ECD-hormone complex could be detected (21), with rbPRLR-ECD, most of the hormones reacted similarly with the exception of bovine placental lactogen (bPL) (20) and ovine (o)PL (23), in which a 2:1 stoichiometry was found. In contrast to the interactions of rPRLR-ECD with hGH, oPRL, bPL (22), and oPL,[2] which yielded a 2:1 stoichiometry, homologous rPRL

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[1] The abbreviations used are: PRL, prolactin; PRLR-ECD, prolactin receptor-extracellular domain; GHR-ECD, growth hormone receptor-extracellular domain; GH, growth hormone; PL, placental lactogen; h, human; b, bovine; o, ovine; r, rat; rb, rabbit; mAbs, monoclonal antibodies; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide; RU, resonance unit.

[2] A. Gertler, J. Grosclaude, C. J. Strasburger, S. Nir, and J. Djiane, unpublished observations.

formed only a 1:1 complex. A similar 2:1 stoichiometry for the complex between rPRLR-ECD expressed in mammalian cells and oPRL was also reported (24). Moreover, we found that homodimerization of rPRLR-ECD by hGH, and even more so by oPRL, is concentration-dependent, and upon dilution from micromolar to nM concentrations, the complex dissociated from a 2:1 to a 1:1 form (22). Recent crystallographic studies of the complex between hGH and hPRLR-ECD also revealed a 1:1 stoichiometry and documented the formation of the complex through site I of hGH (25). Furthermore, our research indicated that despite the differences in the binding properties and stoichiometries of interactions of the respective PRLR-ECDs and lactogenic hormones, the biopotencies of these hormones, as determined in several homologous or heterologous *in vitro* bioassays, were, surprisingly, almost identical (26–28).

The present work attempts to clarify this situation by making use of a novel method for real-time kinetic measurements of the interactions between macromolecules, based on surface plasmon resonance (29). This technique enables a determination of kinetic constants and the stoichiometry of interaction of a complex in which one component is immobilized on a flexible dextran matrix, whereas the other is free in solution. It has been used successfully for studies of hGH (30), epidermal growth factor (31), and interleukin 2 (32) receptors. Using this method, we detected weak dimeric complexes that had not been identified by classical binding, gel filtration, or crystallographic studies, and the kinetics of their formation and dissociation was assessed. Preliminary results of this study were reported at the Endocrine Society meeting (33).

EXPERIMENTAL PROCEDURES

Materials—Recombinant bPL was prepared as described previously (34), and recombinant hGH was obtained from Biotechnology General Inc. (Rehovot, Israel). Recombinant nonglycosylated rb, b, and r PRLR-ECDs and hGHR-ECD were prepared as described previously (15, 20–22). Ovine PRL (NIDKK AFP-8277E) and rPRL (NIDKK rPRL-B3) were from the National Hormone and Pituitary Program (Bethesda, MD), and rbPRL (AFP7730B) was from Dr. A. F. Parlow (Harbor-UCLA Medical Center, CA). Human PRL was obtained from Dr. V. Goffin (Liege, Belgium). Monoclonal anti-hGH antibodies (mAbs) 10A7 and 7B11 were prepared as described elsewhere (35). SPR reagents including CM5 sensor chips, Hepes buffer saline (HBS), *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (EDC), 2-(2-pyridinyldithio)ethanamine hydrochloride, and ethanolamine hydrochloride were obtained from Pharmacia Biotech Inc. (Uppsala, Sweden).

Coupling of hGHR-ECD and rbPRLR-ECD to Dextran Matrix via Thiol Groups —Covalent linking of hGHR-ECD and rbPRLR-ECD was performed according to Johnsson *et al*. (29). The immobilization process was performed with 25 mM Hepes buffer, pH 7.4, containing 150 mM NaCl and 0.05% (w/v) P20 surfactant (HBS) at a flow rate of 5 μl/min. The matrix was activated for 3 min with 0.05 M EDC/NHS in HBS, followed by an 8-min injection of 80 mM 2-(2-pyridinyldithio)ethanamine hydrochloride in 0.1 M sodium borate buffer, pH 8.5, and then rbPRLR-ECD (50 μg/ml) in 5 mM sodium borate buffer, pH 5, for 5 min, or hGHR-ECD (70 μg/ml) in 5 mM sodium borate buffer, pH 4.7, for 7 min. Unreacted matrix groups were deactivated through a 6-min injection of a 50 mM L-cysteine, 1 M NaCl solution in 0.1 M sodium formate buffer, pH 4.3. This procedure ensured an immobilization of 4000 RU for hGHR-ECD and 800 RU for rbPRLR-ECD. Binding capacities of the immobilized R-ECDs were checked by sequential injections of 2.5 μM hormone solutions in HBS for 6 min followed by a regeneration pulse of 4.5 M $MgCl_2$ for 1 to 2 min.

Coupling of Various Hormones to a CM-Dextran Matrix via Amino Groups—The hormones were covalently linked according to Johnsson *et al.* (29). HBS was injected at 5 μl/min, and activation with 0.05 M EDC/NHS in HBS was carried out for 7–8 min. The various hormones were then injected at concentrations ranging from 50 to 100 μg/ml in 10 mM sodium acetate buffers at pH values of 4.5–5, based on the optimal electric charge for electrostatic preconcentration on the matrix surface, preservation of the R-ECD binding capacities for the appropriate levels of hormone immobilization: hGH, pH 4.5 (1400 RU), oPRL, pH 4.7 (3100 RU), rbPRL, pH 4.7 (6800 RU), rPRL, pH 4.7 (2500 RU), bPL, pH 5 (5400 RU), and hPRL, pH 5 (3900 RU). Unreacted sites were blocked with an 8-min injection of 1 M ethanolamine hydrochloride at pH 8.5. Binding capacities were checked by repeated injections of 5 μM R-ECDs in HBS. All immobilized hormones except rbPRL could be regenerated over 50 runs with 4.5 M $MgCl_2$ pulses (1–2 min). Rabbit PRL was regenerated by washing out the bound R-ECDs with HBS for 20 min at 20 μl/min.

Presentation of hGH by Anti-hGH mAb Capture—Rabbit anti-mouse IgG-purified immunoglobulins were covalently linked to the matrix gel via amino-group coupling as recommended by Pharmacia Biosensor. Two anti-hGH mAbs (33) were used as capture antibodies: mAb 10A7, which binds hGH at a distance from both receptor interaction sites, and mAb 7B11 which binds to site II of hGH, thereby allowing the receptor to bind only to site I. Each of the mAbs prepared in HBS at 100 μg/ml was captured by a 6-min injection at a flow rate of 5 μl/min, which ensured immobilization of 2000 RU on rabbit anti-mouse IgG-purified immunoglobulins. For R-ECD-hGH interaction studies, saturating levels (2.5 μM) of hGH were injected for 6 min, and about 500 RU of the hormone were thus captured in each case. For kinetic characterization of hGH capture by the two mAbs, serial runs were performed with 0–1000 nM hormone solution. Regeneration at the end of each run was achieved by a 2-min pulse of 100 mM HCl.

Kinetic Measurements of R-ECD-Hormone Interactions—All experiments were performed at a flow rate of 5 μl/min in HBS at 25 °C. Once the hormone being tested was immobilized, either covalently through amino-group coupling or via mAb capture (for hGH), serial dilutions ranging from 1000 to 62.5 nM of each R-ECD were injected for 6 min and then washed out for 10 min prior to regeneration. Because the recombinant R-ECDs were lyophilized with sodium bicarbonate buffers at a salt:protein ratio of 1:2 ratio, bulk refractive indexes varied with sample dilution and were corrected by injections of the same dilutions into flow cells where unrelated ligands had been immobilized. In experiments in the presence of Zn^{2+}, hormones were immobilized in HBS as described earlier. Then the 15 μM $ZnCl_2$ was added to the HBS running buffer, and all subsequent dilutions were performed in 15 μM $ZnCl_2$-containing HBS.

Data Analysis and Calculation of Kinetic Constants—BIAcore incorporated software (BIA Evaluation and BIA Simulation) allowed us to 1) fit experimental curves with 1:1 or 1:2 association/dissociation models and calculate the probabilities of either one of these being the most accurate representation of reality, and 2) calculate kinetic constants with standard deviations; reverse verification of calculated data was performed by simulating the interaction assuming a variable relative occupation of sites.

Theoretical Analysis—The solution concentration of the receptor at time t is denoted $R(t)$, whereas its concentration in the flowing solution is R_o. Eventually, *i.e.* in a steady state, $R(t) = R_o$. The hormone can exist in any of the four states H(0,0), H(1,0), H(0,1) and (H1,1), which denote, respectively, the concentrations of hormone molecules without bound receptors, or with one receptor at site I or site II or sites I and II. The set of nonlinear differential equations describing the process of receptor binding is given in Appendix I. Schematically, in the case of independent binding sites, the binding reactions considered are:

$$H(0,0) + R \underset{k_{I,\mathrm{off}}}{\overset{k_{I,\mathrm{on}}}{\rightleftharpoons}} H(1,0) \qquad \text{(Eq. 1)}$$

$$H(0,0) + R \underset{k_{II,\mathrm{off}}}{\overset{k_{II,\mathrm{on}}}{\rightleftharpoons}} H(0,1) \qquad \text{(Eq. 2)}$$

$$H(1,0) + R \underset{k_{II,\mathrm{off}}}{\overset{k_{II,\mathrm{on}}}{\rightleftharpoons}} H(1,1) \qquad \text{(Eq. 3)}$$

$$H(0,1) + R \underset{k_{I,\mathrm{off}}}{\overset{k_{I,\mathrm{on}}}{\rightleftharpoons}} H(1,1) \qquad \text{(Eq. 4)}$$

The depleted concentration of the receptor is continuously replenished due to the flow, but depending on hormone concentration, the flow rate, and the reaction rate constants, $R(t)$ may be severalfold smaller than R_o. We assumed that the interaction follows a sequential pattern, whereby H(0,1) cannot be formed from H(0,0), *i.e.* the forward direction in Equation 2 is ignored, but H(0, 1) can be formed by dissociation of H(1,1), *i.e.* the reverse reaction in Equation 4. The steady state solutions turn out to be independent of the flow rate and are the same as those obtained by assuming constant receptor concentrations. For the

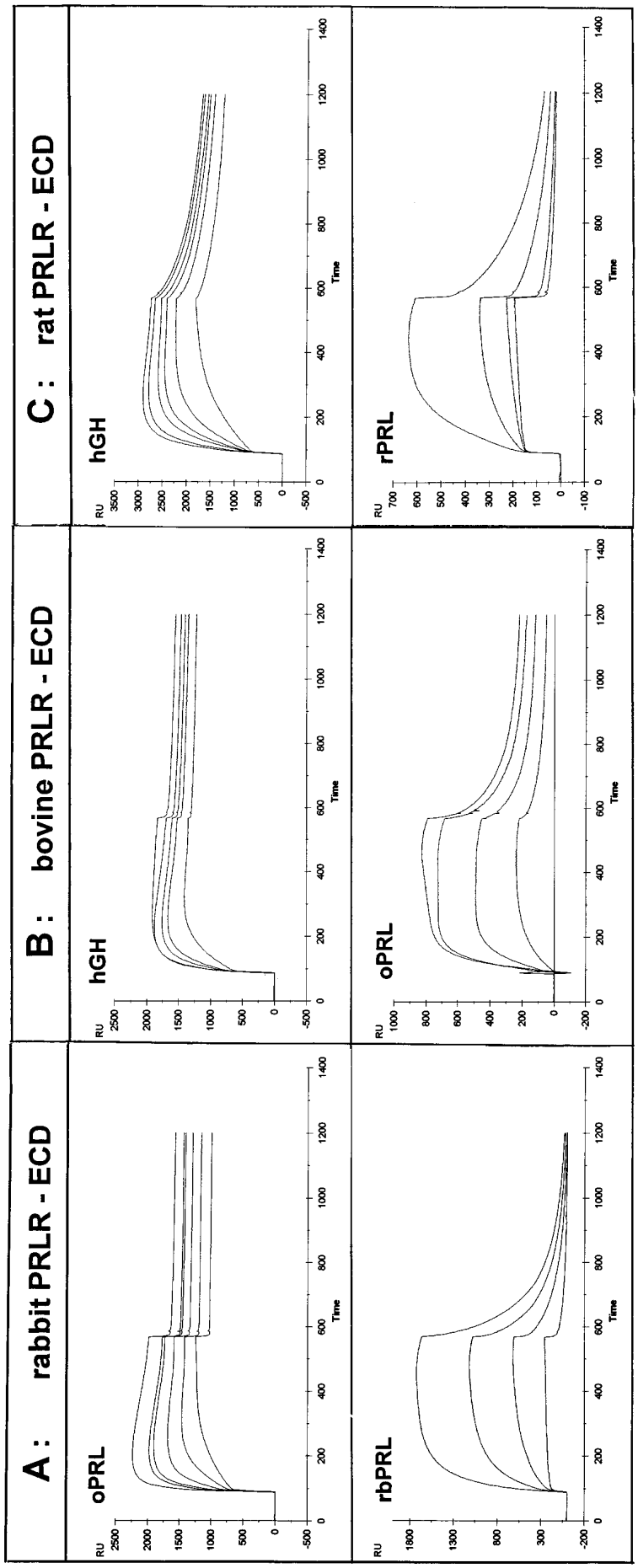

FIG. 1. **Association and dissociation kinetics of rbPRLR-ECD (*A*), bPRLR-ECD (*B*), and rPRLR-ECD (*C*) with heterologous (*upper panel*) or homologous (*lower panel*) lactogenic hormones covalently linked to CM-dextran through amino groups.** Resonance signals (*RU*) were plotted as a function of time for several concentrations (62.5–1000 nM) of various PRLR-ECDs. The association phase was followed through 600 s after which the infusion of the soluble PRLR-ECDs was stopped, and the dissociation phase in continuous buffer flow was monitored for additional 600 s.

TABLE I
Calculation of kinetic and thermodynamic constants of sites I and II for the interaction between rabbit PRLR-ECD and several lactogenic hormones

Hormone (sites I and II)	k_{on} ($mol^{-1} \cdot s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_d	ECD:hormone (molar ratio) SPR[a]	Half-life
			μM		*min*
hGH[b] I	3.9	0.5	1.3	1:1	231
hGH II	0.4	520	13000		0.2
hGH[c] I	7.2	0.8	1.2	1:1	144
hGH II	5.4	446	826		0.3
hGH[d] I	ND[e]	ND	ND	ND	ND
oPRL I	16	1.5	0.9	1:1	77
oPRL II	3.5	546	1580		0.2
bPL I	4.2	1.6	3.8	2:1	72
bPL II	14	10	7.1		12
rbPRL I	0.8	51	653	1:1	2.3
rbPRL II	1.9	185	975		0.6
rPRL I	20	32	16	2:1	3.6
rPRL II	43	414	96		0.3
hPRL I	1.4	3.8	28	2:1	30
hPRL II	0.6	240	4000		0.5

[a] Apparent stoichiometry as determined by surface plasmon resonance.
[b] Bound covalently.
[c] Bound via captured mAb 10A7.
[d] Bound via captured mAb 7B11.
[e] ND, not determined.

case of independent sites we found an analytic solution for the steady state stage:

$$H(0,0) = 1/(1 + (K_I + K_{II})R_o + K_I K_{II} R_o^2) \quad \text{(Eq. 5)}$$

$$H(1,0) = K_I R_o/(1 + (K_I + K_{II})R_o + K_I K_{II} R_o^2) \quad \text{(Eq. 6)}$$

$$H(0,1) = K_{II} R_o/(1 + (K_I + K_{II})R_o + K_I K_{II} R_o^2) \quad \text{(Eq. 7)}$$

$$H(1,1) = K_I K_{II} R_o^2/(1 + (K_I + K_{II})R_o + K_I K_{II} R_o^2) \quad \text{(Eq. 8)}$$

in which the bars indicate the fractions of hormones in the different states and $K_I = k_{I,\,on}/k_{I,\,off}$ and $K_{II} = k_{II,\,on}/k_{II,\,off}$. These equations show that in the limit of $R_o \to 0$, $H(0,0) = 1$, whereas the other quantities vanish, and when R_o tends to infinity, $H(1,1) = 1$ and the other quantities vanish. The steady state solutions are independent of hormone concentration.

RESULTS

Optimization of the Immobilization Procedure—Initially, rb, r, and bPRLR-ECDs were immobilized via their single free cysteine residue. Despite reasonably good covalent binding (2000–3000 RU/mm^2), the immobilized ECDs totally lost their ability to bind to the different PRLs, bPL, or hGH. Alternative immobilization of PRLR-ECDs using a cross-linker acting through amino groups also failed, and the binding capacity of the immobilized ECDs was drastically reduced. This most likely happened because of heterogeneous immobilization via

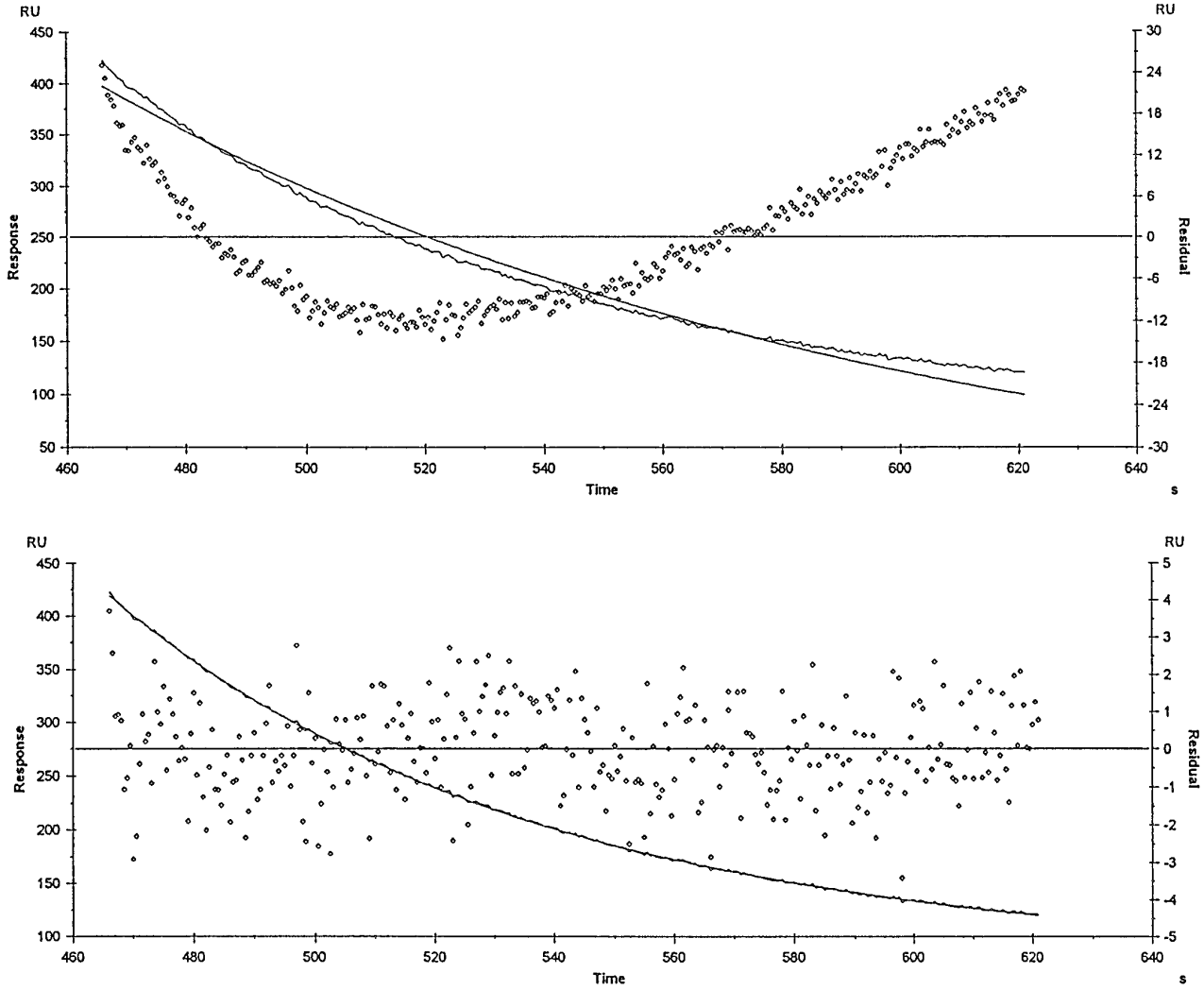

FIG. 2. **Fitting analysis by BIAcore software of the dissociation kinetics of rbPRLR-ECD bound to immobilized rbPRL (see Fig. 1*A*, *lower panel*) for one-site model (*upper panel*) and two-site model (*lower panel*).** The theoretical predicted dissociation curve is shown by a *solid line* (—), and the experimental results by *points* (· · ·). The residual difference between theoretical and experimental curves are represented on expanded scale (*right*). The signal noise in BIAcore corresponds to 4–6 RU.

TABLE II
Calculation of kinetic and thermodynamic constants of sites I and II for the interaction between bovine PRLR-ECD and several lactogenic hormones

Hormone (sites I and II)	k_{on} ($mol^{-1} \cdot s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_d	ECD:hormone (molar ratio) SPR[a]	Half-life
			nM		*min*
hGH[b] I	7.4	0.8	1.1	1:1	144
hGH II	4.9	68	138		1.7
hGH[c] I	5.0	4.4	8.7	1:1	26
hGH II	12	540	450		0.2
hGH[d] I	2.1	173	823	1:1	0.7
oPRL I	4.9	7.5	15	1:1	15
oPRL II	3.2	95	297		1.2
bPL I	0.6	14	233	1:1	8.3
bPL II	16	238	149		0.5

[a] For footnotes, see Table I.

TABLE III
Calculation of kinetic and thermodynamic constants of sites I and II for the interaction between rat PRLR-ECD and several lactogenic hormones

Hormone (sites I and II)	k_{on} ($mol^{-1} \cdot s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_d	ECD:hormone (molar ratio) SPR[a]	Half-life
			nM		*min*
hGH[b] I	3.0	8.9	30	2:1	13
hGH II	3.9	129	332		0.9
hGH[c] I	1.4	7.2	50	2:1	16
hGH II	4.3	142	330		0.8
hGH[d] I	1.3	105	785	2:1	1.1
hGH[e] I	1.8	0.5	2.8	2:1	230
hGH II	1.8	306	1700		0.4
oPRL I	2.4	25	104	2:1	4.6
oPRL II	9.6	490	510		0.2
bPL I	3.6	2.0	5.5	2:1	58
bPL II	2.1	10	48		12
rPRL I	2.6	26	100	1.2:1	4.4
rPRL II	0.7	160	2285		0.7
hPRL I	2.1	27	129	2:1	4.4
hPRL II	3.4	150	441		0.7

[a] For Footnotes *a–d* see Table I.
[e] As in Footnote b but the experiment was performed in the presence of 0.015 mM $ZnCl_2$.

several amino groups, some of them probably being important for hormone binding. An alternative protocol was employed to overcome this difficulty; the respective hormones were immobilized via their amino groups. This method yielded promising results; in all cases, the immobilized hormones retained a high capacity of interaction with soluble PRLR-ECDs or, in the case of hGH, with hGHR-ECD. As shown in Tables I–IV, the immobilized hormones were capable of binding the soluble R-ECDs with apparent stoichiometries ranging from 1:1 to 1:2, under conditions in which the ligands were applied at 0.25–1 μM.

Interaction of rb, b, and rPRLR-ECDs with Several Lactogenic Hormones—The interactions of rbPRLR-ECD with oPRL and hGH yielded similar kinetic parameters (Fig. 1*A* and Table I). The apparent stoichiometry indicated a 1:1 interaction at equilibrium. However, analysis of the kinetic data by fitting to a theoretical model (Fig. 2) showed to be unsuited to the 1:1 model and strongly favoring the two-site model. Similar results were also obtained with hPRL; although here, the apparent stoichiometry of interaction was 2:1. Interaction with rPRL also yielded a 2:1 apparent stoichiometry, but in this instance the k_{on} values were ~10-fold higher than those of aforementioned complexes. The interaction with bPL was much stronger, mainly due to a relatively low k_{off} for site II, and an apparent 2:1 complex was observed by both SPR and gel filtration analysis (20). As can be seen, the interaction of rbPRLR-ECD with the putative site I of all hormones was always much stronger than with site II, although the relative affinities varied to a large extent. The lower affinity for site II unanimously originated from elevated k_{off} values, whereas the k_{on} values had hardly any effect. In contrast, the interaction with the homologous hormone rbPRL was characterized by a rather weak interaction with both sites I and II.

Interaction of bPRLR-ECD with oPRL, bPL, and hGH indicated formation of an apparent 1:1 complex as determined by both SPR (Fig. 1*B* and Table II) and gel filtration (21). However, analysis of association and dissociation kinetics, performed as shown in Fig. 2 (data not presented), indicated a complete lack of fit to the presumed one-site model and a very good fit to the two-site model. Simulation analysis also favored the 50/50 double-site model with the exception of hGH. As in the binding of hGH, the different affinities for sites I and II could be attributed to the 10–20-fold higher k_{off} values for site II, whereas the differences in the k_{on} values were relatively small. Similar to the aforementioned case of homologous interaction, the interaction of bPRLR-ECD with oPRL (identical to bPRL) and bPL, both homologous hormones, was also characterized by a relatively lower affinity of the homologous hormones, not only for site II but also for site I. Here again, the lower affinity resulted mainly from the increase in k_{off}.

The interaction of rPRLR-ECD with different hormones (Fig. 1*C* and Table III) yielded in SPR experiments an apparent stoichiometry of 2:1, except with rPRL in which only a 1:1 stoichiometry has been observed, as also shown in gel filtration experiments (22). Kinetic analysis revealed a very good fit to the two-site model and a poor fit to the one-site model. All kinetic parameters also gave an optimal 50/50 two-site simulation analysis. Bovine PL exhibited the highest affinity to both sites I and II, although the latter was relatively lower, mainly due to a 5-fold higher k_{off} value as compared with site I. Other hormones, such as h or oPRL, exhibited lower affinities for binding to both sites, again mainly due to higher k_{off} values. The affinity of rPRLR-ECD for the homologous rPRL was weaker than for other hormones. Whereas the affinities and kinetic constants for site I resembled those of oPRL and hPRL, the affinity to site II was 6–15-fold lower, primarily because of a lower k_{on}.

Presentation of hGH by Anti-hGH mAbs—Direct, covalent immobilization of the hormones via their amino groups raised the question of whether the immobilization yielded homogeneous or heterogeneous populations. The latter could affect the calculation of association and dissociation kinetic constants. To answer this question, an alternative method of homogeneous immobilization of hGH was employed using two anti-hGH mAbs, mAb 10A7 which binds to hGH in a way that does not interfere with the latter's ability to form a 1:2 homodimeric complex with hGHR-ECD, and mAb 7B11 which interacts with an epitope located within site II of hGH and, as such, enables only a 1:1 interaction through site I (35). Both mAbs were captured by a covalently immobilized rabbit anti-mouse IgG-purified immunoglobulins. The association of hGH with mAb 7B11 was ~20-fold slower than with 10A7 ($k_{on} = 0.98 \times 10^4$ and 2.05×10^5 mol^{-1} s^{-1}, respectively), and the dissociation was also ~3-fold slower ($k_{off} = 4.5 \times 10^{-5}$ and 14.1×10^{-5} s^{-1}, respectively). The resultant $K_{d/}$ values for mAbs 7B11 and 10A7 were, correspondingly, 4.6 and 0.68 nM and their respective calculated half-lives were 257 and 82 min.

Binding of Soluble hGHR-ECD and rPRLR-ECDs to hGH Immobilized via Anti-hGH mAbs—Direct immobilization of hGH *versus* its presentation via mAb 10A7 yielded very similar results in terms of its interaction with hGHR-ECD (Fig. 3 and Table IV). The kinetics of association and dissociation were

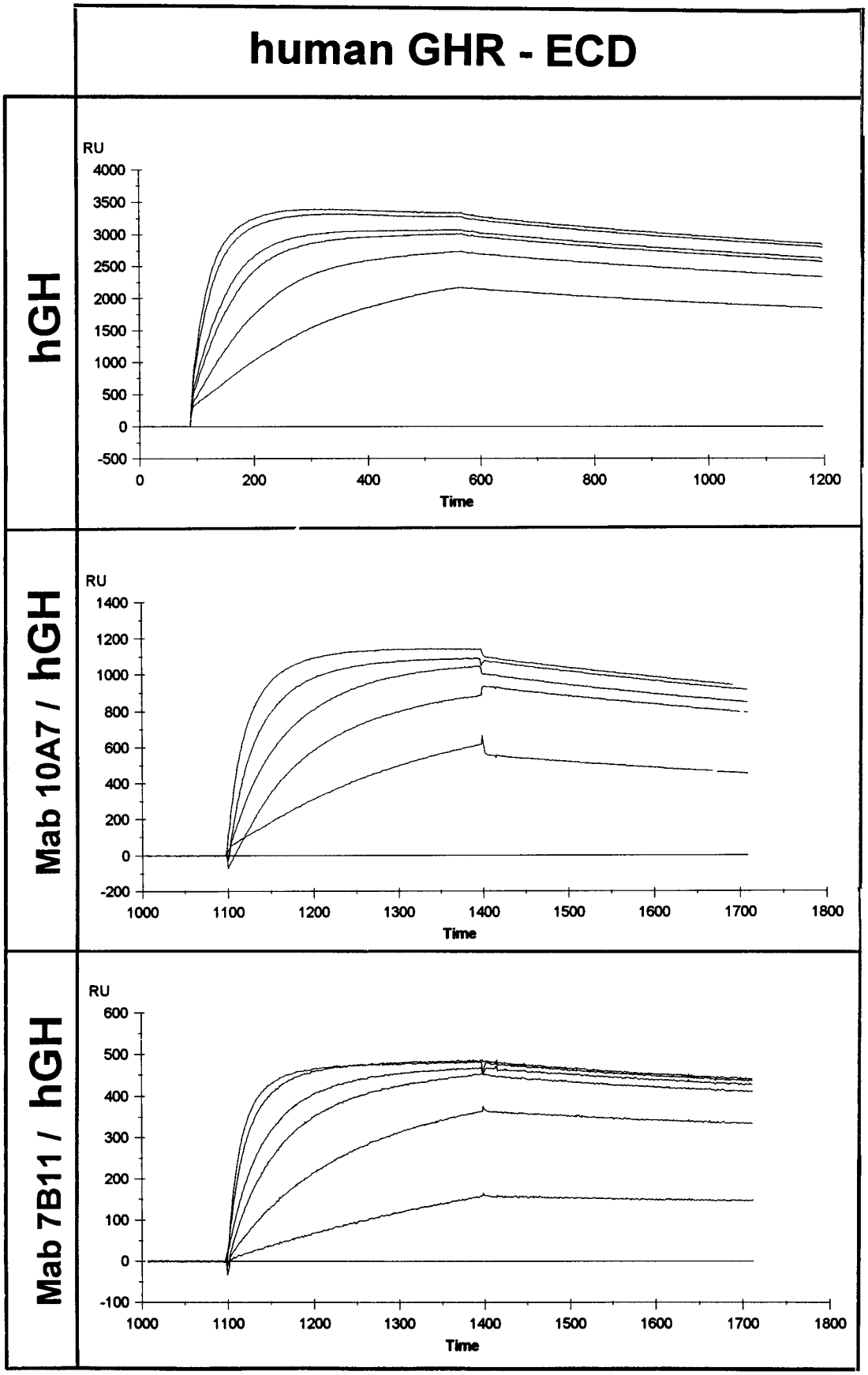

FIG. 3. **Association and dissociation kinetics between hGHR-ECD and hGH covalently linked to CM-dextran through amino groups (*upper panel*), presented via capture of mAb 107A (*middle panel*) or via capture of mAb 7B11 (*bottom panel*).** In experiments in which hGH was presented via capture of mABs the association phase was preceded by a step of successive capture of the respective mAb and hGH. For other details see the legend to Fig. 1.

TABLE IV
Calculation of kinetic and thermodynamic constants of sites I and II for the interaction between hGHR-ECD and hGH

Hormone (sites I and II)	k_{on} ($mol^{-1} \cdot s^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_d	ECD:hormone (molar ratio) SPR[a]	Half-life
			nM		*min*
hGH[b] I	6.9	3.8	5.6	2:1	30
hGH II	1.0	2.3	23		50
hGH[c] I	9.3	5.5	5.5	2.2:1	21
hGH II	2.5	5.0	20		23
hGH[d] I	4.8	2.6	5.6	1.2:1	44
hGH[e] I	2.3	4.3	18	2:1	27
hGH II	0.7	3.0	43		38

[a] For Footnotes *a–d* see Table I.
[e] As in Footnote b but the experiment was performed in the presence of 0.015 mM $ZnCl_2$.

analyzed using both 2:1 and 1:1 models of interaction. The results did not fit the one-site model with either direct or indirect immobilization, whereas a good statistically significant ($p < 0.05$) fitting was obtained with the two-site model. Moreover, calculating the kinetic constants by BIAcore simulation analysis also confirmed the 50/50 double-site model.

A comparison of the kinetic and thermodynamic constants for the binding of rb, b, and rPRLR-ECDs by hGH that was either directly immobilized or presented via mAb 10A7 yielded a high degree of similarity, as found with hGHR-ECD (see Fig. 4 and the four top lines in Tables I–III). In the case of rbPRLR-ECD (Fig. 4 and Table I), the interaction with site I of hGH yielded almost identical kinetic constants, whereas the association to site II was enhanced ~10-fold in hGH presented via mAb 10A7. Although the apparent stoichiometry was 1:1 in both cases, as in gel filtration experiments (19, 20), neither the association nor the dissociation data fit the 1:1 model. In contrast, analysis using the two-site model yielded a much better fit. The discrepancy between the apparent 1:1 stoichiometry and the putative 2:1 stoichiometry (obtained by calculation) can most likely be attributed to the 3 to 4 orders of magnitude difference in the corresponding K_d values for the interaction with sites I and II of the hormone. These differences indicate that the interaction with site II is less stable, as determined by measuring the k_{off} constants; correspondingly, the half-life of the complex formed by interaction with site II of hGH was extremely short (<0.2 min). Similar results were also obtained with bPRLR-ECD (Fig. 4 and Table II), although in this case the dissociation rate of the complex formed with hGH presented by mAb 10A7 was ~5-fold higher than that with the directly immobilized hormone. Here again, an apparent 1:1 stoichiometry was observed, whereas kinetic analysis favored the 2:1 model. Similarly no differences were observed in the interaction of rPRLR-ECD with hGH immobilized directly or indirectly via both mAbs (Fig. 4 and Table III). In this case, however, not only was the stoichiometry calculated from the kinetic data 2:1, but the apparent stoichiometry was also 2:1, as found previously in gel filtration experiments (22).

Presentation of hGH via mAb 7B11 resulted in the formation of 1:1 complex with all three PRLR-ECDs (see Tables I–III, line 5). However, in contrast to the binding of hGHR-ECD in which the kinetic and thermodynamic parameters were close to those calculated for the interaction with site I of the hormone, the corresponding parameters calculated for interaction with b and rPRLR-ECDs were in all cases similar to the values calculated for the interaction with site II. These results indicate that in the latter case, occupation of site II by mAb 7B11 also weakens the apparent interaction of site I of hGH with the respective PRLR-ECDs. This occurs as a result of destabilization of the complex, rather than of changes in the rate of its formation, as evidenced by a ~100- to 1000-fold increase in k_{off} values. In the case of interaction with rbPRLR-ECD, kinetic constants could not be calculated because the latter's interaction with hGH presented by mAb 7B11 destabilized the mAB·hGH complex, causing its dissociation (Fig. 4).

Effect of Zn^{2+} on the Interaction of hGH with rb, b, and Rat PRLR-ECDs—The cation Zn^{2+} has been implicated in the interaction of hGH with hPRLR-ECD by forming a coordinate bond between His^{19} and Glu^{174} of the hormone and Asp^{217} and His^{218} of the receptor (25). To study whether such an effect also exists in the interaction of hGH with other PRLR-ECDs and hGHR-ECD, binding experiments were carried out in the presence of 15 μM $ZnCl_2$. The addition of Zn^{2+} had only a minor effect. With hGHR-ECD, no major change was noted in the dissociation kinetics, but the association was clearly slower (see Table IV, last two lines). With rPRLR-ECD (see Table III, line 6), the addition of Zn^{2+} increased the dissociation of 2:1 complex to 1:1 complex but rendered the latter more stable. Similar results were obtained with rbPRLR-ECD and bPRLR-ECD (not shown).

Evaluation of "Sequential" Versus "Two Independent Site" Models—Since the BIAcore software does not include the analysis of a sequential binding model, a set of equations aimed at calculating the fractional occupancy of the immobilized hormone by the R-ECD throughout the experiment was developed and utilized to compare the two possible models. A representative experiment in which the fractional occupancy during the first 10 min of interaction was calculated from the respective kinetic constants presented in Table III is shown in Fig. 5. A logarithmic time scale was used for better visualization of the differences occurring during the initial reaction period. The results indicate that the predicted fractional occupancy of sites I and II only differs slightly at the first 2 min, whereby the two independent site model predicts slightly faster simultaneous occupation of both sites than the sequential model. At later times, both models predict almost identical results. Calculation of other interactions yielded a similar picture (not shown).

DISCUSSION

The main question raised in the present work was whether the interaction of various PRLs with their homologous or heterologous receptors leads to the formation of a homodimer, as in a well-documented GH model. Kinetic experiments using three different PRLR-ECDs and several lactogenic hormones strongly suggested that lactogenic hormone-induced PRLR-ECD dimerization does occur as follows: (*a*) no good fit to the one-site model was found in any case; (*b*) a good fit enabling a calculation of kinetic rate constants was obtained only using the two-site model; (*c*) analyzing the kinetic rate constants by BIAcore simulation program confirmed binding to two independent sites, in a 50/50 double-site model. All three criteria were met for all interactions except for that of hGH with bPRLR-ECD, where the fit criteria were indicative of a two-site interaction but the third criterion indicated unequal occupation of sites I and II. The reasons for this exception are unclear. Since the mathematical model presently used in BIAcore software does not allow an analysis of sequential binding, we developed a mathematical model to analyze the results accordingly. We found the difference between the two independent sites model and the sequential model to be rather small, the latter shifting the fractional occupancy curve slightly to the right, especially early on (Fig. 5*B*). The validity of the sequential model is supported by the findings that (*a*) hGH immobilized via various mAbs directed to site I is unable to bind any hGHR-ECD,[3] and (*b*) antibodies to site I abolish binding of hGHR-ECD to both site I and site II (35), whereas mAb 7B11,

[3] C. J. Strasburger, unpublished data.

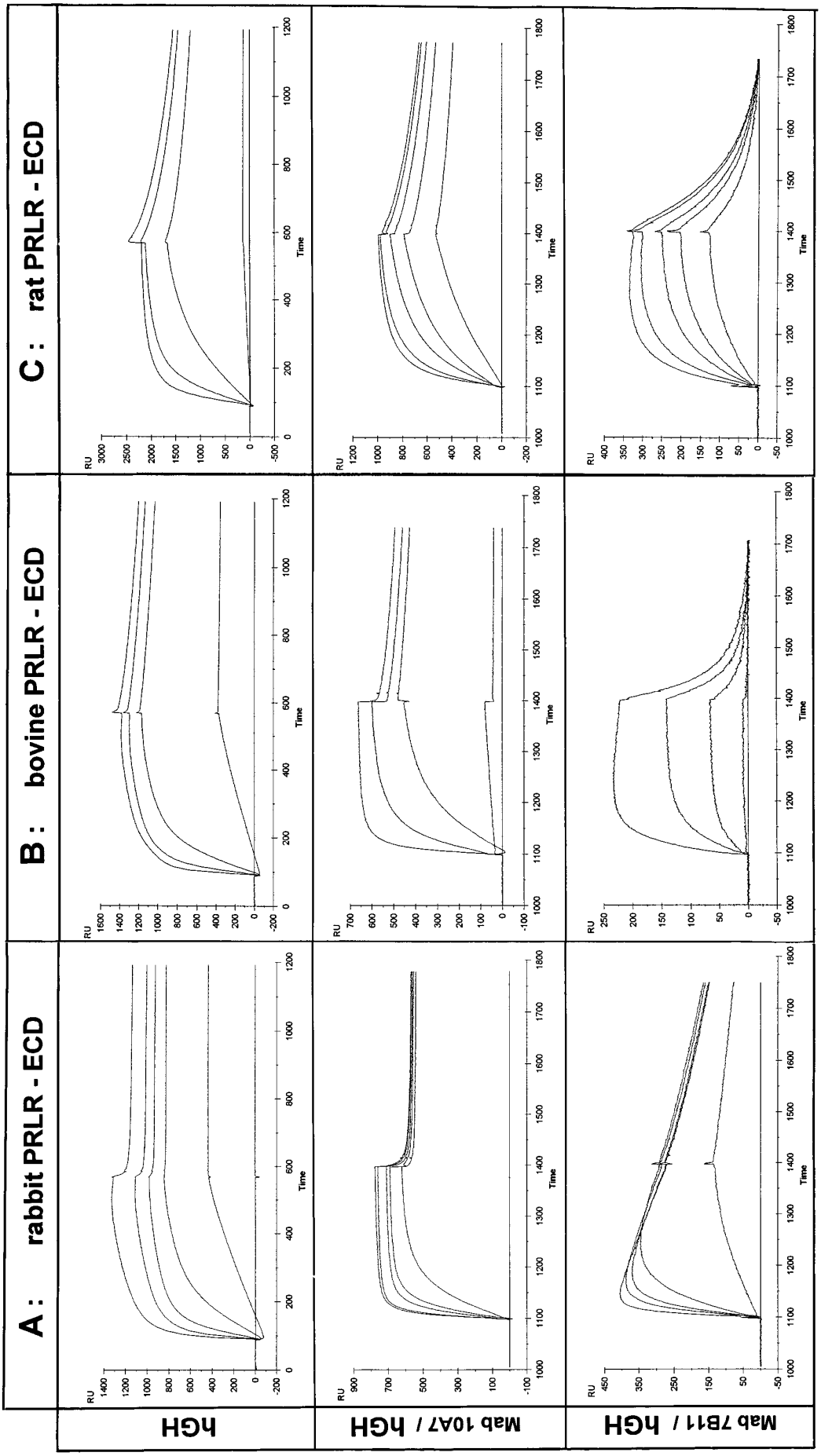

FIG. 4. **Association and dissociation kinetics between rbPRLR-ECD (*A*), bPRLR-ECD (*B*), and rPRLR-ECD (*C*) and hGH covalently linked to CM-dextran through amino groups (*upper panel*), presented via capture of mAb 10A (*middle panel*), or via capture of mAb 7B11 (*bottom panel*).** For other details see the legends to Figs. 1 and 3.

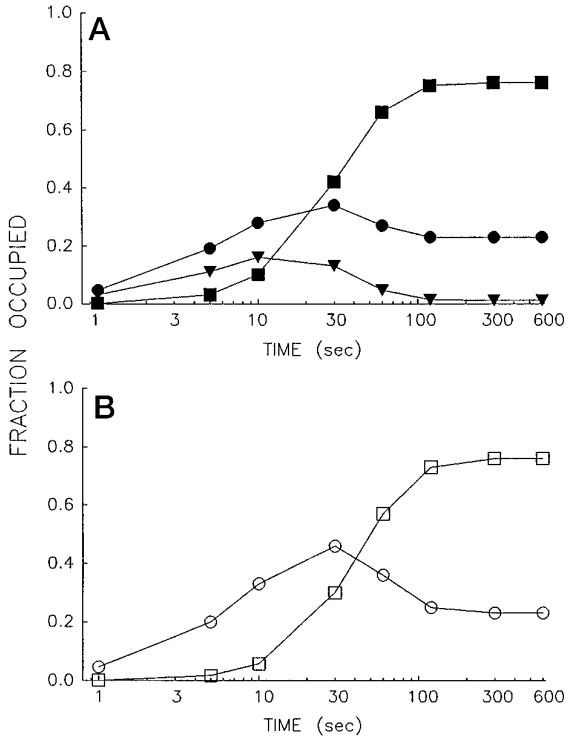

FIG. 5. **Comparison of two independent site (*solid symbols*) (*A*) with sequential (*open symbols*) models for binding of rPRLR-ECD to hGH (*B*).** *Squares*, simultaneous occupation of site I + site II, H(1,1). *Circles*, occupation of site I only, H(1,0). *Inverted triangles*, occupation of site II only, H(0, 1).

which binds to site II of hGH, did not interfere with binding to site I, as documented in Fig. 3 and Table IV.

The universal feature of a strong binding site (designated site I) and a loose binding site (designated site II), the latter usually exhibiting 5- to 100-fold lower affinity, was observed in most of interactions between lactogenic hormones and PRLR-ECDs studied in this work. Although the present results do not enable topological identification of sites I and II, the recently published three-dimensional structure of hPRLR-ECD·hGH complex (25) suggests that the strong binding site I most likely corresponds to the site participating in that interaction, namely the site designated as site I of hGH (10, 25). This situation differs from that observed for the hGH-hGHR interaction, in which a stable 2:1 hGHR-ECD·hGH complex could be isolated (11, 16) and crystallized (10), and most likely indicates formation of transient homodimeric complexes that rapidly dissociate to 1:1 complexes. The only exceptions were interactions of rb and rPRLR-ECDs with bPL, in which the 2:1 complexes were more stable (see Tables I and III). The interaction of rPRLR-ECD with hGH and oPRL also yielded a 2:1 complex, although it was less stable than that formed with bPL (22). These differences parallel the $K_{d\,II}$ values in Table III. Although no structural interpretation can be made as yet, the extended N-terminal portion of bPL may stabilize the homodimer as truncation of 13 of the N-terminal bPL amino acids has a strongly destabilizing effect (20).

The transient nature of the hormone-receptor complexes was even more pronounced in homologous interactions involving hormones and receptors from the same species. These interactions are generally characterized by faster dissociation of the homodimeric complexes or even the 1:1 complexes (see Tables I–III), and the differences between the homologous and heterologous interaction can be mainly attributed to higher k_{off} constants. This observation explains the paradoxical situation faced by many investigators of PRLRs who were unable to perform binding experiments using homologous hormones and used hGH or other heterologous lactogenic hormones as the ligand (26–28). It also explains why the large differences in affinity constants, as calculated by classical binding experiments, were not always paralleled by corresponding differences in biological activity. This seems to be particularly true for the homologous hormones, which often exhibit low thermodynamic association constants (K_a) in contrast to high biological activity (36, 37).

The present results strongly suggest that the use of gel filtration or binding experiments as tools for determining binding constants and stoichiometries of interaction may be misleading, since sometimes the 2:1 PRLR-ECD·hormone complex cannot be identified due to its rapid dissociation to a 1:1 complex. The use of real-time kinetic analysis based on SPR allows a more accurate interpretation. The currently described progress in the elucidation of PRLR-mediated signal transduction enables us to understand why transient homodimerization of the receptor, lasting only a few seconds, may be sufficient to initiate the biological signal by transphosphorylation of Jak2 or other associated kinases (4, 5, 38, 39). Once these enzymes are activated, the receptor dimer is no longer required. In fact, rapid dissociation of a 2:1 complex to a 1:1 complex or to its components may be advantageous in that it can augment receptor re-availability or permit activation of other receptors. The very recently reported erythropoietin-induced transient homodimerization of erythropoietin receptor, characterized by one high affinity and one low affinity interaction (40), further supports our present suggestion. Our results also have an impact on the strategy for preparing the so-called second generation of recombinant hormones. In the case of hormones that stimulate signal transduction by homo- or heterodimerization of receptors, a high affinity recombinant agonist in which the elevated K_a is achieved mainly by lowering the k_{off} will not necessarily be advantageous. Indeed, high affinity analogues of hGH (41) do not exhibit higher biological activity.[4] In contrast, this is not the case when designing hormone antagonists in which prolonged life of the complex is highly beneficial.

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APPENDIX I

The differential equations describing Equations 1–4 are:

$$dH(0,0)/dt = -(k_{I,on} + k_{II,on}) \cdot R \cdot H(0,0) + k_{I,off} \cdot H(1,0) + k_{II,off} \cdot H(0,1) \quad \text{(Eq. 9)}$$

$$dH(1,0)/dt = k_{I,on} \cdot R \cdot H(0,0) - k_{I,off} \cdot H(1,0) - k_{II,on} \cdot R \cdot H(1,0) + k_{II,off} \cdot H(1,1) \quad \text{(Eq. 10)}$$

$$dH(0,1)/dt = k_{II,on} \cdot R \cdot H(0,0) - k_{II,off} \cdot H(0,1) - k_{I,on} \cdot R \cdot H(0,1) + k_{I,off} \cdot H(1,1) \quad \text{(Eq. 11)}$$

$$dH(1,1)/dt = k_{I,on} \cdot R \cdot H(0,1) + k_{II,on} \cdot R \cdot H(1,0) - (k_{I,off} + k_{II,off}) \cdot H(1,1) \quad \text{(Eq. 12)}$$

Generally, R is a function of the time, t.

[4] K. Pearce and J. A. Wells, personal communication.

In a steady state, *i.e.* where $dH(0.0)/dt = dH(1,0)/dt = dH(0,1)/dt = dH(1,1)dt = 0$, Equations 9–12 yield Equations 5–8. The time derivative of R is given by

$$\begin{aligned} dR/dt = & -(k_{I,on} + k_{II,on}) \cdot R \cdot H(0,0) \\ & - k_{I,on} \cdot R \cdot H(0,1) - k_{II,on} R \cdot H(1,0) \\ & + k_{I,off} \cdot H(1,0) + k_{II,off} \cdot H(0,1) \\ & + (k_{I,off} + k_{II,off}) \cdot H(1,1) + \alpha \cdot (R_o - R) \end{aligned} \qquad \text{(Eq. 13)}$$

in which the quantities H(0,0), H(1,0), H(0,1), and H(1,1) denote, respectively, the concentrations of the hormone in the four possible states of occupation. The term $\alpha(s^{-1})$ is the flow-rate constant of the receptor, volume flowing per s relative to the volume where the fixed hormone resides. Equation 13 slightly overestimates $R(t)$ at very short times when lateral diffusion of the receptor molecules significantly lags behind replenishment of the depleted R-ECD from the solution near the hormone molecules. Under our experimental conditions, the assumption $R(t) = R_o$ is adequate at $t \geq 5$ s. In the case of sequential binding, the terms involving $k_{II,\,on} \cdot R \cdot H(0,0)$ in Equations 9, 11, and 13 are ignored. These nonlinear differential equations were solved by Taylor expansion as described previously (42).

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