CHAPTER V
RESULTS AND DISCUSSION OF BINDING EXPERIMENTS

Binding studies were conducted as described in section 2.11 to correlate with information from the rapid-binding studies of the microphysiometer. Binding studies are conducted at 4°C since cellular processes are slowed and receptor internalization becomes negligible (Lauffenburger and Linderman, 1993). Binding of receptors with ligand then allows the calculation of cell surface receptor number and of ligand affinity for the receptor. The relation used in the following calculations takes into account total binding which consists of the two components specific and nonspecific binding:

Total Bound Ligand = $(R_t * L_o) \div (K_d + L_o) + K_n * L_o$

Where

$(R_t * L_o) \div (K_d + L_o)$ = specific binding
$K_n * L_o$ = nonspecific binding

R_t = Receptor number
L_o = free ligand available for binding (we assume no depletion)
K_d = Dissociation constant = $k_{reverse}/k_{forward}$
K_n = Nonspecific binding constant

Mathematica® software was used to evaluate parameters using nonlinear fit (Appendix A)

This binding relation is based on a simple monovalent model of ligand binding to a receptor where a single ligand attaches reversibly to a single receptor. The above equation is derived from an assumption of steady state for the kinetic equation for specific binding:

$dC/dt = 0 = k_f*R*L - k_r*C$

Where

C = number of ligand/receptor complexes
k_f = association rate constant
R = number of receptors
L = concentration of ligand
k_r= dissociation rate constant

To determine nonspecific binding in the experiments, wells were plated with radiolabeled tracer and an excess of unlabeled ligand. By adding an excess of unlabeled ligand, the amount of labeled ligand binding to receptors becomes insignificant. In these studies, an excess of 1 ml of 800 ng/ml cold (i.e. unlabeled ligand) was added with tracer to the wells to determine nonspecific binding.

To verify that 800 ng/ml of cold ligand was sufficient to inhibit specific binding, steady state is assumed for both radiolabeled ligand binding with receptors and competing cold ligand binding with receptors:

$DC_1/dt = 0 = k_f*R*L_1 - k_r*C_1$
$DC_2/dt = 0 = k_f*R*L_2 - k_r*C_2$

Where
Subscript 1 denotes radiolabeled ligand or ligand/receptors complexes
Subscript 2 denotes unlabeled “cold” ligand or ligand/receptor complexes

We assume that the radiolabeled and non-radiolabeled ligand have the same binding affinity for the receptor. Using the relation for total receptors $R = R_t - C_1 - C_2$, and rearranging, the relation derived is:

$$C_1 = R_t * (1 - 1 \div (K_D + Lo_2)) \div (K_D + L_1(1 - 1 \div (K_D + Lo_2))$$

From this it can be seen that $C_1 = L_1 R_t \div (L_1 + K_D)$ when $Lo_2 >> K_D$

For our studies, this means 800 ng/ml IGF-I = 1.0×10^{-7} M >> estimated $K_D = 1.0 \times 10^{-10.}$ M. For IGFBP-3, 800 ng/ml = 1.7×10^{-8} M >> estimated $K_D = 1.0 \times 10^{-10.}$ M. Two orders of magnitude are typically considered sufficient. This establishes 800 ng/ml of cold ligand to be sufficient for determining nonspecific binding.

5.1 Binding study of SV40-IGF-I cells with ^{125}I radiolabeled IGF-I and IGFBP-3

It was found from the microphysiometer studies that SV40-IGF-I cells responded to IGF-I alone, but not to IGFBP-3 alone. To further investigate this occurrence, traditional binding studies as described in Section 2.11 were conducted. Each experimental point is the average of a triplicate. Total binding was determined experimentally for IGF-I and IGFBP-3 binding to SV40-IGF-I cells at 8 different concentrations of ligand. Using the binding model described previously, values of receptor number and the dissociation constant were determined using a nonlinear fit with Mathematica® software. Values for these numbers are listed in Table 5.1. Figure 5.1 shows the close fit of the experimental data and the binding model. Figure 5.2 shows that IGF-I does indeed have specific binding to the cells as was expected. The calculated curve as shown in Figure 5.1 is repeated for Figure 5.2. Figure 5.2 shows experimental data for specific and non-specific binding at 2 and 8 ng/ml IGF-I and IGFBP-3.

Table 5.1 Calculated Cell Binding Parameters

	Receptors (# cell)		K_d (M)	
Cell line	**SV40-IGF-I**	**BAE**	**SV40-IGF-I**	**BAE**
IGF-I	102,000	15,000	5.17×10^{-10}	3.66×10^{-10}
IGFBP-3	6000	3000	6.37×10^{-11}	4.65×10^{-11}

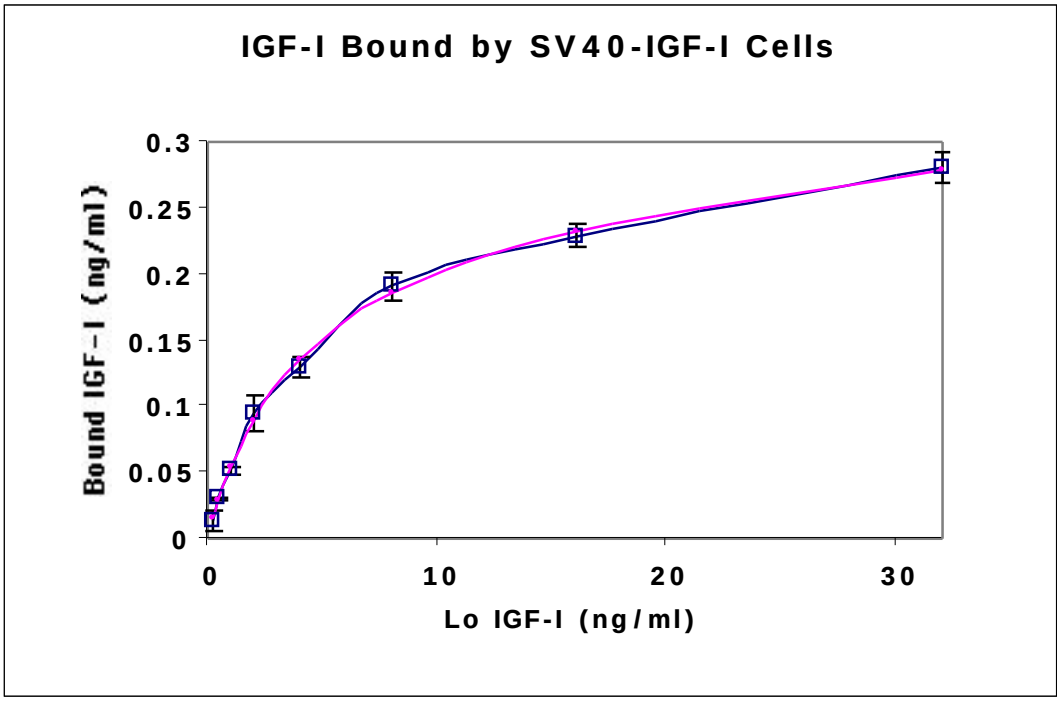

Figure 5.1 *Total binding: SV40-IGF-I (passage 16) cells bound by ^{125}I-IGF-I (□) found experimetally and the calculated bound points (−) from Rt and Kd values calculated from nonlinear regression on Mathematica® software (Table 5.1) according to the binding model. Experimental points are the average of triplicates. Standard error bars are shown.*

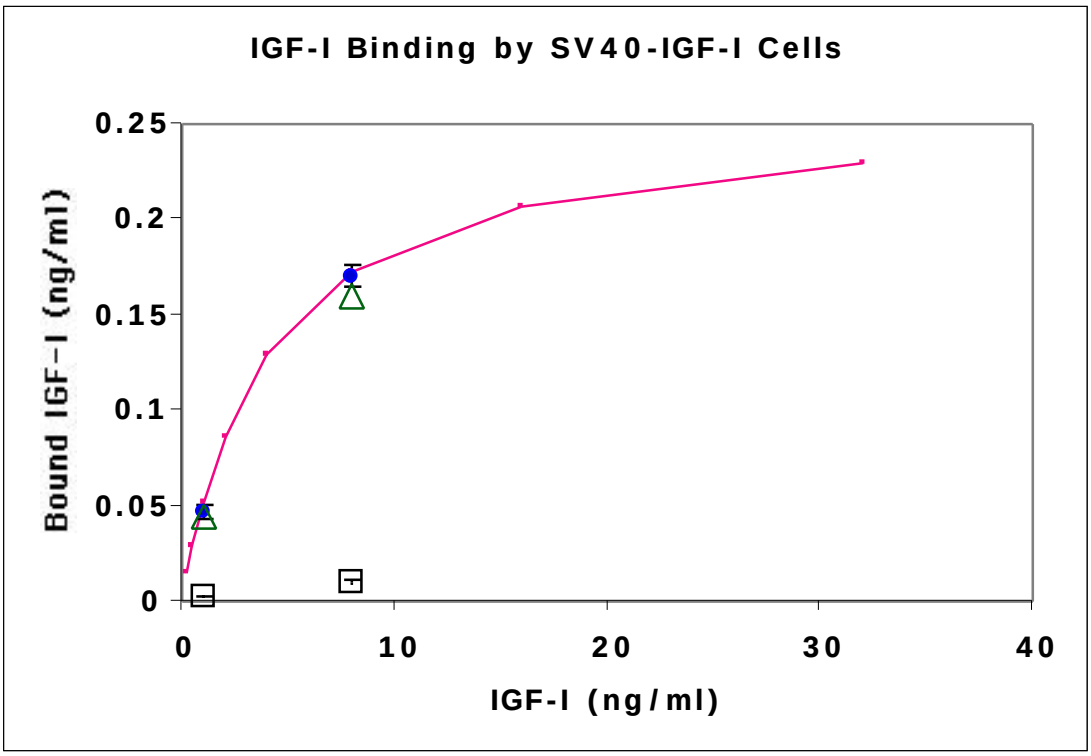

Figure 5.2 *The calculated curve for specific IGF-I binding* (−) *is presented with two data points (2 ng and 8 ng/ml) of total binding* (●), *specific binding* (Δ), *and non-specific binding* (□). *Standard error bars for averaged triplicate experimental data shown.*

Figures 5.3 and 5.4 repeat the same presentation for IGFBP-3 binding to the SV40-IGF-I cells. Calculated values for receptor number and dissociation constant from the nonlinear fit of the experimental data are listed in Table 5.1. IGFBP-3 appears to bind to SV40-IGF-I with high affinity and very few receptor sites as compared to IGF-I. This was not revealed in the microphysiometer experiments, as there was no apparent response to IGFBP-3 alone. It is possible that either the IGFBP-3 ws not binding under these flow conditions or binding was not reflected in the %ECAR response. An alternate explanation is that the very few receptor sites calculated may be insignificant since the

number of cells per well was an estimate, and therefore may have caused the calculated number of receptors to be erroneously high.

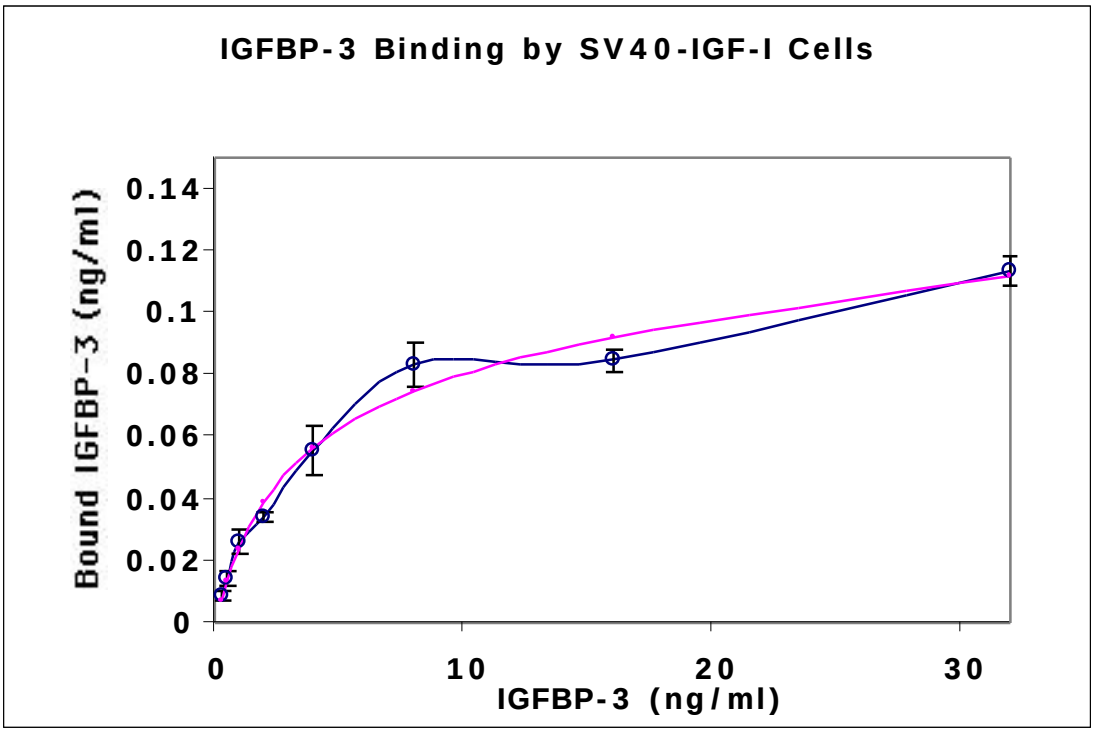

Figure 5.3 *Total binding: SV40-IGF-I cell binding by* ^{125}*I-IGFBP-3 (○) compared with the calculate (–)values from Kd and Rt parameters found using a nonlinear fit of the data. Error bars for duplicate experimental points are shown.*

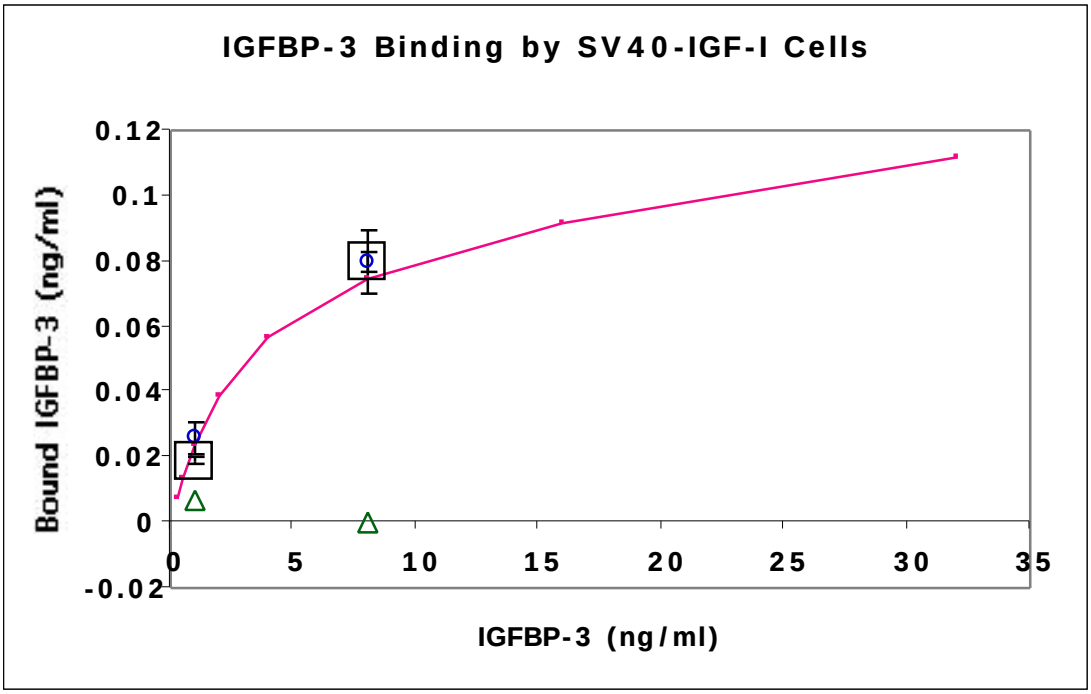

Figure 5.4 *Calculated specific binding curve* (−) *for SV40-IGF-I cells by* ^{125}*I-IGFBP-3 from the calculated values from Kd and Rt parameters found using a nonlinear fit of the data. Experimental values for total* (○), *specific* (Δ) *and non-specific* (□) *binding are represented as labeled. Error bars for duplicate experimental points are shown.*

5.2 Binding study of SV40-IGF-I cells with ^{125}I radiolabeled IGF-I preincubated with 50 ng/ml unlabeled IGF-I

Figure 5.5 shows data from SV40-IGF-I (passage16) cells seeded at 2x10^5 cells/ml into a 24 well plate. Each well held 1 ml of cell suspension. Cells were serum starved for 72 hrs. Preincubated cells (two wells for each tracer concentration) received 50 ng/ml unlabeled IGF-I and were incubated at 37°C for 1 hr. Control wells (one well for each tracer concentration) continued to be serum starved. Cells were then bound with ^{125}I IGF-I. Nonspecific binding was neglected in the binding model, and using nonlinear

regression in Mathematica® (Appendix A), the values for surface receptors and affinity were calculated.

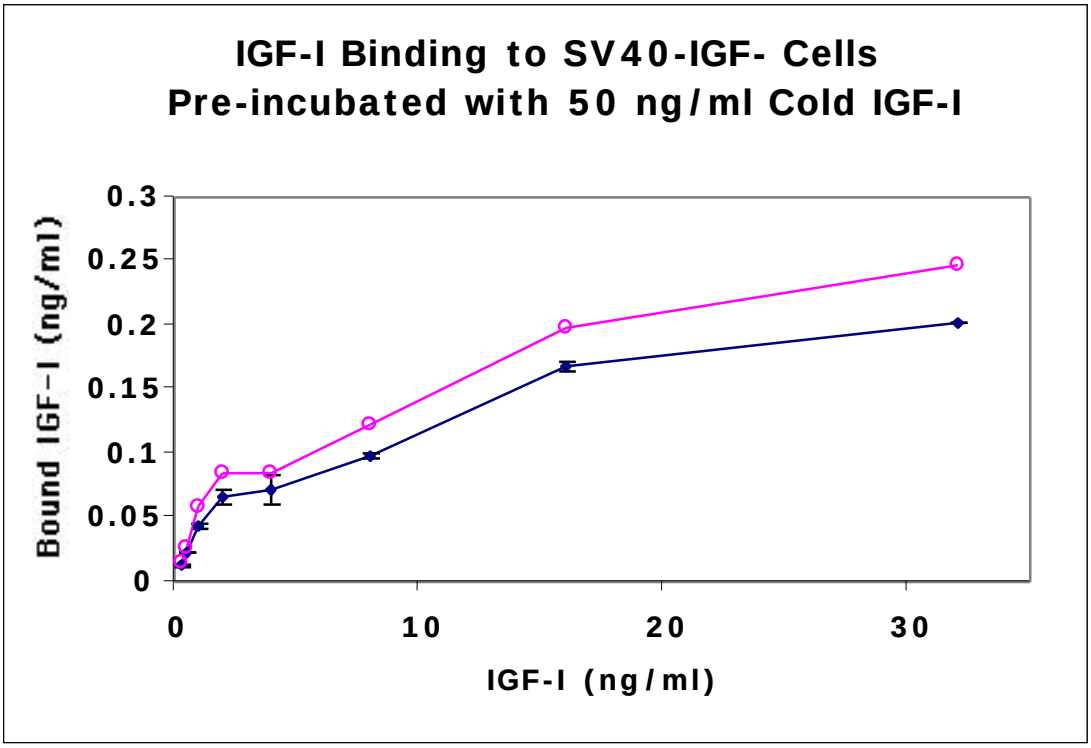

Figure 5.5 *Binding studies of SV40-IGF-I cells (passage16) seeded at 2x10^{5}cells/ml and serum starved for 72 hrs. Binding of the control (○) with ^{125}I radiolabeled IGF-I shows a higher binding compared with pre-incubation of the cells with 5 ng/ml IGF-I (◆) prior to binding with ^{125}I radiolabeled IGF-I. Control points represent single data points. Preincubated cell data points are the average of two measurements with error bars as shown.*

Table 5.2 SV40-IGF-I cells pre-incubated with IGF-I

	Receptors (# cell)	K_d (M)
Control wells	117,000	1.08×10^{-9}
Preincubated wells (50ng/ml IGF-I)	99,000 ± 11,000	$1.13 (\pm .32) \times 10^{-9}$

These results show a possible reduction in cell surface receptors with preincubation with IGF-I, however the sample size is too small to draw conclusions. K_d values are essentially the same. If the reduction in cell surface receptors proved to be significant with repeat experiments, this would suggest down-regulation or desensitization of the cell surface receptors as a result of the 1 hr pre-incubation with IGF-I. This investigation was done after noting decreased response to IGF-I with each subsequent challenge of the same cells on the microphysiometer.