1991

Characterization of the mitogen regulated protein receptor

Joseph T. Nelson
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Biochemistry Commons, and the Cell Biology Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Characterization of the mitogen regulated protein receptor

Nelson, Joseph T., Ph.D.
Iowa State University, 1991
Characterization of the mitogen regulated protein receptor

by

Joseph T. Nelson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
For the Major Department

Signature was redacted for privacy.
For the Graduate College

Iowa State University
Ames, Iowa

1991
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Explanation of Thesis/Dissertation Format</td>
<td>5</td>
</tr>
<tr>
<td>SECTION I. MITOGEN REGULATED PROTEIN (MRP; PROLIFERIN)</td>
<td></td>
</tr>
<tr>
<td>BLOOD CLEARANCE AND TISSUE UPTAKE DURING MURINE GESTATION</td>
<td>7</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>9</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>10</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>12</td>
</tr>
<tr>
<td>Materials</td>
<td>12</td>
</tr>
<tr>
<td>Methods</td>
<td>12</td>
</tr>
<tr>
<td>Radioiodination</td>
<td>12</td>
</tr>
<tr>
<td>SDS polyacrylamide gel electrophoresis (SDS-PAGE)</td>
<td>13</td>
</tr>
<tr>
<td>Trichloroacetic acid (TCA) precipitation</td>
<td>14</td>
</tr>
<tr>
<td>Radioimmunoassay (RIA)</td>
<td>15</td>
</tr>
<tr>
<td>Western blot</td>
<td>15</td>
</tr>
<tr>
<td>Mannose-6-phosphate receptor column chromatography</td>
<td>16</td>
</tr>
<tr>
<td>Placental extract preparation</td>
<td>17</td>
</tr>
<tr>
<td>In vivo $^{125}$I-MRP clearance</td>
<td>17</td>
</tr>
<tr>
<td>RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>Plasma and Placental MRP Concentrations During Gestation</td>
<td>19</td>
</tr>
<tr>
<td>Mannose-6-Phosphate Receptor Binding</td>
<td>19</td>
</tr>
<tr>
<td>Comparison of MRP and BSA Clearance In Vivo</td>
<td>22</td>
</tr>
<tr>
<td>MRP Clearance at Mid-gestation</td>
<td>25</td>
</tr>
<tr>
<td>MRP Stability in Blood</td>
<td>28</td>
</tr>
<tr>
<td>MRP and BSA Uptake by Tissues</td>
<td>29</td>
</tr>
<tr>
<td>MRP Degradation in Tissues at 11 Days of Gestation</td>
<td>32</td>
</tr>
</tbody>
</table>
### Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DISCUSSION</td>
<td>37</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>42</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>43</td>
</tr>
<tr>
<td>SECTION II. CHARACTERIZATION OF THE MITOGEN REGULATED PROTEIN (MRP; PROLIFERIN) RECEPTOR FROM UTERINE MEMBRANES</td>
<td>45</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>47</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>48</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>50</td>
</tr>
<tr>
<td>Materials</td>
<td>50</td>
</tr>
<tr>
<td>Methods</td>
<td>50</td>
</tr>
<tr>
<td>Membrane preparation</td>
<td>50</td>
</tr>
<tr>
<td>Radioiodination</td>
<td>51</td>
</tr>
<tr>
<td>TCA precipitation</td>
<td>51</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>52</td>
</tr>
<tr>
<td>Receptor binding assay</td>
<td>52</td>
</tr>
<tr>
<td>Chemical crosslinking</td>
<td>53</td>
</tr>
<tr>
<td>Protein Determination</td>
<td>53</td>
</tr>
<tr>
<td>RESULTS</td>
<td>54</td>
</tr>
<tr>
<td>Receptor Binding at Mid-gestation</td>
<td>54</td>
</tr>
<tr>
<td>Receptor Characterization</td>
<td>54</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>66</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>71</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>75</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>80</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>84</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

### SECTION I

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>Mannose-6-phosphate content of MRP</td>
<td>22</td>
</tr>
<tr>
<td>Table 2.</td>
<td>$^{125}$I-MRP clearance at different stages of gestation</td>
<td>28</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Comparison of tissue associated radiolabel four hours after injection of $^{125}$I-protein into nonpregnant mice</td>
<td>30</td>
</tr>
<tr>
<td>Table 4.</td>
<td>Chloroquine treatment of pregnant mice</td>
<td>36</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

SECTION I

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gestational profile of MRP concentrations in the maternal circulation</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Clearance profiles of BSA and MRP in nonpregnant mice</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Clearance profiles of MRP at 11 days of gestation showing fast and slow clearance</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>Tissue distribution of MRP</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>Tissue/blood ratios of injected $^{125}$I-MRP</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>Percentage of TCA precipitable label associated with each tissue</td>
<td>34</td>
</tr>
</tbody>
</table>

SECTION II

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Receptor binding of MRP</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>Concentration dependence of MRP binding</td>
<td>57</td>
</tr>
<tr>
<td>3</td>
<td>Receptor binding activity as a function of membrane concentration</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>Receptor association with time</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>MRP dissociation from the receptor with time</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>Receptor binding as a function of pH</td>
<td>62</td>
</tr>
<tr>
<td>7</td>
<td>Receptor binding in the presence of prolactin</td>
<td>63</td>
</tr>
<tr>
<td>8</td>
<td>Chemical crosslinking of the receptor-MRP complex</td>
<td>64</td>
</tr>
</tbody>
</table>
INTRODUCTION

Normal fetal development requires the coordination of specific events at precise times during gestation. The coordination of these events occurs as a result of communication between mother and fetus via hormones and other paracrine factors. Hormones are intercellular communicators that are synthesized in a specific tissue, secreted into the bloodstream, and received by a different tissue. The receipt of the hormone signal by a specific receptor initiates a process that eventually results in some metabolic change in the receiving tissue.

Prolactin is a pituitary hormone secreted during gestation with several functions. The major role of prolactin in gestation appears to be as a lactogenic stimulus in mammary gland tissues (1). There are several prolactin-like hormones synthesized in the placenta of the developing fetus and secreted into the maternal blood. These include the placental lactogens (2-8), the prolactin-like proteins A and B (9-13), and mitogen regulated protein (MRP) (14) also called proliferin (PLF) (15,16). All of these secreted glycoproteins are similar in their core polypeptide molecular weight and their homology to prolactin. Furthermore, the placental lactogens have lactogenic activity (17) and have been implicated in mammary gland preparation for lactation and fetal growth (18). One current hypothesis explains that placental hormone expression marks a shift in the control of pregnancy from the pituitary to the placenta and, in the case of placental lactogens, allows more precise developmental regulation of prolactin-like hormones (18-21). The
placental lactogens have been characterized in much greater detail than either prolactin-like proteins A and B or MRP with respect to possible functions in fetal development and the maternal response to fetal development. The Nilsen-Hamilton research group is currently studying MRP in an effort to fully characterize this secreted glycoprotein and define its function during murine gestation.

MRP represents a family of glycoproteins of 34 kDa average molecular weight originally discovered as a secretion product of growth factor stimulated mouse embryo 3T3 cells (22). MRP is identical to PLF (23), described by Linzer and Nathans in Balb/c 3T3 cells and expressed in the mouse placenta (15). MRP has 32 percent homology to mouse prolactin according to sequence analysis of the cDNA (24) and has a polypeptide molecular weight of about 22 kDa. MRP exhibits a distinct temporal profile of synthesis and secretion during murine gestation with peak levels occurring in the placenta and maternal serum at mid-gestation with a subsequent rapid decline at late gestation (16,25,26). The appearance in the blood at mid-gestation, its synthesis in the placenta, and its homology to prolactin indicates that MRP is, like the placental lactogens, a hormone involved in fetal development.

If MRP is a placental hormone, it should bind to its target tissue via a receptor. Lee and Nathans (27) showed that recombinant PLF binds to the cation-independent mannose-6-phosphate receptor in murine liver and placental membrane preparations during late gestation. Approximately 80-90 percent of the cation-independent mannose-6-phosphate receptors are located intracellularly (28) and participate in translocating proteins to
the lysosomes (29,30). The other 10-20 percent are located on the cell surface. The mannose-6-phosphate residue recognized by the receptor is a high mannose type that is N-linked through asparagine to the polypeptide backbone (31,32). The phosphate is transferred to the mannose by the addition of N-acetylglucosaminyl 1-phosphate followed by hydrolysis of the N-acetylglucosamine (33). Cation-independent mannose-6-phosphate receptors are present in the Golgi (30). When cells are incubated with antibodies to the mannose-6-phosphate receptor, the efficiency of lysosomal targeting is decreased (29) and it is thought that ligand binding occurs in the trans Golgi (34). The receptor is a 300 kDa glycoprotein (34) and there is evidence that it is the same receptor as the insulin-like growth factor II (IGF-II) receptor (35-37). This is interesting with respect to the cell surface mannose-6-phosphate receptor because it indicates the receptor could mediate growth factor stimulated events. IGF-II is not glycosylated, however, and this indicates that the mannose-6-phosphate receptor must have separate binding sites for IGF-II and mannose-6-phosphorylated glycoproteins because the presence of mannose-6-phosphate is absolutely required for lysosomal targeting (38).

MRP binding to the mannose-6-phosphate receptor could be a mechanism used intracellularly to direct MRP to the lysosomes or to mediate a functional response to MRP bound to receptor at the cell surface. Recent evidence shows that MRP binding to the cation-independent mannose-6-phosphate receptor on canine kidney basolateral membranes activates phospholipase C (38). Furthermore, anti-mannose-6-phosphate receptor abolished this activation. This could reflect a signal transduction
mechanism of the bound MRP at the cell surface. However, results from this laboratory show that MRP circulating in the maternal bloodstream does not contain mannose-6-phosphate. Thus, any signal transduction events attributed to the MRP-mannose-6-phosphate receptor interaction at the cell surface cannot occur in vivo on a tissue other than the placenta.

To characterize a specific receptor for MRP, several criteria must be established (39). One very important factor in this characterization is that the receptor must be saturable to ensure a specific interaction between ligand and receptor. A receptor is further characterized by its dissociation constant, $K_D$, and maximal binding, $B_{\text{max}}$, according to analysis by a Scatchard plot (40). The $K_D$ allows an assessment of the affinity of the ligand for the receptor and $B_{\text{max}}$ allows an assessment of the size of the receptor population. Furthermore, the $K_D$ obtained by the above steady-state method should be consistent with the $K_D$ obtained from kinetic studies. The shape of the Scatchard plot will also help to determine whether there are receptor populations with different affinities for the ligand. A plot resulting in a straight line indicates a single affinity receptor. The kinetic studies involve determining association and dissociation rate constants. One defines an association rate constant, $k_1$, by conducting receptor binding over a number of consecutive time points. The measurement of $k_1$ is completed by solving the second order rate equation at each time point in the linear range of the association time course and calculating an average $k_1$. The dissociation rate constant, $k_{-1}$, is determined by following the time course of
dissociation of the receptor-ligand complex. A logarithmic transformation of the resultant data allows the calculation of $k_{-1}$ by measuring the slope of the line of this transformation. A straight line shows a first-order reaction and presumably a reaction involving a receptor with a single affinity. The value of $k_{-1}/k_1$ should be consistent with the $K_D$ obtained from a Scatchard analysis. The final criterion used to establish the existence of a receptor is its specificity. One must test the receptor preparation for binding to its presumed ligand in the presence of competing ligands to insure a specific ligand-receptor interaction.

My dissertation research hypothesis is that MRP is a new placental hormone involved in fetal development that exerts its functional effects via a specific receptor on a tissue separate from placenta. I tested this hypothesis by identifying and characterizing a tissue receptor that specifically binds MRP.

Explanation of Thesis/Dissertation Format

I am using the alternate format for this dissertation as outlined in the Iowa State University Graduate College Thesis Manual. The introduction provides a background into the research and describes the research motivation. The first section entitled, "Mitogen regulated protein (MRP; Proliferin) blood clearance and tissue uptake during murine gestation" describes the different blood clearance profiles of tracer radioiodinated MRP at multiple stages of murine gestation and provides evidence of uterine and mammary gland tissue uptake and utilization at mid-gestation.
I conducted all the experimental studies involving in vivo clearance of radioiodinated MRP, tissue uptake and utilization of radioiodinated MRP, tissue utilization of radioiodinated MRP after chloroquine treatment of pregnant mice, radioiodination of MRP, and mannose-6-phosphate receptor affinity column chromatography of radioiodinated MRP. Yu Fang conducted the experimental studies concerning the quantitation of MRP in maternal plasma and Adnan Mubaidin contributed the experimental data concerning the mannose-6-phosphate content of placental and plasma MRP. I also performed the literature review and prepared the manuscript. I performed the research outlined in the first section to determine the most appropriate period of gestation to investigate the existence of a proposed MRP receptor and to identify a tissue most likely to contain the MRP receptor. In the second section entitled, "Characterization of the mitogen-regulated protein (MRP; Proliferin) receptor from uterine membranes," I conducted all of the experimental studies, performed the literature review, and prepared the manuscript. The summary describes my dissertation conclusions and describes ideas for future research. The references following the summary are a list of the references used in the introduction and summary. The references following sections one and two are listed in accordance with the format of the journal in which the papers will be submitted. The references following the summary are listed in accordance with the format of the approved style manual for the Department of Biochemistry and Biophysics as recommended in the *Iowa State University Graduate College Thesis Manual*. 
SECTION I. MITOGEN REGULATED PROTEIN (MRP; PROLIFERIN) BLOOD CLEARANCE AND TISSUE UPTAKE DURING MURINE GESTATION
Mitogen Regulated Protein (MRP; Proliferin) Blood Clearance and Tissue Uptake During Murine Gestation

Joseph T. Nelson¹, Yu Fang¹, Adnan Mubaidin, Christopher Gabel², Richard Einstein², and Marit Nilsen-Hamilton¹

¹Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

and

²Department of Anatomy and Cell Biology, College of Physicians and Surgeons of Columbia University, 630 W. 168th Street, New York, NY 10032
ABSTRACT

Mitogen regulated protein (MRP; also called proliferin) is a 34 kDa glycoprotein secreted during murine gestation. Its temporal profile of synthesis and secretion and its homology to prolactin indicate an hormonal role in fetal development. To identify its target tissues in vivo, we injected tracer amounts of $^{125}$I-labeled MRP into nonpregnant mice and mice at various days of gestation. At mid-gestation the clearance profile was biphasic and provided evidence for two half-lives for MRP of 0.6 hour and about 8 hours. Both half-lives of MRP were significantly shorter than the half-life of 10 hours measured for serum albumin. Our data show that mammary gland and uterus take up and degrade MRP whereas MRP is taken up but not degraded by liver, heart, spleen, and kidney. MRP degradation in the mammary gland and uterus is reduced by prior treatment with chloroquine which inhibits lysosomal-mediated degradation. We propose that MRP is cleared from the blood by receptor-mediated endocytosis with subsequent degradation by mammary gland and uterus. Because degradation by their target cells is a fate of many hormones and growth factors, we suggest that mammary gland and uterus are also target tissues for MRP action.
INTRODUCTION

Mitogen Regulated Protein (MRP) is a 34 kDa glycoprotein first identified as a protein secreted by growth factor-stimulated mouse embryo 3T3 cells (1). MRP is identical to proliferin (PLF), a prolactin-related protein found in murine placenta during gestation (2). The temporal profile of MRP synthesis in trophoblastic giant cells, its appearance in maternal blood during a well-defined gestational period, and its similarity to prolactin indicate that MRP might be a placental hormone involved in fetal development (3-6). MRP produced from a recombinant plasmid by transformed Chinese Hamster Ovary (CHO) cells binds to the cation-independent mannose-6-phosphate receptor in maternal and fetal liver and in placental membranes (7). Although this interaction with the mannose-6-phosphate receptor can initiate signal transduction (8), our results suggest that this is not the means by which MRP acts in vivo because we show that MRP in the maternal blood does not possess mannose-6-phosphate.

Here, we report the in vivo clearance of radiolabelled MRP at different stages of gestation and compare this with the clearance of serum albumin. Our results show that tracer MRP is cleared from the blood more rapidly than serum albumin in nonpregnant mice. The mammary gland and uterus also specifically degrade MRP. This degradation may be due to the presence of a specific receptor for MRP in these tissues that
is involved in its clearance. Furthermore, the specific degradation of MRP in the mammary gland and uterus may be a consequence of these tissues being targets for this newly-discovered placental hormone-like protein.
MATERIALS AND METHODS

Materials

MRP was purified in our laboratory from conditioned medium of a Swiss 3T3 cell line stimulated by fibroblast growth factor (FGF) and phorbol myristate acetate in the presence of 10 mM ammonium chloride. All chemicals used were reagent grade or more pure. Bovine serum albumin (BSA) was purchased from Sigma Chemical Company. $^{125}\text{I}$ was purchased from Amersham, enzymobeads from Bio-Rad, $^{125}\text{I}$-protein A from ICN, and pansorbin from Calbiochem. Rabbit anti-MRP was prepared in our laboratory using purified MRP from conditioned medium of Swiss 3T3 cells (9). All other chemical were purchased from Fisher Scientific or Sigma Chemical Company.

Methods

Radioiodination

MRP and BSA were radioiodinated using immobilized glucose oxidase and lactoperoxidase (Enzymobeads, BioRad Company). We used the lactoperoxidase labeling method because the requisite enzymes catalyze fewer side reactions than in other labeling methods and the protein is more likely to retain biological activity (10). Three to six micrograms per milliliter of MRP or 130 $\mu$g/ml of BSA was incubated with 50 $\mu$l of washed Enzymobeads, 6.5 mCi/ml of Na$^{125}$I, and 323 $\mu$g/ml of $\alpha$-D-glucose in 65 mM sodium phosphate buffer, pH 7.2 for 25 minutes at 22°C in a total volume of 155 $\mu$l. Fifty millimolar sodium phosphate buffer, pH 7.4 was added to the reaction mixture to a final volume of one milliliter. The mixture
was microfuged for five minutes at 4°C. The supernatant was loaded onto a 10 DG column (BioRad) previously washed with one percent BSA in 50 mM sodium phosphate buffer, pH 7.4 and eluted with 50 mM sodium phosphate buffer, pH 7.4. One milliliter fractions to which 100 μl of one percent BSA was previously added were collected. Fractions 4, 5, and 6 were pooled, loaded onto a second 10 DG column, and 1 ml fractions collected. Each fraction was analyzed by trichloroacetic acid (TCA) precipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing greater than 75 percent TCA precipitable protein were used for experiments. The amount of TCA precipitable protein declined in radioiodinated MRP preparations with storage at 4°C. Therefore, we often passed stored radioiodinated MRP through a desalting column prior to use for in vivo clearance studies. The reason for the rapid decline in TCA precipitable cpm is believed to be adsorption to the walls of the vessel. We found the decline could be prevented by including one percent BSA or 140 mM NaCl in the MRP stock solution. Radioiodinated BSA was quite stable with storage at 4°C.

**SDS polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed according to Nilsen-Hamilton and Hamilton (11). The resolving gel consisted of a 7.5-15 percent linear polyacrylamide gradient and the stacking gel was five percent polyacrylamide. Electrophoresis was completed at 12.5 mAmps for 4.5-5 hours at 22°C.
Trichloroacetic acid (TCA) precipitation

Two methods of acid precipitation were used. Ascending thin layer chromatography was used to separate labeled protein from free label when it was possible to use small volumes of radiolabeled protein. Silica gel-impregnated glass fiber strips (4.8 cm x 1 cm) were spotted 1 cm from the bottom with an equal volume of two percent potassium iodide and radiolabeled protein and developed in 30 percent (v/v) methanol, 10 percent (v/v) glacial acetic acid and 10 percent (v/v) trichloracetic acid, and dried at room temperature. The developed strips were cut in half to separate the acid soluble and insoluble material. Each half was counted in a Tracor Analytic Gamma Trac 1191 gamma counter. To obtain the fraction of the TCA precipitable protein, the counts remaining at the origin were divided by the total counts spotted.

The second method of precipitation, which involved filtering the sample through borosilicate microfiber filters, was used for large sample volumes. Potassium iodide was added to each radiolabeled sample to one percent (v/v) and the samples were incubated at 4°C in 10 percent TCA for 30 minutes. To determine the total cpm, each sample was counted in a gamma counter before filtering. The solution was filtered under vacuum, the filter was washed four times with 10 percent TCA and counted in a gamma counter. To obtain the fraction of TCA precipitable cpm, the cpm remaining on the filter were divided by the total cpm in the radiolabelled sample.
Radioimmunoassay (RIA)

To each duplicate 50 μl sample or standard containing MRP, anti-MRP was added to a final dilution of 1/5000 and incubated in 10 mM sodium phosphate, 150 mM sodium chloride, 10 mM EDTA, and one percent BSA at pH 7.5 for 6 hours at 4°C in a total volume of 230 μl. Radiolabelled MRP was added to 5-7 pg/ml (60,000-80,000 cpm/ml) to a total volume of 250 μl and incubated for 12 hours at 4°C. Pansorbin was added to one percent (v/v) and allowed to stand for one hour at 22°C. The mixture was spun in a microfuge at 4°C for 10 min, the supernatants aspirated, and the pellets counted in a gamma counter. Nonspecific binding was calculated to be the amount of label in the pellet in the presence of 1,000 ng/ml of unlabeled MRP and was subtracted from all samples to obtain specific binding. The linear range of the assay was 0.2-20 ng MRP.

Western blot

After resolution by SDS-PAGE, the gel containing the MRP was soaked in 25 mM Tris, 192 mM glycine, 20 percent methanol, pH 8.0 for 30 minutes at 22°C. The proteins were transferred to a nitrocellulose membrane with a Hoefer TE 50 apparatus at 90 V for four hours at 22°C. The membrane was stained with Ponceau S to verify that proteins were transferred. After destaining, the membrane was incubated for 12 hours at 4°C in a solution containing five percent (w/v) nonfat milk, 140 mM sodium chloride, 5 mM potassium chloride, 0.4 mM dibasic sodium phosphate, 25 mM Tris, pH 7.4 containing 0.02 percent (w/v) sodium azide. The solution was poured off and a fresh five percent nonfat milk solution containing
anti-MRP to a final dilution of 1/200 was added and the membrane was incubated for 90 min at 22°C. This solution was poured off and the membrane was washed for 10 minutes at 22°C with buffer I (140 mM sodium chloride, 5 mM potassium chloride, 0.4 mM sodium phosphate, 25 mM Tris, pH 7.4). This was followed by a second wash for 10 min in buffer I containing 0.05 percent (v/v) NP-40 and a final 10 min wash in buffer I. A solution of $3.6 \times 10^5$ cpm/ml of $^{125}$I-protein A in five percent nonfat milk in buffer I was added to the membrane for 30 minutes at 22°C. The $^{125}$I-protein A solution was then poured off and the membrane was washed three times with buffer I as just described. The membrane was dried and the radioactivity detected by autoradiography.

**Mannose-6-phosphate receptor column chromatography**

Purified MRP and MRP in samples of plasma and placental extract were analyzed for their content of mannose-6-phosphate by their ability to bind to the mannose-6-phosphate receptor. The samples were applied to a mannose-6-phosphate receptor affinity column as described (12). The column was washed with 15 ml of column buffer (50 mM sodium phosphate, 150 mM sodium chloride, 5 mM β-glycerophosphate, 2 mM EDTA, and 1 mM PMSF) and 1 ml fractions were collected. Fifteen milliters of column buffer containing 5 mM mannose-6-phosphate was then added to elute the mannose-6-phosphate-containing compounds and 1 ml fractions collected. For studies in which unlabeled MRP was passed through the column for later analysis by radioimmunoassay, a standard mixture of $^{131}$I-ligands containing mannose-6-phosphate were mixed with the samples prior to
resolution through the column. The mannose-6-phosphate-containing ligands were obtained from secretions of J774 macrophage cells (12). When radioiodinated MRP was analyzed for mannose-6-phosphate content, a mixture of $^{125}$I-ligands were resolved separately through the column. The amount of MRP in each fraction from the column was determined by radioimmunoassay for unlabeled samples of MRP or by TCA precipitation followed by scintillation spectroscopy for $^{125}$I-labeled MRP. The percentage of the MRP containing mannose-6-phosphate was determined by dividing the total MRP in the bound fractions by the total MRP that passed through the column.

**Placental extract preparation**

Placentae were dissected from CF-1 outbred mice, weighed, and immediately frozen by immersion in liquid nitrogen. The frozen tissue was stored at -70°C until ready for use. The frozen tissue was pulverized with mortar and pestle and homogenized at 9 ml/g tissue in homogenizing buffer (10 mM sodium phosphate, 150 mM sodium chloride, 0.2 mM magnesium sulfate, 1 mM EDTA, pH 7.5) using a Tekmar Tissuemizer at 4°C. The homogenate was spun at 13,000 g for 10 min at 4°C, the supernatant was aspirated and adjusted to 10 mM EDTA, and stored at -70°C until needed.

**In vivo $^{125}$I-MRP clearance**

Outbred CF-1 female mice were anaesthetized with ether and 100-360 ng/ml radiolabelled MRP (10-20 μCi/μg) or 6.7 μg/ml BSA (2.3 μCi/μg) was injected directly into the heart. Blood and tissue samples were
collected at several time intervals after injection. For the zero-time point, blood was collected at 2-4 minutes after injection. Blood was collected from the orbital sinus. Immediately after collection, each sample was put on ice and counted for gamma emission. Tissue samples were weighed and homogenized using the Tekmar Tissuemizer at high setting for 20-40 seconds. All samples were analyzed for TCA precipitable protein. Clearance rates were assumed to be first order and calculated from the slopes of semi-logarithmic plots of the log % dose vs. time using the method of least squares where log % dose = log [(sample TCA precipitable cpm/TCA precipitable cpm injected/ml blood)×100]. Half-lives were calculated using the equation, $t_{1/2} = \ln 1/(2.303 \times \text{clearance rate})$.

For mice treated with chloroquine diphosphate, mice were injected intraperitoneally with chloroquine in sterile water at a dose of 60 μg/gm mouse weight. Injections were given daily from day 6 through 11 days of gestation. At 11 days of gestation, the mice were injected with $^{125}\text{I}$-MRP and sacrificed after two hours for analysis of the distribution of MRP in the tissues. Mice receiving daily injections of sterile water were used as controls.
RESULTS

Plasma and Placental MRP Concentrations
During Gestation

To understand the relative contribution of the placenta and maternal tissues to the level of MRP in the blood, we compared the plasma profile of MRP with the profile of MRP mRNA in the placenta. The plasma concentrations of MRP peaks on day 10 of gestation and falls at a rate of 20 percent per day through day 15 (Figure 1a). By day 17, only three percent of the peak levels of MRP remain in the blood. This temporal profile of MRP blood concentration is qualitatively in agreement with Lee et al. (3) as measured by radioimmunoassay, although they did not estimate the rate of decline of MRP after its peak. The decline in MRP levels is first order and can be represented by a half-life of 33 hours (Figure 1b). As well, using data published elsewhere (4), we have estimated that the rate of decline of MRP mRNA in the placenta as a function of gestation can also be represented by a first order decay curve with a calculated half-life of 48 hours.

Mannose-6-Phosphate Receptor Binding

Lee and Nathans (7) found that recombinant MRP expressed in CHO cells bound to the cation-independent mannose-6-phosphate receptor. We tested MRP obtained from maternal plasma and placental extracts, as well as radioiodinated MRP, for binding to the mannose-6-phosphate receptor (Table 1). Plasma samples from day 9 through day 17 of gestation did not bind but most of the placental MRP from day 13 pregnant mice bound the
Figure 1. Gestational profile of MRP concentrations in the maternal circulation. a) The relative amount of MRP in 2 µl plasma samples were measured by a densitometric scan of a Western blot. Each point represents the average and standard deviation of blood plasma obtained from two mice (days 9-16) or one mouse (day 17, 18). b) semi-logarithmic plot of decline of (*) MRP in plasma and (Δ) mRNA starting at day 10 of gestation. The clearance rate was determined from the slope of the plot and half-life calculated using equation, $t_{1/2} = \frac{0.693}{2.3 \times \text{slope}}$. Slope/correlation coefficient: -0.009/-0.974 and $t_{1/2} = 34$ hours (MRP) and -0.006/-0.981 and $t_{1/2} = 48$ hours (m-RNA).
Table 1. Mannose-6-phosphate content of MRP

<table>
<thead>
<tr>
<th>Source of MRP</th>
<th>% bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma - gestation day 9-17^a</td>
<td>0</td>
</tr>
<tr>
<td>Placenta - gestation day 13</td>
<td>88</td>
</tr>
<tr>
<td>Swiss 3T3 cells</td>
<td>38</td>
</tr>
<tr>
<td>Radioiodinated from Swiss 3T3 cells</td>
<td>43</td>
</tr>
<tr>
<td>Radioiodinated and mouse plasma at 37°C for 10 min</td>
<td>12</td>
</tr>
</tbody>
</table>

**NOTE.** Samples to be loaded on the mannose-6-phosphate receptor column were prepared as described in Materials and Methods. For unlabeled samples from plasma, placenta, and Swiss 3T3 cells, the percentage of MRP bound to the mannose-6-phosphate receptor was measured by RIA. For radioiodinated MRP, the samples were loaded onto the column and the percentage of the TCA precipitable label that bound to the mannose-6-phosphate receptor was calculated based on the total TCA precipitable label recovered in the flow through and mannose-6-phosphate eluted fractions.

^Plasma samples from days 9, 11, 14, 15, 16, and 17 were separately analyzed.

Incubating radioiodinated MRP for 10 minutes in mouse plasma reduced binding to the mannose-6-phosphate receptor from 43 percent to 12 percent bound.

**Comparison of MRP and BSA Clearance In Vivo**

The clearance profiles of MRP and BSA were measured after injecting tracer amounts of each radioiodinated protein into nonpregnant mice (Figure 2a). Homogeneous radioiodinated protein preparations were
Figure 2. Clearance profiles of BSA and MRP in nonpregnant mice. a) Nonpregnant mice were injected with a) $^{125}$I-BSA (•) or $^{125}$I-MRP (△) directly into the heart and blood was collected at times after injection. % dose = (TCA precipitable cpm in sample/TCA precipitable cpm injected/ml serum) × 100. Clearance rates and correlation coefficients were calculated as described in Materials and Methods. The calculated clearance rates/correlation coefficients were: MRP: -0.0625 hour/-0.974 (between 1.5 and 16 hours); BSA: -0.0296/-0.983. The value at each point is the average of the results obtained from two mice for BSA and two to six mice for MRP. b) Radioiodinated MRP (M) and BSA (B).
used in these studies (Figure 2b). Because it is heterogeneously glycosylated, radioiodinated MRP migrates on an SDS-PAGE as a broad, diffuse band (1).

Radioiodinated serum albumin exhibited a monophasic blood clearance profile while radioiodinated MRP exhibited an initial accumulation in the blood followed by a monophasic clearance profile between 1.5 and 16 hours after injection. Clearance rates were calculated to be 0.0625 hour\(^{-1}\) and 0.0296 hour\(^{-1}\) for MRP and BSA, respectively. The calculated half lives of MRP and BSA were 4.8 and 10.2 hours, respectively. The clearance profiles of MRP at 6 and 15 days of gestation were very similar to that of nonpregnant mice.

**MRP Clearance at Mid-gestation**

Clearance of MRP on day 11 was biphasic and did not include an initial period of slow release into the blood. Figure 3a shows the initial fast clearance profile of MRP after injecting tracer radioiodinated MRP into mice at 11 days of gestation. This fast rate was observed between 0 and 90 minutes after injection. A slower clearance rate was observed between 90 minutes and 16 hours after injection (Figure 3b). This biphasic profile was only observed with mice at 11 days of gestation. At other times of gestation the initial 90 min period was marked by a slow rise in the plasma \(^{125}\)I-MRP level. A comparison of the calculated clearance rates, correlation coefficients, and half-lives of MRP at different stages of gestation is given in Table 2.
Clearance profiles of MRP at 11 days of gestation showing fast and slow clearance. Mice at 11 days of gestation were injected with $^{125}$I-MRP directly into the heart and blood was collected at times after injection. % dose = (TCA precipitable cpm in sample/TCA precipitable cpm injected/ml blood)×100. The results for the first 2 hours (a) are plotted separately from the results for the 0-16 hour period (b). Clearance rates and correlation coefficients were calculated as described in Materials and Methods. The calculated clearance rates/correlation coefficients were: initial fast rate (0-90 minutes) = -0.467 hour$^{-1}$/0.979; slow rate (1.5-16 hour) = -0.0371/-0.948. Values at each point are the average of blood samples from eight mice at t=0-60 minutes and at least two mice at t=1.5-16 hours. △ nontransformed, ○ logarithmically transformed.
Table 2. $^{125}$I-MRP clearance at different stages of gestation

<table>
<thead>
<tr>
<th>Day of gestation</th>
<th>Clearance Rate</th>
<th>Correlation Coefficient</th>
<th>Half-life (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonpregnant</td>
<td>-0.0625</td>
<td>-0.974</td>
<td>4.8</td>
</tr>
<tr>
<td>6 day</td>
<td>-0.0637</td>
<td>-0.999</td>
<td>4.7</td>
</tr>
<tr>
<td>11 day fast</td>
<td>-0.467</td>
<td>-0.979</td>
<td>0.6</td>
</tr>
<tr>
<td>slow</td>
<td>-0.0371</td>
<td>-0.948</td>
<td>8.1</td>
</tr>
<tr>
<td>15 day</td>
<td>-0.0709</td>
<td>-0.987</td>
<td>4.2</td>
</tr>
</tbody>
</table>

**NOTE.** Radioiodinated MRP was injected into the heart of nonpregnant, 6, 11, and 15 day pregnant mice. Serum samples (50-100 µl) were collected at various times after injection and the percentage of TCA precipitable protein was determined. The % dose was calculated at each sample time by the equation: $% \text{dose} = \frac{(\text{TCA precipitable cpm in sample/} \text{TCA precipitable cpm injected/ml blood}) \times 100}{}$. The clearance rate and half-lives were calculated as described in Materials and Methods. For all but day 11 of pregnancy, there was a slow increase in $^{125}$I-MRP blood levels over the first two hours after injection of $^{125}$I-MRP. MRP clearance rates were calculated using time points following the initial increase in blood MRP content.

**MRP Stability in Blood**

To determine if the rapid clearance of MRP at day 11 of gestation is due to MRP degradation in the blood and subsequent clearance of a radiolabelled breakdown product, we incubated $^{125}$I-MRP with serum obtained from nonpregnant and 11-day pregnant mice at 37°C. The samples were then resolved by SDS-PAGE. There were no detectable breakdown
products in serum from nonpregnant or 11-day pregnant mice over a two-hour incubation period as analyzed by autoradiography after SDS-PAGE. In addition, there was no change in the intensity of $^{125}$I-MRP after incubation with serum at 37°C for up to two hours when compared with the intensity of $^{125}$I-MRP at 0 time as measured by densitometric scanning of the autoradiogram. There was no change in the percentage of TCA precipitable protein over the same two hour incubation period.

**MRP and BSA Uptake by Tissues**

We surveyed tissues for uptake of radiolabel after injection of $^{125}$I labeled serum albumin and MRP. Table 3 shows the tissue accumulation, tissue/blood ratio, and the percentage of TCA precipitable protein in each tissue four hours after injection. Similar results were found from two to 16 hours after injection. With the exception of mammary gland and uterus, there was no significant difference in radiolabel associated with the tissues surveyed. For all tissues, the tissue/blood ratio for MRP was higher than for BSA. There was also a much lower percentage of TCA precipitable radiolabel in mammary gland and uterus for the MRP-injected mice than for MRP in other tissues and for BSA in these tissues.

We examined the tissue uptake of radioiodinated MRP at 11 days of gestation (Figure 4). Maximal MRP content is achieved within one to two hours of its injection for all tissues. There is a general trend toward decreasing amounts in all tissues after reaching its peak with time after injection with the exception of the spleen. There was significantly (p<.01) higher tissue associated radiolabel in the liver and uterus one
Table 3. Comparison of tissue associated radiolabel four hours after injection of $^{125}$I-protein into nonpregnant mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% of total dose injected/g tissue</th>
<th>tissue/blood</th>
<th>% TCA precipitable protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BSA (n=2)</td>
<td>MRP (n=4)</td>
</tr>
<tr>
<td>liver</td>
<td>2.6±0.8</td>
<td>2.6±0.4</td>
<td>0.27</td>
</tr>
<tr>
<td>heart</td>
<td>5.1±2.6</td>
<td>3.5±1.2</td>
<td>0.53</td>
</tr>
<tr>
<td>spleen</td>
<td>1.1±0.1</td>
<td>1.5±0.6</td>
<td>0.12</td>
</tr>
<tr>
<td>kidney</td>
<td>4.7±1.5</td>
<td>3.3±2.7</td>
<td>0.49</td>
</tr>
<tr>
<td>mammary gland</td>
<td>2.0±1.5</td>
<td>0.8±0.3</td>
<td>0.21</td>
</tr>
<tr>
<td>uterus</td>
<td>2.9±1.5</td>
<td>1.4±0.4</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**NOTE.** Radioiodinated protein was injected into the heart of nonpregnant mice and the mice were sacrificed four hours later. Tissues were dissected, counted for gamma emission, and TCA precipitation performed as described in Materials and Methods. The tissue/blood ratio is the percent of the total dose of the tissue divided by the percent of the total dose in the blood at four hours. The percentage of TCA precipitable protein is the TCA precipitable cpm associated with the tissue divided by the total cpm associated with the tissue.
Figure 4. Tissue distribution of MRP. Total tissue-associated $^{125}$I-MRP in 11 day mice for kidney (K), spleen (S), liver (L), mammary gland (MG), and uterus (U) at 30 minutes and 1, 2 and 4 hours after injection. % dose = (tissue associated cpm/total cpm injected/g tissue) x 100. Values are the average and standard deviation of three to eight mice at each time. Bars: open, 30 minutes; close hatches, 1 hour; wide hatches, 2 hours; stippled, 4 hours.
hour after injection in 11 day pregnant mice when compared with nonpregnant mice.

The tissue-blood ratios of $^{125}$I-MRP were surveyed in nonpregnant mice and mice at 11 days of gestation (Figure 5). The ratios were consistently higher in 11 day pregnant mice than the same ratios in nonpregnant mice. Another major difference between nonpregnant and 11 day pregnant mice was the rate of increase in tissue/blood ratios of MRP in 11 day pregnant mice with time after injection. The increase in the tissue/blood ratio between 30 and 60 minutes after injection was 19-fold in uterus, 10-fold in mammary gland, and 9-fold in liver of 11 day pregnant mice. For nonpregnant mice, the increase in tissue/blood ratio over the same time period was 1.6-, 1.4-, and 1.9-fold in uterus, mammary gland, and liver, respectively. The highest tissue/blood ratios at 11 days of gestation occurred at two hours after injection of $^{125}$I-MRP for all tissues except heart.

MRP Degradation in Tissues at 11 Days of Gestation

We examined the percentage of TCA precipitable radiolabel in tissues at 11 days of gestation (Figure 6). With time after injection, the percent of TCA precipitable $^{125}$I-cpm decreases in most tissues with the largest decline occurring in uterus and mammary gland at one and two hours after injection. Mammary gland and uterus only have nine percent and 15 percent of the total label associated with the tissue that is TCA precipitable after two hours.
Figure 5. Tissue/blood of injected $^{125}$I. The tissue/blood ratio was calculated as the % dose of the total tissue associated cpm divided by the % dose of the total blood cpm. Tissue/blood ratios are shown for samples taken at 30 minutes, 1 and 2 hours after injection of $^{125}$I-MRP in nonpregnant mice and mice at 11 days of gestation (n = 2-4 mice at each time). Key: L, liver; MG, mammary gland; U, uterus.
Figure 6. Percentage of TCA precipitable label associated with each tissue. The percentage of TCA precipitable label was determined at 30 minutes, 1 and 2 hours after injection of $^{125}$I-MRP. The percentage of TCA precipitable label is the TCA precipitable cpm associated with the tissue divided by the total cpm associated with the tissue*100. Values are the average and standard deviation of 3-5 mice at each time and normalized to the TCA precipitable label injected. Key: K, kidney; S, spleen; L, liver; MG, mammary gland; U, uterus.
To determine whether the greater decrease in TCA-insoluble MRP in the mammary gland and uterus was due to lysosomal degradation we treated pregnant mice with chloroquine for six days prior to $^{125}$I-MRP injection at 11 days of gestation. Chloroquine inhibits lysosomal proteolysis by accumulating in the lysosomes and increasing the intralysosomal pH. In chloroquine-treated mice there was an increase in the percentage of TCA precipitable label in mammary gland and uterus when compared with control mice two hours after injection (Table 4). The six-fold increase in mammary gland and three-fold increase in uterine content of acid-insoluble MRP compares with minor increases in other tissues. All tissues show less tissue-associated label, but this decrease is prominent in only mammary gland and liver which show a 4.4-fold and 2.3-fold decrease, respectively.
Table 4. Chloroquine treatment of pregnant mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% TCA precipitable</th>
<th>cpm/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=3)</td>
<td>Chloroquine (n=3)</td>
</tr>
<tr>
<td>liver</td>
<td>66±6</td>
<td>60±8</td>
</tr>
<tr>
<td>heart</td>
<td>56±6</td>
<td>66±13</td>
</tr>
<tr>
<td>spleen</td>
<td>46±6</td>
<td>49±13</td>
</tr>
<tr>
<td>kidney</td>
<td>37±3</td>
<td>44±2</td>
</tr>
<tr>
<td>mammary gland</td>
<td>8±0</td>
<td>46±2</td>
</tr>
<tr>
<td>uterus</td>
<td>17±1</td>
<td>49±1</td>
</tr>
</tbody>
</table>

NOTE. Mice at 6 days of gestation were treated daily until 11 days of gestation with 60 ug/g weight of chloroquine diphosphate or sterile water. At 11 days 125I-MRP was injected into the heart and the mice were sacrificed after two hours. Tissues were dissected, weighed, counted, and TCA precipitation performed. The % TCA precipitable is the TCA precipitable cpm associated with each tissue divided by the total cpm associated with the tissue*100. The % TCA precipitable is normalized to the TCA precipitable cpm injected. The cpm/mg tissue is the total cpm associated with each tissue normalized to the wet weight of the tissue in mg.
DISCUSSION

Synthesis of MRP occurs only in the placenta (2,3,6). The circulating level of MRP in maternal blood peaks at day 10 of gestation and declines to 30 percent of peak levels at day 13. Comparison of the rates of decline of MRP in the blood and of MRP mRNA in the placenta shows that the plasma protein declines more rapidly than the placental mRNA. This suggests that an event such as clearance, beyond those that regulate MRP mRNA levels, determines the rate of decline of MRP in the plasma. Clearance from the blood might involve rapid uptake by target tissues via a specific receptor or rapid removal for the purpose of clearing excess MRP from the bloodstream.

The rate of clearance of MRP at all stages of gestation and in nonpregnant mice was much higher than the rate of clearance of serum albumin. Serum albumin has been shown to bind several tissues and this binding has been associated with distinct membrane proteins of 73, 56, 31 and 18 kDa (13). Once bound, the albumin appears to be taken up by an endocytic mechanism (14). Maleylated albumin binds with high affinity to 85, 35 and 15 kDa hepatic membrane proteins which have been partially purified under reducing conditions (15). From these results it seems that MRP is cleared by a different receptor than that which recognizes BSA.

Recombinant glycosylated MRP possesses mannose-6-phosphate and binds to the mannose-6-phosphate receptor (7). Here, we have shown that MRP from placental extracts but not MRP in the blood contains mannose-6-
phosphate. Also, short incubation of phosphorylated MRP with blood plasma results in the removal of the phosphate. Therefore, it seems the mannose-6-phosphate initially on MRP when it is in the placenta is soon removed after MRP is secreted. The function of the mannose-6-phosphate is probably to guide MRP during intracellular trafficking within the placental giant cells. Also, MRP binds specifically to 11-day uterine membrane preparations in the presence of excess mannose-6-phosphate (Nelson and Nilsen-Hamilton, manuscript in preparation). Thus, it appears that MRP uptake from the blood does not involve the mannose-6-phosphate receptor.

At day 11 of gestation we observed a biphasic clearance profile of MRP with half-lives of 36 minutes and 8.1 hours. These biphasic clearance profiles are similar to the clearance profiles of placental lactogen in sheep (16) and rats (17,18) and of prolactin in rats (19), but MRP was cleared considerably slower than these other hormones. The 36-minute short half-life of MRP is longer than that found for other members of this hormone family. For example, the half-lives of placental lactogen in rats is 19.5 or 1.2 minutes, depending on the time in gestation (18), or 5.4 minutes (17). The half-life of prolactin is five minutes in euthyroid (19) and lactating rats (20,21). If MRP is cleared by the same receptor as these other proteins, then the receptor must have a lower affinity for MRP than for placental lactogen or prolactin. Alternately, MRP may be taken up by different receptors from the currently recognized lactogenic hormone receptors. We have evidence for a unique population of receptors for MRP that do not recognize other lactogenic hormones.
The population of these MRP receptors may be much smaller than the population of lactogenic receptors.

The temporal profile of \(^{125}\)I-MRP clearance was different at day 11 than in nonpregnant mice and mice at the other times of gestation. At all other stages tested (nonpregnant, days 6 and 15) tracer MRP was slowly released into the bloodstream for the first several hours after injection. The most likely explanation for this result is that the heart contains saturable binding sites for MRP. The slow release is proposed not to be a significant factor at day 11 because the MRP binding sites are expected to be saturated by endogenous MRP which is present in the bloodstream at a concentration of about 7.5 \(\mu g/\)ml. Therefore, less of the injected tracer is likely to be trapped in the heart at 11 days of gestation. Because of this slow release of MRP by the heart at all other stages of development, we were only able to measure the short half-life of MRP in 11 day pregnant mice. In vitro studies with plasma from 11 day pregnant and nonpregnant mice showed MRP is not significantly degraded by plasma components and that the clearance rates we have measured can be attributed to clearance of intact MRP from the blood.

We found no consistent significant differences in the association of MRP with different tissues that would indicate a selective accumulation. But we did find that MRP tended to distribute into the tissues more than BSA. This was evident from the much higher tissue/blood ratios for MRP than BSA. The differences in tissue/blood ratios for MRP vs. BSA varied from four-fold in mammary gland to 13-fold in spleen.
The mammary gland and the uterus rapidly take up radioiodinated MRP immediately after it appears in the bloodstream. Simultaneously with this uptake is a large decrease in the percentage of radiolabel that is TCA precipitable in these tissues compared with the radiolabel in the blood. At 11 days of gestation, this large decline in the percentage of TCA precipitable label occurs in the uterus and mammary gland between 30 minutes and two hours after injection of $^{125}$I-MRP. Coincident with this decline in acid-insoluble radiolabel is a very large increase in the tissue/blood ratio of label in each tissue. This suggests that these tissues take up and degrade MRP.

To determine whether the uptake and degradation of MRP by the uterus and mammary gland might involve receptor-mediated endocytosis, we tested the effect of chloroquine, an inhibitor of lysosomal degradation on accumulation of TCA-insoluble MRP in the tissues. When mice were treated with chloroquine prior to injection of MRP there was a selective increase in the percentage of TCA precipitable label in mammary gland and uterus two hours after $^{125}$I-MRP injection when compared with control mice. This suggests that receptor-mediated endocytosis and subsequent degradation of MRP probably occurs in these tissues. The observation that chloroquine treatment had little effect on the percent of acid-precipitable MRP in the other tissues tested suggests these tissues do not actively degrade MRP by a similar lysosome-mediated mechanism. A characteristic of a number of hormones and growth factors is degradation after receptor-mediated endocytosis. Thus, these results suggest the mammary gland and the uterus are MRP target tissues.
The temporal profile of its secretion by the placenta and its homology with prolactin and growth hormone indicates MRP may be a hormone involved in fetal development. We have shown that MRP is rapidly cleared from the bloodstream and that the rate of clearance is likely to be faster than the rate of its synthesis and secretion by the placenta.

The rapid clearance of MRP observed in mid-gestation may be mediated by a specific receptor. Although all tissues analyzed took up MRP, mammary gland and uterus were different because they also rapidly degraded the protein. The early increase in accumulation of MRP-associated radiolabel in the mammary gland and uterus correlates well with the short half-life of MRP measured in the blood at mid-gestation. From the evidence we have presented, we proposed MRP is rapidly cleared from the blood via specific receptors on the mammary gland and uterus. The protein is then proposed to be endocytosed and degraded. It is expected that MRP delivers a signal to some cells in these tissues. We are currently conducting receptor binding and crosslinking studies to show the existence of a specific MRP receptor.
ACKNOWLEDGEMENT

This work was funded in part by a grant from the Iowa State University Biotechnology Council. This is Journal Paper No. J of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 2620.
REFERENCES


SECTION II. CHARACTERIZATION OF THE MITOGEN REGULATED PROTEIN (MRP; PROLIFERIN) RECEPTOR FROM UTERINE MEMBRANES
Characterization of the mitogen-regulated protein (MRP; Proliferin) receptor from uterine membranes

Joseph T. Nelson and Marit Nilsen-Hamilton

Department of Biochemistry and Biophysics
Iowa State University, Ames, Iowa 50011
Mitogen regulated protein (MRP; also called proliferin) is a proposed new placental hormone of the prolactin-growth hormone family involved in fetal development. MRP is taken up and degraded by the uterus and mammary gland at mid-gestation in the mouse and this uptake correlates with its temporal profile of synthesis and secretion. To determine if the uterus is a target tissue for MRP, we tested uterine membrane preparations at mid-gestation for MRP binding. We found peak binding activity at 11 days of gestation. Scatchard analysis of 11 day uterine membranes showed a $K_D = 3.5 \times 10^{-10}$ M and $B_{\text{max}} = 72$ fmoles/mg membrane protein. The proposed receptor on uterine membranes showed a single affinity for MRP and prolactin or mannose-6-phosphate did not compete for binding. Chemical crosslinking of the receptor-MRP complex with disuccinimidyl suberate and SDS-PAGE analysis under reducing conditions revealed a 57 kDa complex which results in a receptor $M_r = 23$ kDa. We propose that MRP is a new placental hormone which exhibits functional effects on the uterus for the purpose of coordinating fetal development and maternal preparation for birth.
Mitogen Regulated Protein (MRP) is a 34 kDa glycoprotein secreted by Swiss 3T3 cells after stimulation by growth factors (1). It is a member of the prolactin-growth hormone family of proteins and has also been called proliferin (2,3). MRP is synthesized in murine placenta and secreted into the maternal bloodstream during gestation (4-7). The temporal profile of the placental mRNA content and maternal MRP blood concentrations over gestation and its similarity in sequence to prolactin show that MRP is a developmentally regulated protein with a possible hormonal function (7). Studies of the rates of its in vivo clearance using radioiodinated MRP show MRP to be cleared from the maternal bloodstream most efficiently at mid-gestation and utilized most efficiently by mammary gland and uterus (Nelson et al., in preparation). This suggests there is a receptor that binds and takes up MRP on these tissues.

The receptors that could bind MRP include the mannose-6-phosphate and asialoglycoprotein receptors. However, MRP found in the blood is sialylated (Y. Fang, unpublished results) and does not contain mannose-6-phosphate (Nelson et al., in preparation). This suggests that these receptors do not bind MRP in vivo. To determine if there is a MRP-specific receptor, we tested uterine membrane preparations at mid-gestation for their ability to bind MRP. Our results show uterine
membranes contain a specific receptor that is developmentally regulated. This provides additional evidence that MRP is a hormone involved in fetal development.
MATERIALS AND METHODS

Materials

MRP was purified in our laboratory from conditioned medium of a Swiss 3T3 cell line stimulated by phorbol myristate acetate. Na\(^{125}\)I was purchased from Amersham. Enzymobeads and desalting columns (10-DG) were purchased from Bio-Rad. Glucose was purchased from Fisher. BSA (Cohn fraction V) and mannose-6-phosphate were purchased from Sigma. Disuccinimidyl substrate was purchased from Pierce. Prolactin was a generous gift of Dr. A. F. Parlow. All other chemicals used were reagent grade or more pure.

Methods

Membrane preparation

The procedure for membrane preparation is a modification of Shiu et al. (8). Uterine tissue was dissected from pregnant mice at 11 days of gestation and washed in 0.3 M sucrose. All subsequent steps are completed at 4°C. The tissue was minced into small pieces and ice-cold 0.3 M sucrose was added to 10 ml/g tissue. The suspension was homogenized for four minutes at a medium setting in the Tekmar Tissuemizer followed by a one-minute homogenization at the highest setting. The homogenate was centrifuged at 1500 g for 20 minutes and the pellet was discarded. The supernatant was centrifuged at 15,000 g for 20 minutes and the pellet was discarded. The resultant supernatant was centrifuged at 100,000 g for 90 minutes. The pellet was resuspended in 250 mm tris, 10 mm MgCl\(_2\), pH 7.6 and stored in aliquots at -70°C until ready for use.
Radioiodination

MRP was radioiodinated using immobilized glucose oxidase and lactoperoxidase (Enzymobeads, Bio-Rad Company). Two to three micrograms per milliliter of MRP was incubated with 50 µl of washed Enzymobeads, 6.5 mCi/ml of Na$^{125}$I, and 323 µg/ml of α-D-glucose in 65 mM phosphate buffer, pH 7.2 for 25 minutes at 22°C in a total volume of 155 µl. Fifty millimolar phosphate buffer, pH 7.4 was added to the reaction mixture to a final volume of one milliliter. The mixture was microfuged for 5 minutes at 4°C and the supernatant was loaded onto a Bio-Rad 10 DG column previously washed with one percent BSA in 50 mM phosphate buffer, pH 7.4 and eluted with 50 mM phosphate buffer, pH 7.4. One milliliter fractions containing 100 µl of one percent BSA were collected. Fractions 4, 5 and 6 were pooled, loaded onto a second desalting column, and one milliliter fractions were again collected. Analysis of each fraction by trichloroacetic acid (TCA) precipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then completed. Fractions containing greater than 75 percent TCA precipitable protein were used for experiments.

TCA precipitation

Silica gel impregnated glass fiber strips (4.8 cm x 1 cm) were spotted 1 cm from the bottom with an equal volume of two percent potassium iodide and radioiodinated MRP and developed in 30% (v/v) methanol, 10% (v/v) glacial acetic acid and 10% (v/v) trichloroacetic acid. The developed strips were air-dried, cut in half to separate acid soluble and
insoluble material, and counted in a Tracor Analytic Gamma Trac 1191 gamma counter. The acid insoluble counts remaining at the origin were divided by the total counts spotted to obtain the TCA precipitable protein fraction.

**SDS-PAGE**

SDS-PAGE was completed according to Ornstein (9) and Davis (10) as modified by Nilsen-Hamilton and Hamilton (11). The resolving gel consisted of a 7.5-15 percent linear polyacrylamide gradient and the stacking gel was five percent polyacrylamide. Electrophoresis was completed at 12.5 mamps for 4.5-5 hours at 22°C.

**Receptor binding assay**

Binding assays were modified as described (8) by including 1-10 mM mannose-6-phosphate in the assay mixture. Briefly, 0.5-1.0 mg/ml uterine membrane protein was incubated in 250 mM Tris, 10 mM MgCl₂, pH 7.6 containing one percent BSA, 1-10 mM mannose-6-phosphate, 0.1 mM PMSF and 0.1-1.0 ng/ml ¹²⁵I-MRP for 90 minutes at 24°C. Nonspecific binding was determined by the addition of 100-fold excess unlabeled MRP to the above reaction mixture and was subtracted from total binding to obtain the specific binding of ¹²⁵I-MRP. One milliliter of ice cold 250 mM Tris, 10 mM MgCl₂, pH 7.6 buffer was then added and the mixture was immediately filtered under vacuum using either a mixed ester membrane (Millipore RAWP 02500, 1.2 μm pore size) or Durapore membrane (Millipore GVWP 02500, 0.2 μm) to separate free and bound ¹²⁵I-MRP. The filter was washed six times with 3 ml of ice cold buffer and counted in a Tracer Analytic Gamma Trac
Chemical crosslinking

Uterine membranes were incubated with $^{125}$I-MRP in the presence or absence of excess unlabeled MRP or unlabelled prolactin. After incubation, disuccinimidyl suberate was added to the mixture to 0.2 mM and incubated at 4°C for 20 minutes. An equal volume of ice cold 250 mM tris, 10 mM MgCl$_2$, pH 7.6 was added and the mixture was microfuged at 4°C for 10 minutes. The pellet was washed one time with ice cold buffer and microfuged. The washed pellet was suspended in electrophoresis buffer and analyzed by SDS-PAGE and autoradiography.

Protein determination

Protein concentrations were determined using the Coomassie blue G-250 Protein Assay Reagent from Pierce Chemical Co. The assay is based on the Bradford method (12) and bovine serum albumin was used as the standard.
RESULTS

Receptor Binding at Mid-gestation

We conducted receptor binding assays on uterine and mammary gland membrane preparations from day 10-14 (Figure 1). To eliminate any potential binding contribution to the mannose-6-phosphate receptor, we determined that mannose-6-phosphate in concentrations of 1 mM completely blocked binding to the presumed mannose-6-phosphate receptor and included 1 mM mannose-6-phosphate in the reaction mixtures. For uterine membranes there was a peak of receptor binding at day 11 of gestation with a rapid decline between day 11 and 12 of gestation. Almost no binding activity was found in day 14 uterus. Mammary gland membrane preparations showed greatest binding at day 10 and with little or no binding after day 11 of gestation. However, we experienced problems with filtering mammary gland membrane preparations to separate bound and unbound $^{125}\text{I-MRP}$. The mammary gland mixtures generally filtered more slowly than uterine membrane mixtures and it was difficult to wash the filters as extensively. For this reason, we used uterine membrane preparations for subsequent studies.

Receptor Characterization

We used uterine membrane preparations from 11 days of gestation to characterize the MRP receptor. The binding isotherm (Figure 2a) shows saturable kinetics with half maximal binding at $1.28 \times 10^{-10}$ M. The Scatchard plot (13) shows a single affinity receptor population with an average $K_D = 3.5 \times 10^{-10}$ M and maximal binding of 72 fmoles/mg membrane
Figure 1. Receptor binding of MRP. Receptor binding was measured in membrane preparations from uterus (□) or mammary gland (●) isolated from mice at day 10 through day 14 of gestation as described in Materials and Methods. Specific binding was measured by subtracting the cpm bound in the presence of a 100-fold excess of unlabeled MRP. Values are the average and standard deviation of 2 (day 10, 13, 14), 3 (day 13), and 4 (day 11) different membrane preparations.
Figure 2. **Concentration dependence of MRP binding.** a) Membranes prepared from uterine tissue at 11 days of gestation were incubated with increasing concentrations of $^{125}$I-MRP in a total volume of 250 µl as described in Materials and Methods. Specific binding was determined in the presence of excess unlabeled MRP at each concentration of $^{125}$I-MRP for duplicate samples. Half-maximal binding was found at $1.28 \times 10^{-10}$ M. b) Scatchard plot of MRP binding with membranes prepared from 11 days of gestation. $K_D = 3.5 \times 10^{-10}$ M and $B_{max} = 72$ fmole/mg protein.
protein (Figure 2b). Nonspecific binding with 11 day uterine membranes represented about 7% of the total TCA precipitable label in these assays. Receptor binding is linear up to about 0.8 mg/ml of membrane protein with uterine membranes from 11 days of gestation (Figure 3).

Receptor association is rapid and shows association kinetics indicative of a second order bimolecular process (Figure 4). Using the linear portion of the association curve and the equation for a second order process, \( k_1 = \ln \frac{B(A-x)/A(B-x)}{t(A-B)} \), where A, B, and x are the ligand, receptor, and receptor-ligand concentrations, we calculated an association rate constant of \( 1.35 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1} \). The receptor concentration was obtained from the \( B_{\text{max}} \) of the Scatchard analysis and it was assumed there was only one binding site per receptor molecule.

The receptor shows reversible binding kinetics indicative of a first order dissociation process with half maximal dissociation after 12 minutes (Figure 5). The calculated first order dissociation rate constant, \( k_{-1} \), is \( 4.18 \times 10^{-4} \text{ sec}^{-1} \). MRP binding reaches equilibrium after 30 minutes. The \( K_D \) calculated from the association and dissociation rate constants is \( 3.1 \times 10^{-10} \text{ M} \) using the formula \( K_D = k_{-1}/k_1 \).

Optimal binding of MRP occurred at pH 7.6 (Figure 6) with a significant decline in binding around the pH optimum. There was no binding at very acidic (pH 4.4) or basic (pH 8.3) conditions.

Prolactin did not compete for binding with MRP at a concentration up to 45 nM (Figure 7).

We used the chemical crosslinker, disuccinimidyl suberate, to crosslink MRP with its receptor (Figure 8). The autoradiogram shows a
Figure 3. Receptor binding activity as a function of membrane concentration. Receptor binding was measured with increasing concentrations of uterine membranes prepared from 11 days of gestation. Specific binding was determined in the presence of excess unlabeled MRP for duplicate samples. Average specific binding: 2.69 ± 0.27 fmole/mg membrane protein.
Figure 4. **Receptor association with time.** Uterine membranes prepared from mice at 11 days of gestation were incubated with $^{125}$I-MRP in the absence or presence of excess unlabeled MRP as described in Materials and Methods. Duplicate samples were taken at various times of incubation and the specific binding determined. The rate of association, $k_1 = 1.35 \times 10^6 \pm 2.5 \times 10^5 \text{ M}^{-1} \text{sec}^{-1}$ was calculated using the six points in the linear range and the second order rate equation, $k_1 = \ln \left( \frac{B(A-x)/A(b-x)}{t(A-b)} \right)$. 
Figure 5. **MRP dissociation from the receptor with time.** Uterine membranes prepared from mice at 11 days of gestation were incubated with $^{125}$I-MRP for 60 minutes in the absence or presence of excess unlabeled MRP in a total volume of 250 μl as described in Materials and Methods. Excess unlabeled MRP was then added and the incubation continued. Specific binding for duplicate samples was determined at the indicated times. Specific binding at 0 minutes was determined without the addition of unlabeled MRP. The plot is a logarithmic transformation of the data. $k_{-1} = 4.18 \times 10^{-4}$ sec$^{-1}$ calculated by the method of least squares.
Figure 6. Receptor binding as a function of pH. Specific binding to 11 day uterine membranes was determined at pH 4.4, 6.6, 7.2, 7.6, and 8.3. The pH dependency was tested using the following buffers: 250 mM Tris, 10 mM MgCl₂ pH 8.3; 250 mM Tris, 10 mM MgCl₂ pH 7.6; 250 mM Tris, 10 mM MgCl₂ pH 7.2 (pH 7.2T); 200 mM sodium phosphate, 10 mM MgCl₂ pH 7.2 (pH 7.2S); 250 mM sodium citrate, 10 mM MgCl₂ pH 6.6; 250 mM sodium acetate, 10 mM MgCl₂ pH 4.4.
Figure 7. Receptor binding in the presence of prolactin. MRP binding to 11 day uterine membranes was determined in the presence of increasing concentrations of unlabeled MRP (•) or prolactin (▲) as described in Materials and Methods.
Figure 8. Identification of MRP-binding protein by crosslinking.
Receptor binding was performed as described in Materials and Methods in the absence or presence of excess unlabeled prolactin or MRP. Disuccinimidyl suberate was added and incubated for 20 minutes in electrophoresis buffer. SDS-PAGE was completed under reducing conditions. Lane 1, no unlabeled MRP; Lane 2, + unlabeled prolactin; Lane 3, + unlabeled MRP. Arrow points to the position of 57 kDa receptor-MRP complex.
distinct band at 57 kDa that is diminished in the presence of excess unlabeled MRP but not in the presence of prolactin. We also performed the crosslinking of $^{125}$I-MRP in the absence of uterine membranes and found no 57kDa band. Using a Mr of 34 kDa for MPR, the receptor or binding unit for MRP is about 23 kDa.
DISCUSSION

MRP binds to the cation-independent mannose-6-phosphate receptor in liver and placental membranes with maximal binding occurring at late gestation (14). Most of the cation-independent mannose-6-phosphate receptor is located intracellularly (15) and serves to target polypeptides to the lysosomes. Although there is evidence that ligand binding to the plasma membrane cation-independent mannose-6-phosphate receptor activates phospholipase C and that MRP is one of those ligands (16), we doubt that this receptor is involved in transducing a signal from MRP in vivo because circulating MRP does not contain mannose-6-phosphate (Nelson et al., in preparation).

Our recent findings showed that radioiodinated MRP is rapidly degraded by mammary gland and uterine tissue in a chloroquine-sensitive manner (Nelson et al., in preparation). Based on these findings, we collected membranes from mammary gland and uterus in mid-gestation. We found peak binding activity for mammary gland and uterine membranes prepared from mice at days 10 and 11 of gestation, respectively. Studies in our laboratory with placental homogenates also showed specific MRP binding. MRP binding in the placenta peaked on day 12 (data not shown). Two of these three tissues (mammary gland and uterus) were tissues identified by in vivo clearance studies as those likely to express a receptor for MRP (Nelson et al., in preparation). We elected to use 11-day uterine membranes for receptor characterization because separation of unbound from bound label in mammary gland incubations was a slower
process.

The developmental profile of expression of the MRP receptor parallels that of MRP (17). However, the circulating level of MRP at mid-gestation is extremely high (4-20 μg/ml at peak levels) and declines with an apparent half-life of 33 hours. Thus, a high affinity receptor such as we have identified would be capable of being stimulated by MRP throughout much of development. The developmentally-regulated expression of this receptor suggests that the ability of MRP to initiate an intracellular signal in the uterine cells is determined by the presence of the receptor rather than its ligand.

After day 11 of gestation, the MRP concentration in the blood declines at a constant rate with a half-life of 33 hours. Over the same period the level of MRP mRNA in the placenta decreases with a half-life of 48 hours. Therefore, it seems that MRP is rapidly removed from the blood. Uterine and mammary gland receptors are unlikely to be involved in this clearance because their gestational profile of expression is not coincident with the period of decline. This suggests that, as well as the uterine and mammary gland receptors, there is another receptor for MRP involved in its clearance.

Other prolactin-like proteins are synthesized and secreted by rodent placentae during gestation (18-23). Two of these, placental lactogens I and II, have receptors that have been characterized (24-28). The MRP receptor is similar to these receptors in that binding activity changes over gestation. At 11 days of gestation, receptors on the uterine membranes have an average $K_D = 3.5 \times 10^{-10} \text{ M}$ and show maximal MRP binding
of 72 fmoles/mg membrane protein. At day 10 of gestation the $K_D$ was 6.5 $x 10^{-10}$ M and maximal MRP binding was 130 fmoles/mg protein, respectively, while we could not measure the $K_D$ at day 13 due to low specific binding. There was no significant difference between receptor affinities measured at days 10 and 11 of gestation.

The MRP receptor displays a single affinity for its ligand. This indicates the uterus contains a single population of active receptors. Unlike placental lactogen receptors (24,26,27) prolactin does not compete for binding to the MRP receptor at concentrations up to 45 nM. These data indicate the MRP receptor is not the same as the placental lactogen I receptor for which placental lactogen I binding is effectively competed for by prolactin.

The MRP receptor has a sharp pH-optimum for binding MRP. We speculate the decreased binding at low pH may be part of a mechanism employed to separate MRP from its receptor during internationalization. This model has been proposed for receptor-ligand complexes that are endocytosed. Our previous results suggest the uterus and mammary gland degrade MRP by a lysosomal-mediated mechanism which is common for ligands that are taken up by receptor-mediated endocytosis (Nelson et al., in preparation).

The uterine MRP receptor shows rapid association kinetics with an association rate constant, $k_1$, of $1.35 \times 10^6$ M$^{-1}$ sec$^{-1}$ assuming second order rate kinetics. MRP binding is reversible and occurs at a dissociation rate constant of $4.18 \times 10^{-4}$ sec$^{-1}$, assuming first order kinetics apply. The calculated dissociation constant from these data is $3.1 \times$
M, which matches well with the $K_D = 3.5 \times 10^{-10}$ M calculated from the Scatchard plot.

The molecular weight of the receptor-MRP complex which could be identified by crosslinking was 57 kDa under reducing conditions. The calculated $M_r$ for the receptor is thus 23 kDa after subtracting 34 kDa as representative of the $M_r$ of MRP. Small molecular weight "receptors" have also been found for the placental lactogens. For example, the fetal sheep liver placental lactogen receptor was identified as $M_r$ of 38 kDa (29), the receptor in murine ovaries and liver was 42 and 50 kDa (24), and the murine liver receptor was 45 kDa (27). Proteins of 28-93 kDa have been identified as prolactin binding lactogen receptors in a number of tissues in rats, rabbits, and pigs (30-38). Thus, the MRP receptor is substantially different from the receptor that binds the placental lactogens at mid- and late-gestation. However, the $M_r$ of the MRP receptor is similar to the prolactin binding lactogen receptors found in rat kidney (34) and rabbit mammary gland (36). The receptor to which MRP binds is not likely to be the lactogenic receptor because MRP binding is not competed for by prolactin.

In summary, we have characterized a high-affinity receptor for MRP from murine uterine membrane preparations. Receptor binding peaks at 11 days of gestation and the affinity for MRP differs at different days of gestation. Prolactin does not compete for binding. Based on its specificity and molecular weight, we conclude the receptor is not related to previously identified prolactin or placental lactogen receptors. This
finding supports the hypothesis that MRP is a new, placentally-derived, and developmentally-regulated hormone with a different function from prolactin and the placental lactogens.
REFERENCES


Nilsen-Hamilton (41) proposed a model for the role of MRP during mammalian development. She proposed that transforming growth factor α (TGF-α) is released from the decidual cells of the developing placenta and then stimulates placental giant cells to secrete MRP. This MRP then binds to nearby fetal cells and is also secreted into the maternal blood where it binds to a target tissue. The result of the endocrine action is to stimulate the release of insulin-like growth factor which then acts in an autocrine factor to stimulate proliferation. It is this proliferation that initiates the next phase in a coordinated development process. The focus of my research was to identify the MRP target tissue and to characterize a specific MRP receptor on that tissue. MRP synthesis occurs in the placenta and reaches maximum levels during mid-gestation. The temporal profile of MRP synthesis correlates with the maternal blood concentration during gestation. I’ve determined that tracer radiiodinated MRP is cleared from the maternal blood circulation rapidly at mid-gestation. Furthermore, I have identified two tissues that rapidly take up and degrade MRP.

The binding of a ligand to a receptor at the cell surface may involve internalization of the ligand with subsequent transfer to the Golgi and lysosomes (42). Epidermal growth factor (EGF) binds its receptor and is transported to the lysosome for degradation (43). Prolactin is a hormone internalized and found associated with the lysosomes (44). Human growth hormone is transported to the lysosomes in
human lymphocyte cultures and lysosomotropic agents such as $\text{NH}_4\text{Cl}$ and chloroquine inhibit degradation of growth hormone (45). My results show decreased degradation of radioiodinated MRP in uterus and mammary gland after treatment of pregnant mice with chloroquine. This suggests that MRP is degraded in the lysosomes and may follow a similar internalization pathway as EGF, growth hormone, prolactin, and other ligands such as insulin (46) and low density lipoproteins (47).

Preparations of murine uterine membranes from mid-gestation show receptor binding activity to radioiodinated MRP. Binding peaks on day 11 and declines over the next few days of gestation. The average $K_D$ at 11 days of gestation obtained by Scatchard analysis was $3.5 \times 10^{-10}$ M. This compares favorably with the $K_D$ calculated from the kinetic studies of $3.2 \times 10^{-10}$ M. The average $B_{\text{max}}$ was 72 fmole/mg protein. These binding characteristics at 11 days of gestation compare with a $K_D = 6.5 \times 10^{-10}$ M and $B_{\text{max}} = 130$ fmole/mg protein at day 10 of gestation. I could not determine the $K_D$ or $B_{\text{max}}$ at day 13 due to low specific binding. The difference in receptor affinities and maximal binding was not significant between days 10 and 11. The placental lactogens exhibit different binding affinities and maximal binding dependent on the day of gestation (48), but this change occurred over a four-day period. The amount of MRP binding decreases significantly ($p<.005$) between days 11 and 14 of gestation and this change may reflect a requirement for MRP at a specific time of gestation.

I have demonstrated that the MRP receptor is specific for MRP. Prolactin at concentrations up to 45 nM does not compete for binding with
MRP on uterine membrane preparations. Mannose-6-phosphate does not block MRP binding at concentrations up to 10 mM. Thus, MRP does not bind to the mannose-6-phosphate receptor at this stage of gestation. Prolactin competes with placental lactogens for binding to placental lactogen receptors (48-50). Recombinant MRP does not bind to the prolactin receptor (16) or to Nb2 lymphoma cells (K. Ebner, personal communication), a cell line known to bind lactogenic hormones. This evidence supports the existence of a specific MRP receptor that is not related to the placental lactogen receptor.

MRP does not bind to its receptor under acidic conditions. This is in agreement with the mannose-6-phosphate and other receptors that transport ligands to intracellular compartments which also release their ligands at acidic pH conditions (30, 51). This is an interesting finding if the MRP-receptor complex follows an internalization process similar to other internalized hormones. The change in pH that occurs in the endocytotic vesicles could allow dissociation of the receptor-MRP complex with subsequent MRP degradation in the lysosomes. The fact that in vivo degradation declines upon treatment with the lysosomotropic agent, chloroquine, suggests that MRP may be degraded in the lysosomes. However, the receptor may not be recycled because receptor numbers decrease with advancing gestation. This suggests the receptor may also be degraded upon internalization.

The molecular weight of the receptor of 23 kDa is small when compared with receptors of prolactin and the placental lactogens. However, several groups identified prolactin binding subunits of 28-40
kDa (52-56) and the 23 kDa species may be a subunit of the receptor. Placental lactogen receptors have a range of molecular weights depending on the tissue examined of 38 kDa - 50 kDa (48,50,57). The difference in receptor molecular weight between MRP and placental lactogens also supports the hypothesis of a specific MRP receptor.

I conclude that MRP has a specific receptor that appears in uterine tissue at mid-gestation. The presence of this receptor along with the placental location of its synthesis, its temporal profile of MRP secretion into the maternal bloodstream, and the homology of MRP to prolactin supports MRP being a placental hormone involved in fetal development. Other prolactin-like hormones, the placental lactogens, possess lactogenic activity (17,58). The biological role of MRP is not known, but the relationship to prolactin and its binding to both uterine and mammary gland tissue indicate these are target tissues.

Faria et al. (21) speculated that placental lactogens may also play a role in growth and differentiation of the uterine decidua and in fetal growth. This role reflects a somatotropic effect similar to growth hormone, another hormone of the prolactin-growth hormone family (59). The biological action of MRP on uterine tissue, specifically, may be to stimulate uterine growth and this action may be mediated through the insulin-like growth factors as proposed by Nilsen-Hamilton.

The discovery of an MRP receptor in uterus and mammary gland leads to much future research relating to further receptor characterization, biological function during gestation, and how the MRP binding signal is transduced.
Purification of the MRP receptor can lead to a better understanding of receptor function by characterizing binding, transmembrane, and signal domains. One may also propose a catalytic function, if it exists. The identification and expression of a cDNA for the receptor will assist in primary sequence identification and will allow comparison with other receptor proteins to determine similarity of functional domains.

Prolactin and the placental lactogens promote lactogenic activity and MRP may also stimulate a lactogenic response. One can assay for lactogenic activity in mammary epithelial cell cultures (49) and it will be useful to test MRP for this activity. I have suggested MRP may stimulate somatotropic effects on uterine tissue. One can determine potential somatotropic function by competition assays with growth hormone for somatotropic binding sites on IM-9 lymphocytes (54). Furthermore, it will be useful to establish the relationship between MRP binding and IGF stimulation, if one exists. Growth of uterine cells in primary culture and assay of MRP binding and IGF induction may provide evidence of such a relationship.

Primary cell culture would also be useful in the study of signal transduction mechanisms utilized after MRP-receptor binding, although an immortal cell line that contains the MRP receptor would be even more useful.

This future research will provide additional evidence that MRP is a placental hormone with a defined biological role during gestation and help to elucidate the molecular mechanism of its action.
REFERENCES


ACKNOWLEDGMENTS

I express my sincere gratitude to Dr. Marit Nilsen-Hamilton for her guidance and impressing upon me the philosophy of performing good science and always using the proper experimental controls. I am extremely proud to have been a part of such an international research group and the cultural education that I was afforded.

I thank Ms. Susan Lund for her excellent job of preparing this manuscript.

Most of all, I reserve my highest praise, appreciation, and love to my family and especially my wife, Lynn, who helped me through the tough times and shared all the joyful experiences.